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GESSICA DUTRA GONÇALVES

REPRODUCTIVE SYSTEM DEVELOPMENT IN ADULT RAT OFFSPRING FROM MATERNAL PROTEIN-CALORIC RESTRICTION IS RETARDED AND GENDER DIMORPHIC

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 obtenção do grau do Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Paulo Cezar de Freitas Mathias.

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BIOGRAFIA

Gessica Dutra Gonçalves é filha de Natalia Dutra Gonçalves e Nivaldo Vinhoto Gonçalves nascida em Londrina-PR/Brasil em 24/10/1991. Finalizou o ensino fundamental e ensino médio em escolas Públicas. Obteve em fevereiro de 2014 os títulos de bacharel e licenciatura em Ciências Biológicas pela Universidade Estadual de Londrina. Em fevereiro de 2015 finalizou a especialização em Biologia Aplica a Saúde e início o mestrado em Patologia Experimental, ambos pela Universidade Estadual de Londrina. No ano de 2017 obteve o título de mestre e início o doutorado em Ciências Biológicas (área de concentração: Biologia Celular e Molecular) na Universidade Estadual de Maringá. Durante seu doutorado teve a oportunidade de realizar um ano de doutorado sanduíche (2018-2019) no Laboratório de doenças renais do Prof. Dr. John Bertram (Monash University- Melbourne Austrália). Possui experiência na área de biologia celular, histologia, fisiologia e biologia experimental, atuando principalmente nos temas: toxicologia reprodutiva, programação reprodutiva, desenvolvimento, hormônios sexuais e protocolos histológicos.

“Só há duas maneiras de viver a vida: a primeira é vivê-la como se os milagres não existissem. A segunda é vivê-la como se tudo fosse milagre.”
- Albert Einstein

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APRESENTAÇÃO

Esta tese é composta por três artigos científicos experimentais, em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas (PBC). Todos os artigos foram redigidos de acordo com as normas das revistas escolhidas, e tem como objetivo abordar os efeitos de uma baixa proteína materna durante os doze primeiros dias de lactação na prole de machos e fêmeas, assim como avaliar os danos causados a estes animais quando submetidos à um segundo insulto por uma dieta de alta gordura na vida adulta, confirmando o conceito de Origens Desenvolvimentistas da Saúde e da Doença (DOHaD). Os efeitos e alterações foram avaliados aos 21 e aos 90 dias de vida em ambos os sexos.

O primeiro artigo intitulado, **"Patterns of reproductive development to postnatal low-protein diet in early and late life in male and female offspring",** foi submetido na *Journal Biomedical Science*, de fator de impacto 5,07 e QUALIS A2. Neste trabalho mostramos pela primeira vez alterações na expressão dos genes relacionados ao controle de hormônios sexuais, assim como um atraso na maturação sexual de ambas proles de machos e fêmeas, causados pela exposição maternal á restrição proteica durante a lactação.

O segundo artigo intitulado, **"Shortly postnatal malnutrition by low-protein diet alters spermatic function in the male offspring when or not overfed to a high-fat diet in adulthood"** ao qual será submetido na *Journal of Nutritional Biochemistry*, de fator de impacto 4, 87 e QUALIS A1. Este artigo demonstra uma programação do sistema reprodutor masculino, devido a insultos causados no início da vida. Uma susceptibilidade à danos foi observada nos órgãos sexuais desses animais, quando submetido a um segundo insulto na vida adulta.

O terceiro artigo intitulado, **"Early exposition to low protein during breastfeeding caused reproductive programming in female offspring exposed to a high-fat diet challenge in adulthood. "** deverá ser submetido na revista *Cell and Tissue Research*, de fator de impacto 4,04 e QUALIS A2. Apresentamos pela primeira vez a influência de uma desnutrição proteíca em um curto período durante a lactação no sistema reprodutor feminino. Mostrando que os órgãos femininos reprodutores sofreram uma plasticidade devido ao insulto no início da vida.

Gessica D. Gonçalves; Lucas P. J. Saavedra1; Camila Q. Neves; Kelly V. Prates; Anna R. O. Ferreira; Henrique R. Vieira ; Silvano Piovan1; Pedro L. Zonta2; Leticia F. Barbosa1; Isabela P. Martins1; Scarlett R. Raposo1; Camila B. Zara1; Glaura S. A. Fernandes 4; Nilza C. Buttow2, Paulo C. F. Mathias1. **Patterns of reproductive development to postnatal insults in early life and late in life in male and female offspring**.

Gessica D. Gonçalves; Lucas P. J. Saavedra; Camila Q. Neves; Kelly V. Prates; Anna R. O. Ferreira; Henrique R. Vieira; Silvano Piovan; Pedro L. Zonta; Leticia F. Barbosa; Isabela P. Martins; Glaura S. A. Fernandes; Nilza C. Buttow, Paulo C. F. Mathias. **Shortly postnatal malnutrition by low-protein diet alters spermatic function in the male offspring when or not overfed to a high-fat diet in adulthood.**

Gessica D. Gonçalves; Anna R. O. Ferreira; Camila Q. Neves; Gabrieli D. Gonçalves; Kelly V. Prates; Lucas P. J. Saavedra; Pedro L. Zonta; Silvano Piovan; Leticia F. Barbosa; Henrique R. Vieira; Scarlett R. Raposo; Camila B. Zara; Nilza C. Buttow, Paulo C. F. Mathias. **Early exposition to low protein during breastfeeding caused reproductive programming in female offspring exposed to a high-fat diet challenge in adulthood.**

RESUMO GERAL

INTRODUÇÃO

Artigos demonstrando como insultos no início da vida podem interferir no desenvolvimento de uma vida saúdavel, vêm cada vez mais sendo abordados na literatura através do conceito Origens Desenvolvimentistas da Saúde e da Doença (DOHaD). O fenótipo poupador é uma das hipóteses mais comumente citadadas dentro do conceito DOHaD, no qual indivíduos que sofreram desnutrição nas fases iniciais da vida apresentam maiores risco de desenvolver tardiamente doenças cardiovasculares, diabetes e obesidade. Ainda, pré-gestação, gestação, lactação e adolescência representam importantes fases de plasticidade e insultos nesses períodos podem levar a programação metabólicas e fisiológicas. Juntamente, a exposição a outros insultos na vida adulta ou velhice nestes indivíduos que incluem poluição, fármacos, tabaco, tóxicos e mudanças nutricionais, pode acarretar a danos irreversíveis por um disbalanço na homeostase do organismo.

A fase da amamentação tanto em humanos como em animais, representa uma fase de desenvolvimento onde mudanças fisiológicas, hormonais e conexões neuronais, ainda continuam sua maturação após o parto. Em ratos os 10 primeiros dias após o nascimento representam uma fase crítica de desenvolvimento onde a fisiologia e comportamento sexual finalizam seu desenvolvimento. Dessa forma, o aumento de casos de infertilidade desde 1990, pode apresentar como um dos fatores insultos pré-natais ou pós-natais, no entanto este possível fator para a infertilidade ainda é pouco abordado na literatura.

Por fim, a desnutrição por déficit proteico ainda atinge diversas populações em todo o mundo, atigindo principalmente países em desenvolvimento ou não desevolvidos, consequentemente afetando principalmente pessoas de baixa renda, uma vez que alimentos de alta proteína possuem um valor mais alto que os demais. Da mesma forma, estudos têm apresentado uma relação com pessoas que passaram por períodos de fome no inicio da vida, principalmente na vida pós natal e o aumento de doenças não comunicáveis em adultos. Em adição, a transição nutricional pela mudança abrupta de estilo de alimentação, especialmente pela facilidade em se obter alimentos altamente calóricos mas de baixo valor nutricional, tem sido associada ao aumento da obesidade em jovens, adultos e idosos.

OBJETIVOS:

Manuscrito 1: Avaliar se a ingestão de baixa proteína materna durante o ínicio da lactação irá afetar a maturação sexual e expressão hipotalâmica de genes sexuais de machos e

fêmeas, e exarcebar seus efeitos sob um segundo insulto por uma dieta altamente calórica quando adultos.

Manuscrito 2: Analisar se a restrição protéica materna durante um pequeno período da lactação afetará negativamente a função reprodutiva de ratos, pela alterações nos parâmetros espermáticos e na atividade das enzimas antioxidante após ingestão de dieta rica em gordura na idade adulta.

Manuscrito 3: Examinar os resultados reprodutivos femininos devido à dieta hipoproteica materna durante os primeiros 12 dias de vida, e como os órgãos reprodutivos femininos responderão diante de um uma mudança na homeostase quando adulto por uma dieta hiperlipídica.

MÉTODO

Protocolo Experimental

Ratos e ratas Wistar com 75 e 85 dias de idade, respectivamente, proveniente do Biotério Central da Universidade Estadual de Maringá (UEM), foram adaptados por 7 dias no biotério do Laboratório de Biologia de Secreções (UEM). Após o período de adaptação os animais mantidos na proporção de duas fêmeas e um macho por gaiola, para se acasalarem. E as fêmeas prenhas, foram transferidas para gaiolas individuais e alimentadas com dieta padrão (Nuvital®; Curitiba / PR, Brasil). O dia do nascimento foi considerado o dia pós-natal (PN) 0. No dia PN 1 a ninhada foi padronizada para oito ou nove filhotes por mãe e sempre que possível mantendo a proporção sexual de 1: 1. Também, no PN 1 as mães foram divididas em dois grupos experimentais (n = 14 / grupo) e receberam, nos primeiros 12 dias de lactação, uma dieta proteíca normal (NP, 20% de proteína; 4128 kcal / Kg), ou uma dieta pobre em proteínas (LP, 4% de proteína; 4128 kcal / Kg). Após PN 21, os filhos machos e fêmeas foram desmamados. Os machos e fêmeas foram separados e alocados quatro animais por gaiola. Do PN 21 ao 60, os animais foram alimentados com dieta padrão (3810 kcal / Kg, Nuvital®; Curitiba / PR, Brasil). No PN 60, as fêmeas descendentes de mães NP e LP foram subdivididas e alimentadas com uma dieta de gordura normal (NF, 4% de gordura; 3810 kcal / Kg) ou uma dieta rica em gordura (HF; 35% de gordura; 5370 kcal / Kg) até os 90 dias de idade. Assim, foi composto por quatro grupos: NP / NF, prole controle alimentada com dieta com gordura normal (n = 14-15/9 ninhadas); NP / HF, prole controle alimentada com uma dieta rica em gordura (n = 14-15/9 ninhadas); LP / NF, prole com baixo teor de proteína alimentada com uma dieta com gordura normal (n = 15/9 ninhadas); e LP / HF, prole com baixo teor de proteína alimentada com dieta rica em gordura (n = 15/9 ninhadas). Todos os animais ao longo dos

procedimentos experimentais foram mantidos em condições controladas de temperatura (23 ° C ± 2 ° C) e fotoperíodo (7h00 às 19h00, ciclo de luz). As femêas ao PN 90 foram eutanasiadas apenas em estro, para apresentarem um padrão hormonal similar.

RESULTADOS E DISCUSSÃO

Manuscrito 1: O presente estudo mostrou que a restrição na lactação foi capaz de alterar a composição do leite e, como consequência, a ingestão de leite dos filhotes, sem alterações na produção. Além disso, observamos um atraso no início da puberdade em ambos os sexos. Pela primeira vez, mostramos que a resposta de expressão de *Kiss1* e *Gnrh1* foi sexualmente dimórfico de acordo com a fase da vida e tratamento utilizado, sendo as fêmeas aparentemente mais resistentes às mudanças de expressão gênica causadas no início da vida pela restrição protéica.

Manuscrito 2: Foi observado neste trabalho que a dieta de baixa proteína materna durante a amamentação afetou o número de espermatozoides em testículos e epidídimos, e a produção diária de espermatozóides. Também, alterações nos paramêtros de estresse oxidativo foi observada nos órgãos analisados dos grupos submentidos a baixa proteína e a dieta de alta gordura na vida adulta, além de alterações na morfologia e motilidade espermática porém os testículos apresentaram mais resistencia aos danos oxidativos do que o epidídimo.

Manuscrito 3: Neste estudo a restrição protéica materna durante um curto periodo na lactação induziu uma redução do peso corporal ao longo do período experimental, aumento do número de corpos lúteos e mudanças nos paramêtros de estresse oxidativo dos ovários. Enquanto poucas alterações foram observadas no útero, com diminuição no número de glandulas endotelias aos 21 dias de vida e aumento de lipídeo peroxidação nos três grupos analisados aos 90 dias de idade. A ingestão de uma dieta rica em gordura nos animais que passaram pelo insulto pós natal no início da vida, apresentaram diminuiçao nos dias em estro, apresentando similariedades na estrutura dos ovários e paramêtros de estresse oxidativo ao grupo controle, que não passou por nenhum insulto em nenhuma fase da vida.

CONCLUSÃO

Manuscrito 1: Nós mostramos que a dieta hipoproteica materna afetou a composição do leite como uma resposta adaptativa para evitar danos na ninhada. Nossos resultados também demonstraram uma resposta diversificada na expressão dos genes sexuais sobre diferentes estressores em diversos momentos da vida, assim como essas respostas foram dimorficas entre machos e fêmeas

Manuscrito 2: No presente estudo, podemos concluir que a desnutrição pós-natal por uma dieta pobre em proteínas pode interferir nos parâmetros espermáticos e alterações nos paramêtros de estresse oxidativo após um segundo insulto tardiamente, o que podem afetar a reprodução masculina na vida adulta. Além disso, o período de amamentação representa uma importante fase de plasticidade e o insulto causado pela baixa proteína neste período pode induzir a programação da fisiologia do sistema reprodutivo ao longo da vida animal.

Manuscrito 3: Nossos resultados sugerem que uma dieta de baixa proteína materna pode causar insultos ovarianos e uterinos e uma possível antecipação da menopausa. Surpreendentemente, o consumo da dieta rica em gordura no grupo que passou pela desnutrição proteica no início da vida, somente apresentou alterações no ciclo estral. No entanto essa diminuição no número de estros seria o suficiente para reduzir a capacidade reprodutiva desse animal, explicando as alterações encontradas neste grupo. Dessa forma, o período lactacional representa uma fase importante do desenvolvimento e resultando em adaptações na fisiologia reprodutiva feminina, da mesma forma o segundo insulto pela dieta rica em gordura casou um desregulação na homeostase das fêmeas que receberam a baixa proteína na lactação, resultado em uma diminuição da atividade ovariana.

PALAVRAS-CHAVE: Restrição proteica materna, lactação, programação reprodutiva, fisiologia reprodutiva, obesidade, machos e fêmeas.

ABSTRACT

INTRODUCTION

Some studies have been showing how insults early in life can interfere with the development of a healthy life, and it is being addressed in the literature through the concept of Developmental Origins of Health and Disease (DOHaD). The thrifty phenotype is one of the most commonly cited hypotheses inside the DOHaD concept, in which individuals who suffered malnutrition in the early stages of life present more risk of developing cardiovascular diseases, diabetes, and obesity late in life. Still, pre-pregnancy, pregnancy, lactation, and adolescence represent important phases of plasticity, and insults in these periods can lead to metabolic and physiological programming and exposure to other insults in adulthood or old age in these individuals, which include pollution, drugs, tobacco, toxic and nutritional changes, can cause irreversible damage due to an imbalance in the homeostasis of the organism.

Together, the breastfeeding phase in both humans and animals represents a developmental phase where physiological and hormonal changes and neuronal connections still continue maturation after delivery. In rats, the first 10 days after birth characterizes a critical stage of development where physiology and sexual behavior end their development. Thus, the increase in infertility cases since 1990, may present as one of the factors prenatal or postnatal insults, however, this possible factor for infertility is still little addressed in the literature.

Finally, malnutrition due to protein deficit still affects several populations around the world, affecting mainly developing or undeveloped countries, mostly affecting low-income people, since high-protein foods have a higher value than the others. Likewise, studies have shown a relationship between people who went through periods of hunger early in life, especially in post-natal life, and the increase in non-communicable diseases in adults. In addition, the nutritional transition due to the abrupt change in the style of eating has also been growing in recent years, especially due to the ease in obtaining high-calorie foods, but with low nutritional value, resulting in obesity in young people, adults, and the elderly.

AIMS

Manuscript 1: Assess whether the ingestion of low maternal protein during early lactation will affect sexual maturation and hypothalamic expression of male and female sexual genes and exacerbate its effects under a second insult for a high-calorie diet when adults.

Manuscript 2: To analyze whether maternal protein restriction during a short period of lactation will negatively affect the reproductive function of male rats, due to changes in sperm parameters and oxidative stress parameters after overfed in adulthood.

Manuscript 3: Examine the female reproductive results due to the maternal low-protein diet during the first 12 days of life, and how the female reproductive organs will respond to a change in homeostasis as an adult by a high-fat diet.

METHODS

Experimental Protocol

Female and male Wistar rats, 75 and 85 days old, respectively, from the animal house central of the State University of Maringá (UEM), were adapted for 7 days in the animal house of the Secretion Biology Laboratory (UEM). After the adaptation period, animals kept in the proportion of two females and one male per cage, to mate. And the pregnant females were transferred to individual cages and fed a standard diet (Nuvital®; Curitiba / PR, Brazil). The day of birth was considered the postnatal day (PN) 0. On day PN 1 the litter was standardized to eight or nine puppies per mother and the sex ratio of 1: 1, if possible. Also, in PN 1 the mothers were divided into two experimental groups (n = 14/ group) and received, in the first 12 days of lactation, a normal protein diet (NP, 20% protein; 4128 kcal/kg), or a low protein diet (LP, 4% protein; 4128 kcal / Kg). After PN 21, the male and female children were weaned. Males and females were separated, and four animals were allocated per cage. From PN 21 to 60, the animals were fed a standard diet (3810 kcal / Kg, Nuvital®; Curitiba / PR, Brazil). In PN 60, females descended from mothers NP and LP were subdivided and fed a normal fat diet (NF, 4% fat; 3810 kcal / Kg) or a high-fat diet (HF; 35% fat; 5370 kcal / Kg) up to 90 days of age. Composing four groups: NP / NF, control offspring fed a normal fat diet (n = 14-15 / 9 litters); NP / HF, control offspring fed a high-fat diet (n = 14-15 / 9 litters); LP / NF, low-protein offspring fed a normal fat diet (n = 15/9 litters); and LP / HF, low-protein offspring fed a high-fat diet (n = 15/9 litters). All animals throughout the experimental procedures were kept under controlled conditions of temperature (23 ° C ± 2 ° C) and photoperiod (7 am to 7 pm, light cycle). The females at PN 90 were euthanized only in estrus, to present a hormonal pattern.

RESULTS AND DISCUSSION

Manuscript 1: The present study showed that the lactation restriction was able to alter the composition of the milk and, as a consequence, the milk intake of the puppies, without changes in production. In addition, we observed a delay in the onset of puberty in both sexes.

For the first time, we showed that the expression response of Kiss1 and Gnrh1 was sexually dimorphic according to the stage of life and treatment used, with females apparently more resistant to changes in gene expression caused early in life by protein restriction.

Manuscript 2: It was observed in this study that the low maternal protein diet during breastfeeding affected the number of sperm in testicles and epididymis and caused a daily in the sperm production. Also, changes in the oxidative stress parameters were observed in the organs analyzed organs of the groups submitted to low protein and the high-fat diet in adulthood, in addition to changes in sperm morphology and motility, being the testicles more resistant to oxidative damage than the epididymis. Still the male reproductive organs analyzed showed to suffer a plasticity due the insult in early life, since the oxidative stress parameters was altered in these animals, and the intake of high-fat caused an organism homeostasis disbalance, which can be observed by the reduction in the sperm quality.

Manuscript 3: In this study, maternal protein restriction during a short period during lactation induced a reduction in body weight throughout the experimental period, an increase in the number of corpus luteum, and changes in the oxidative stress parameters of the ovaries. While few changes were observed in the uterus, with a decrease in the number of endometrial glands at 21 days of life and an increase in lipid peroxidation in the three groups analyzed at 90 days of age. The intake of a high-fat diet in animals that went through the postnatal insult early in life, showed a decrease in estrus days, being similar in the ovaries structure and oxidative stress parameters to control group, which did not undergo any insult at any stage of life.

CONCLUSION

Manuscript 1: We showed that the maternal protein restriction diet affected the composition of the milk as an adaptive response to prevent damage to the litter. Our results also demonstrated a diversified response in the expression of sexual genes over different stressors at different times in life, as well as these responses were dimorphic between males and females.

Manuscript 2: In the present study, we can conclude that postnatal malnutrition from a low protein diet can interfere with sperm parameters and changes in the oxidative stress parameters after a second insult late, which can affect male reproduction in adulthood. In addition, the breastfeeding period represents an important phase of plasticity and the insult caused by low protein in this period can induce the programming of the physiology of the reproductive system throughout animal life.

Manuscript 3: Our results suggest that a low maternal protein diet can cause ovarian and uterine insults and possible anticipation of menopause. Surprisingly, the consumption of a high-fat diet in the group that experienced protein malnutrition in early life only showed changes in the estrous cycle. However, this decrease in the number of estrus would be enough to reduce the reproductive capacity of this animal, explaining the changes found in this group. Thus, the lactation period represents an important stage of development, which results in adaptations in female reproductive physiology. In the same way, the second insult for the high-fat diet caused a dysregulation in the homeostasis of the females who received low protein during lactation, demonstrating a decreased in the ovarian activity.

KEYWORDS: Maternal protein restriction, lactation, reproductive programming, reproductive physiology, obesity, males, and female.

SUMÁRIO

MANUSCRITO 1

Patterns of reproduc development to postnatal low-protein diet in early and late life in male and female offspring

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Abstract

Undernutrition remains a global problem reaching a thousand people including breastfeeding mothers and their newborns. Still, the brain development in humans and rats continues after birth, representing an important programming window. We evaluated whether the low-protein (LP) intake during the lactational period affects the sexual maturation of males and females, and its consequences by facing 'second-hit' at adulthood. Breastfeeding mothers were fed by either LP (4% protein) diet from post-natal days (PND) 1 to 12 or a normal-protein (NP; 20% protein) diet throughout lactation. Both male and female offspring, from NP and LP mothers, received a normal-protein diet from weaning until PND60. During PND 60-90, a batch of animals from both groups was fed either a high-fat (HF; 35% fat) diet or a normal-fat (NF; 4%fat) diet. Maternal protein restriction caused an altered milk composition, as well as a reduction in the pups' milk intake and altered biometric parameters during lactation with a low body weight of offspring until adulthood. Both sexes showed a delay in sexual maturation. The females and males presented a sexual dimorphism in response to LP or HF diet, in the reproductive organs weight, hypothalamus, and *Kiss1* and *Gnrh1* mRNA expression. In conclusion, maternal undernutrition during the first twelve days of lactation can impact the proper reproductive system development being these alterations maintained throughout the animal life.

Introduction

The first signs of sexual structure differentiation between males and females are observed in 12 weeks of gestation in humans and embryogenic day 12 in rats, presenting a complete maturation after birth [15,39]. The hypothalamic-pituitary-gonadal axis (HPG) is responsible by the release of sexual hormones, and its maturation continues after birth until the reproductive capacity is activated [21]. The HPG axis is controlled by Gonadotropin-releasing hormone (GnRH) which is neuromodulated by kisspeptin protein an excitatory neuropeptide released by Kiss1 neurons [22]. So, in response to reproductive hormones, Kiss1 neurons control the GnRH neurons secretion in the hypothalamus, which stimulates the production and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary gland [22]. During the lactational period, a "mini-puberty" seems to have a role in the penile growth and Sertoli and germ cell development in males and increase of mammary gland diameter in females due to the raise of LH and FSH [6]. Later, puberty is going to complete the reproductive maturation with precise hormonal control [22]. Including, delayed puberty in both males and females can be associated to Kiss1 and Gnrh1 deficient expression [22].

Periods of famine in early life were linked to an increase of altered coronary heart disease, breast cancer and infertility in adulthood [19,31,40]. Calories reduction to less than 1500 daily per capita were reported in World War II [34] and great famines such as Dutch [31] and Chinese [23]. This calorie restriction was associated with a reduction in the macronutrients in the diet, affecting pregnant, lactating, neonate, and children [34,44]. Together, proper maternal nutrition is essential to breast milk composition, which is rich in bioactive such as proteins, hormones, anti-inflammatory molecules, and growth factors, responsible for progeny development [3,24,41].

Alteration in body weight (BW) has been closely associated with reproductive dysfunction. The obesity rate has increased in the last decades, principally due to the consumption of food with high energy and low nutrients values [1]. Also, obesity is very well established to cause alteration in the metabolism of individuals, closely related to infertility/subfertility or changes in the reproductive system function of both sex [12,46]. Studies have been linked the alterations in early life with the development of obesity, including the relationship between low caloric intake and increased ratio of obesity development in adolescence and adulthood [36,37].

In addition, the actual COVID-19 disease scenario with the increase in the food insecurity following the augment of food prices may reduce the caloric intake in poor households causing futures health outcomes [28,42]. Also, a sexual dimorphism present by males and females leads

to a variety of diseases responses [7]. Still, there are only a few studies highlighting the importance of nutritional status during the lactational period and how it would interfere in the male and female sexual development in adulthood. Thus, we hypothesized that maternal protein restriction in the lactation period would induce an alteration in the sexual maturation of male and female offspring even when overweighted in adulthood.

Materials and methods

Ethical approval

All experimental procedures in this paper are in accordance with the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation, approved by the Ethics Committee on Animal Use of State University of Maringá, UEM, Maringá-PR, Brazil (CEUA/UEM number 6328301019)

Experimental design and diets

Females and males *Wistar* rats (70 and 80 days of age, respectively) obtained from the central animal facility at State University of Maringá (UEM) were maintained in the animal house of Secretion Biology Laboratory at UEM. Throughout the experimental period, the animals were kept under controlled temperature (22 °C ± 2 °C) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle) conditions. The animals received water and food *ad libitum*. After one week of adaptation, the animals were mated in a ratio of two females and one male. When pregnancy was detected the females were transferred to individual cages. At birth, the litter was standardized to eight-nine pups per dam maintaining as close to a 1:1 sex ratio as possible. The dams were divided into two experimental groups and received a normal-protein diet (20% protein; NP =14 dams), or a low-protein diet (4% protein; LP=14 dams) for the first 12 days of lactation. On postnatal day (PND) 21, the male and female offspring were weaned. The males and females were separated and allocated four per cage. From PND 21 to 60, all animals were fed a standard diet (3810 kcal/Kg, Nuvital®; Curitiba/PR, Brazil). To assess the developmental behavior in face of a 'second-hit, at 60 days of age, offspring from NP and LP dams were subdivided into four groups, fed a normal-fat diet (NF- 4% fat) or a high-fat diet (HF-35% fat) until 90 days of age, as follows: NP/NF, control offspring fed a normal-fat diet (n= 9/9 litters); NP/HF, control offspring fed a high-fat diet (n=9/9 litters); LP/NF, low-protein offspring fed a normal-fat diet (n=9/9 litters); and LP/HF, low-protein offspring fed a high-fat diet (n=9/9

litters). The compositions of the low-protein and high-fat diet has been detailed in Table S1 and S2.

Mother weight monitoring, food intake, offspring body growing, and weight gain during the lactational period

At PND 1 the number of the pups born in the day was noted, such as the sex ratio of each litter. The mother's BW was measured every other day. The mother's food intake was calculated by the weight of the food add previously less the food weighted on the day, during all lactational period. In the offspring, the BW was measured every day during the experimental period, also, the length, head diameter, and waist diameter were measured on PND 7, 14, and 21.

Maternal milk production and absolute and relative pup milk intake

On PND 7, 14, and 21 at 7:00 am, the pups and mothers were weighted, labeled in the tail with a permanent marker, and separated from each other for 4 h. The pups remained without food while mothers ate and drink *ad libitum*. After this time mothers and pups were weighted and put together for 1 h and again weighted after this time. The data was calculated using the mother BW after and before 1h breast-feeding for milk production, the pup body weighted after and before sucking was used for absolute pup milk intake. Relative milk intake was calculated from the absolute food intake/pup BW, as previously described by Bautista, et al. [6]**.**

Measurement of milk composition

A different cohort of n=5/litter per group of dams was used for the collection of breast milk on PND 7, 14, and 21. Mothers separated from the pups was anesthetized with sodium thiopental (45 mg/kg of BW, i.p., Thiopentax®, Cristália, Itapira, São Paulo, Brazil) and received an injection (2.5 UI/kg of BW, i.p.) of oxytocin (Oxytocin®, Chemical Union, Embu, São Paulo, Brazil) for subsequent collection of breast milk samples. Samples were vortexed, divided into aliquots, and frozen at −20°C until analysis. Milk samples were thawed at 37°C and vortexed vigorously before pipetting to ensure sample uniformity. Milk samples were diluted (1:20 v/v) in saline solution (0.9 % NaCl) and used for posterior measurement of total protein, glucose, total cholesterol, and triglycerides by an enzymatic colorimetric method using a commercial kit (Gold Analisa® Belo Horizonte, Minas Gerais, Brazil), according to the manufacturer's instructions [16].

Sexual maturation monitoring

The puberty onset was evaluated from PND 30, daily from 8:00 to 10:00 a.m. All the male pups were examined through manual retraction of the prepuce with gentle pressure until complete preputial separation. Just after preputial separation, the BW was assessed [20].
The same way the female offspring was evaluated daily until the complete vaginal opening [31]. After vaginal opening, rats were weighed and evaluated concerning the day of the first estrus through vaginal fluid cells content abundant cornified vaginal epithelial cells, as previously described by Guerra, et al. [19]. Briefly, saline 0.9% solution was inserted into the vagina and subsequently aspirated. Vaginal fluids were placed into slides and posteriorly analyzed under a lightmicroscope at x400 magnification to evaluation of vaginal epithelial cells.

Body, reproductive organs and hypothalamic weight at PND 21 and 90 from male and female offspring

Body weight at PNDs 21 and 90, were assessed from one male and female of each litter. The rats were anesthetized with inhalation of isoflurane ® (Cristália, Itapira, São Paulo, Brazil), inside of laminar flow chamber, decapitated, and laparotomized to remove their hypothalamus and reproductive organs, such as testis and epididymis in males or ovaries and uterus in females. All the organs were weighed, and the absolute and relative organs were analyzed. The hypothalamus collected was frozen rapidly in liquid nitrogen and stored at -80 ºC freezer for mRNA relative expression. All euthanasia was realized between 7:00 a.m. and 11:00 a.m. and in adult females when in the estrus phase.

Hypothalamic *Kiss1* and *Gnrh1* mRNA relative expression at PND 21 and 90 from male and female offspring

This methodology has been described previously by de Oliveira et al., (2020) and Ivanski et al., (2020). TRIzol® reagent (Life Technologies, Carlsbad, CA, USA) was used for extraction of hypothalamus total RNA in a microtube with a micro pestle, according to the manufacturer's instructions. Total RNA was used for reverse transcription followed by real-time quantitative PCR (RT-qPCR). The total RNA concentration was estimated with a nanospectrophotometer (KASVI model k23-002, Brazil), and the total RNA integrity was analyzed in an electrophoresis 1.2 % agarose gel in TBE buffer (Tris/Borate/EDTA) through visualization of 18S and 28S ribosome bands, stained with ethidium bromide. 2.5 µg of sample was reverse transcribed by the GoScript Reverse Transcription System (Promega, Madison, USA) using oligo (dTs) according to the manufacturer's instructions. Real-time PCR from the product of

reverse transcription (RTqPCR) was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies, Carlsbad, USA) for *Kiss1*, *Gnrh1* and *Actb* according to the manufacturer's instructions. The amplification was performed with the Applied Biosystems QuantStudio3™ Real-Time PCR System (Applied Biosystems, Singapore) and consisted of the following cycle conditions: 50 °C (2 min), 95 °C (2 min), and 40 cycles of 95 °C (15 s) and 60 °C (1min). At the end of the reaction, a melting curve was generated and analyzed to confirm the specificity of the amplification. The average cycle threshold (Ct) was automatically determined using QuantStudio5™ Software v1.5.1. (Applied Biosystems), and quantification was performed by the $2^{-\Delta\Delta C}$T method, as described previously [32]. Cytoplasmatic beta-actin (*Actb*) was used as an internal control. All material used was RNA free to avoid any contamination. The primer sequences and the GenBank access number of genes for each tissue are shown in Table S3.

Statical Analysis

All data were subjected to the D'Agostino Pearson normality test to assess their Gaussian distribution. Statistical analysis was performed using Student's t-tests or two-way ANOVA analysis of variance, followed by Bonferroni's post hoc analyses, according to the group's number. $P<0.05$ was considered statistically significant, and the analyses were performed using GraphPad Prism version 9.0 for IOS (GraphPad Software, Inc. San Diego, CA, USA). Data are presented as means with their standard errors (S.E.M).

Results

Number pups per litter, mothers, and pups weight gain and maternal food intake

The number of total pups per litter (NP: 10.13 ± 0.515; LP: 10.33 ± 0.373, $P>0.05$), such as the number of males and females born in each litter (Males, NP: 4.56 ± 0.73; LP: 4.60 ± 0.58; Females, NP: 5.50 ± 0.54; LP: 5.89 ± 0.56, $P>0.05$), were similar between the groups. Both mother and pups showed reduced BW starting on PND 2 and PND 4, respectively, in the LP group, as showed in Fig. 1A and 1C. As well as the biometric parameters at PND 7, 14 and 21 showed to be altered in LP compared with NP, in both male and females (Table S4). In the same way, the LP mothers showed a reduced intake of food during the LP exposure (Fig. 1B). However, after the introduction of the NP diet the food intake presented an increase in the LP mother group, did not show a significant difference between PND 16 to 19, thus on PND 21, the LP had an augment of 36% in the food intake compared with NP ($p<0.0001$).

Milk intake, production and composition during the suckling period

Fig. 2A and 2B, display the absolute and relative milk intake by the pup. The milk intake was reduced by LP pups in the PND 7 and 14 (73 and 45%, respectively), on the other hand, the relative milk consumption showed similar intake on PND 14 and increased intake on PND 21 (46%), compared with NP. The Milk production was not affected by LP diet (Fig. 2C), conversely, the total protein, glucose, and total cholesterol presented alteration in all evaluated days, with an increase of these macronutrients on the day 7 and 14 and reduction on the 21 (Table 1). The triglycerides did not show any difference between the groups on the observed days.

The puberty onset in male and females offspring

LP animals demonstrate a delay of 4 days in preputial separation, vaginal opening, and the first estrus compared with NP (Fig. 3 A-C - $p<0.001$, $p<0.0001$, $p<0.01$, respectively). Also, the BW on the puberty onset was decreased in males ($p<0.05$) but not in females, on LP groups (Fig 3D-F).

Body weight (BW), absolute and relative weight of reproductive organs at PND 21 and 90

At PND 21 the BW in both males and females was reduced in the LP group compared with NP (Table 2). In the reproductive organs, the absolute weight of testis and epididymis showed a reduction of approximately 30% in both parameters, instead, the ovaries and uterus did not show significant alterations in the absolute weight among the groups. However, in the relative weights, the testis, epididymis, and uterus were similar between the LP and NP animals, while the ovaries were increased in the LP group ($p<0.05$).

These data are shown in Table 3. About the BW, at 90 days old LP/NF groups males and females maintained the BW in 12% lighter than NP/NF groups ($p<0.01$, males and $p<0.05$, females). Thus, NP/HF groups increased the BW in both sexes, compared with NP/NF. Interestingly, the LP/HF male group showed to be augmented compared with LP/NF ($p<0.05$) and diminished compared with NP/HF ($p<0.0001$), being similar to NP/NF. LP/HF females also presented a decrease in the BW compared with NP/HF ($p<0.05$), but not to NP/HF and LP/NF. The testis and epididymis weight were reduced only by the LP diet, being reduced in LP/NF and LP/HF groups, compared with NP/NF. In the relative weights, the testis exhibited a decrease in the NP/HF and LP/HF groups related to NP/NF. The epididymis was similar

between the groups but showed HF factor $p<0.05$. In females, reproductive organs did not change the absolute or relative weights in multiple comparison analysis, however, the ovaries relative weight demonstrated $p<0.05$ in LP and HF factors, and uterus absolute weight presented $p<0.05$ in LP factor.

Dimorphism of absolute and relative hypothalamus weight at PND 21 and 90

At PND 21 days old, the absolute hypothalamus weight was similar between the NP and LP groups, and similarly, the factors showed any significance (Fig. S 1A). On the other hand, the relative weight showed a significant difference of hypothalamus in the LP group in females, such as LP factor $p<0.05$ (Fig.S 1B). Thus, the males and females showed similarities in the hypothalamus weight.

At 90 days old, the groups showed to be similar in the hypothalamus weight among the groups in males and females. But were observed a dimorphism in absolute weight among males and females in NP/NF, LP/NF, and LP/HF with females showing a hypothalamus weightier than males (Fig. S 1C). Likewise, the relative hypothalamus weight was higher in females than males in all groups evaluated. (Fig. S 1D).

Hypothalamic expression of *Kiss1* and *Gnrh1* in male and female offspring at PND 21 and 90

At PND 21 days old males showed an altered *Gnrh1* expression with LP presenting an increase of 53% compared with NP, while no alteration was observed in the hypothalamic expression of *Kiss1*. Females of the same age presented similar expression of both genes between the groups (Fig 4 A and B).

On other hand, at 90 days old males did not show changes in the mRNA expression of *Kiss1* and *Gnrh1*, as the factors observed (Fig. 4 C and E). But, in females hypothalamic *Kiss1* expression in the HF diet augmented in NP/HF group in 85% compared with NP/NF and 91% compared with LP/NF, similarly, the *Gnrh1* expression was approximately 60% higher in NP/HF than NP/NF and LP/NF. The factors LP, HF, and I was significant in both *Kiss1* and *Gnrh1* expression at PND 90 (Fig. 4 E and F).

Discussion

The protein is a macronutrient with high importance to properly development of fetus and newborn. In the gestational period, the restriction of protein intake was related to damages in

544 the brain central nervous system, causing a decrease of essential amino acid for fetus brain
545 development [37]. The current study showed that a restrictive protein diet in the lactation
546 period was able to change the milk composition and as consequence, the milk intake of pups.
547 Also, we observed a delay in the puberty onset. For the first time, we show that
548 the *Kiss1* and *Gnrh1* expression response was sex dimorphic at different times of life and
549 treatment, being the females more resistant to changes caused by the early life protein
550 restriction.
551 Studies in gestation and/or lactation showed that LP food intake caused a reduction in the BW
552 of the mothers [2,40]. The composition of diets LP and NP used in this study has the same
553 amount of calories, however, we observed that LP mothers presented a reduction in the food
554 intake, causing undernutrition of these dams. The alteration in milk composition (i.e. increase
555 of proteins, glucose, and total cholesterol) can be related to the decrease of maternal BW due
556 to the mobilization of nutrients and a possible proteolyze of muscle and lipogenesis [35]. As
557 well as, Bautista, et al. [5] observed a liver lipogenesis and β-oxidation of mother submitted to
558 a 10% of low-protein through gestation and lactation. That way, the reduction of BW in the
559 dams can be a way to maintain the proper nutritional status of the pups, since the BW of the
560 mothers started to decrease at PND 2 while in the pups started after PND 5.
561 In rodents, the intake of 8% protein in the gestational and lactational period decreased the
562 essential amino acids in the mother's milk. As some organs are still developing after birth, and
563 are dependents on growth factors, the absence or diminution of essential amino acids due to
564 the protein restriction would interfere in the correct offspring development maintained until
565 adulthood [3,34]. In the current study, even observing an increase of relative milk intake in the
566 LP litter after 12 days of lactation, the LP intake was sufficient to decrease the offspring
567 biometrics parameters at PND 21, and throughout the life of these male and female offspring.
568 Preputial separation and vaginal opening are markers of sexual development. Zambrano, et al.
569 [52] showed that males offspring from mothers fed at 10% of the protein in the lactational
570 period, had a delay in the testis descent as preputial separation, and the testis weight at PND
571 25 and 70 reduced in the LP group compared with them control. While, in females offspring,
572 8% of protein during preconception, gestation, and lactation caused a decrease in the number
573 of follicles at PND21 and 24 weeks old, but no differences were observed in the ovarian volume
574 in both studies [50]. Here, we showed that the LP intake on lactation presented a reduction in
575 the testis weight and no alteration in ovaries weight, both at PND 21 and 90, but the delay in
576 the sexual maturation was observed. Interestingly, the malnutrition postnatal condition due to
577 the low resources can induce a reproductive delay until the reproduction can be successful [28].

This may explain the delay in sexual maturation, at least in females, when the vaginal opening and first estrus occurred only when the BW was similar to control. On other hand, males can present a delay in puberty that is not related to alterations in the BW, but with hormonal changes, mainly by a decrease of testosterone and LH, as showed by Zambrano, et al. [52]. Also, the normal ovary weight and low testicular weight found in this study can corroborate these hypotheses. Together, the males and females presented a different response caused by the LP diet, being the males more susceptible to changes in the reproductive organs than females.

Both GnRH and Kiss1 neurons are related to sexual hormones release control and puberty maturation. In humans, the delay in puberty was associated with normal GnRH expression but decrease or absence of kisspeptin [24]. However, in 32 days old rats females from large litter malnourished through lactation presented a low density of Kisspeptin neuron fibers, otherwise, the GnRH neuron was not changed by the undernutrition in the hypothalamus [10]. We observed, at PND 21, that there was a similar expression of *Kiss1* mRNA in females and males, however, males presented a high expression of *Gnrh1* mRNA. Contrary to our findings, the undernutrition by reduction of 50% of the daily food intake in gestation and lactation caused a decrease in hypothalamic *Kiss1* mRNA expression in females together with a delay in vaginal opening [27]. As shown before, lactation represents an important period of brain plasticity, and insults can induce changes in neuronal functioning and behavior by epigenetic modifications [9]. It is known that environmental factors, such as diet and epigenetic modulations can affect the activity of GnRH neuron and *Gnrh1* expression, during its development [29]. This way, the alteration in the *Gnrh1* expression can support our finding in the male sexual delay by hormonal disbalance caused by the maternal LP diet.

At 90 days old, no alterations were observed in the *Kiss1* and *Gnrh1* in LP males independently of the exposure to HF diet or not. While, in LP females did not change the sexual maturation gene expression, the HF diet intake alone, increased the expression of both genes. Kisspeptin neurons present receptor to leptin, since the GnRH does not show these receptors [45]. Leptin is a hormone that acts in the body energy expenditure it has been associated with enhanced activity of reproductive system [22]. Recently the association, with the receptors for leptin in Kisspeptin neuron showed a possible action of an obesogenic environment with disturbs in the reproduction [22]. The female rat exposed to a 45% of high-fat diet post-weaning presented an increase of *Kiss1* expression together with an increase of leptin [15]. Therefore, the increased leptin in these animals may be associated with activation of kisspeptin neurons as a consequence, activation of GnRH neurons, increasing the expression of the *Kiss1* and *Gnrh1*.

On other hand, the 10% LP diet seems to reduce leptin in 110 days old females rats [51]. Since the LP/HF females did not show a difference in the sexual genes expression, the suppression of leptin by LP could reduce the effects of the HF diet. In addition, the males did not show any alterations in the *Kiss1* and *Gnrh1*, as shown before males and females can respond differently to a variety of stressors.

Many studies have shown a dimorphism between males and females in a range of diseases [18,49,52]. Differently than demonstrated in this study, in human, the hypothalamus volume seems to be higher in men than women, still there is sexual dimorphism in the hypothalamus function of males and females [47]. Curiously, the sexually dimorphic nucleus of the preoptic area, are higher in males than in females adult rats, likewise, in young human adults, this area seems to be higher in men than in women, while in neonates and childhood seem to have similar density area [47]. Contrary, the activation in kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) and the preoptic periventricular nucleus (PeN), was 10 fold higher in females rats than in males during puberty [11]. We did not find sexual dimorphism, in the hypothalamus weight at 21 days old, however, the LP females presented an increase in the hypothalamus relative weight, similar to what was found in the ovaries weight at the same age. The hypothalamus plays a key role in the regulation of metabolism and BW [12]. While the animals did not differ between groups, the females presented a hypothalamus heavier than males, representing a possible major regulation in the physiological function than males.

Conclusion

The lactational period is an important phase of development. However, the effects of environmental stressors during this period remain little known, as well as how this period can contribute to reproductive development. In the current study, we showed that the maternal low-protein diet cause milk composition as an adaptive response to avoid damages in the litter. In addition, males and females presented a dimorphic response to alterations caused in the reproductive organs. Interestingly, the high-fat diet did not alter the male hypothalamic expression while females showed an increase in these genes, demonstrating an unlike response to different stressors at different times in life. In contrast, females showed to be less susceptible to damage in early life than males, demonstrating plasticity to prevent damages when exposed to a second insult.

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Author Contributions

G.D.G., R.R. and P.C.F.M. and were responsible for the conception, design of the experiments and in drafting the article. H.R.V. , K.V.P., L.P.J.S., A.R.O.F., M.D.C., S.P., L.F.B. and K.P.R. were responsible for the collection, experimental procedures and analysis and interpretation of the data. All authors were involved in drafting the article and critically revising it for intellectual content.

Conflicts of interest: All authors approved the final version of the manuscript submitted for publication and declare no competing financial interests.

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819 Gonzalez G., Guzman C., Larrea F. and Nathanielsz P. Sex differences in
820 transgenerational alterations of growth and metabolism in progeny (F2) of female
821 offspring (F1) of rats fed a low protein diet during pregnancy and lactation. The Journal
822 of physiology 566(1):225-236, 2005.

823 Table 1S. Composition of the normal and low-protein diet.

	20%		4%	
Ingredients (g)	Normal Protein (NP)	kcal/g	Low-protein (LP)	kcal/g
Casein (88% protein)	233.3	933.2	45.5	182
Soybean oil	48	432	48	432
Fish oil	16	144	16	144
Sucrose	127.2	508.8	200	800
Cornstarch	527.5	2110	642.5	2570
Mineral mix (AIN-93) *	32	0	32	0
Vitamin mix (AIN-93)*	16	0	16	0
Total	1000	4128	1000	4128

824 The dietary component values are presented as g of diet and the energy in Kcal/g. Diet used was previously
825 published by Almeida et al. (2019) and de Oliveira et al. (2011)
826 *The salt and vitamin mixture that was used in the manufactured diet followed the AIN-93 recommendation.

827
828 Table 2S. Composition of the normal and high-fat diet.

	4%		35%	
Ingredients (g)	Normal Fat (NF)	kcal/g	High-fat (HF)	kcal/g
Lard	-	-	312	2808
Soybean oil	40	360	40	360
Casein (88% protein)	200	800	200	800
Sucrose	100	400	100	400
Cornstarch	559.5	2238	247.5	990
Cellulose	50	0	50	0
Mineral mix (AIN-93) *	35	0	35	0
Vitamin mix (AIN-93)*	10	0	10	0
L -cystine	3	12	3	12
Choline bitartarate	2.5	0	2.5	0
Total	1000	3810	1000	5370

829 The dietary component values are presented as g of diet and the energy in Kcal/g. Diet used was previously
830 published by Barella et al. (2012)
831 *The salt and vitamin mixture that was used in the manufactured diet followed the AIN-93 recommendation.

832 Table 3S. Primers used for RT-qPCR analyses and GenBank access number of target genes.

Gene	NCBI Reference Sequence	Primer sequence (5' - 3')
Gnrh1 (Gonadotropin releasing hormone 1)	NM_012767.2	F: AGGAGCTCTGGAACGTCTGAT R: AGCGTCAATGTCACACTCGG
Kiss1 (KiSS-1 metastasis-suppressor)	NM_181692.1	F: GGAGCCACTGGCAAAAATGG R: GCCAGGCATTAACGAGTTCC
Actb (Actin, beta)	NM_031144.3	F: CGCGAGTACAACCTTCTTGC R: CGTCATCCATGGCGAACTGG

833 F, Forward; R, Reverse

834 Table 4S. Biometric parameters from male and female offspring at 7, 14 and 21 days old.

			NP	LP
Male	Height (cm)	7	6.957 ± 0.110	6.302 ± 0.053**
		14	8.565 ± 0.122	7.383 ± 0.134****
		21	10.650 ± 0.174	9.537 ± 0.121****
	Head diameter (cm)	7	1.427 ± 0.029	1.283 ± 0.023**
		14	1.558 ± 0.020	1.404 ± 0.009***
		21	1.551 ± 0.046	1.438 ± 0.018*
	Waist diameter (cm)	7	1.447 ± 0.026	1.309 ± 0.027**
		14	1.558 ± 0.020	1.376 ± 0.025***
		21	1.626 ± 0.052	1.611 ± 0.027
Female	Height (cm)	7	6.797 ± 0.130	6.124 ± 0.036***
		14	8.678 ± 0.142	7.290 ± 0.100****
		21	10.358 ± 0.115	9.430 ± 0.068****
	Head diameter (cm)	7	1.399 ± 0.027	1.259 ± 0.013**
		14	1.549 ± 0.033	1.398 ± 0.011***
		21	1.556 ± 0.023	1.408 ± 0.048**
	Waist diameter (cm)	7	1.444 ± 0.028	1.281 ± 0.029*
		14	1.644 ± 0.034	1.376 ± 0.027****
		21	1.818 ± 0.052	1.601 ± 0.054***

835 Males, n = NP: 9 litters and LP: 9 litters. Females, n = NP: 9 litters and LP: 9 litters. * = $p<0.05$, ** = $p<0.01$, ***
836 = $p<0.001$, and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal protein
837 intake (NP); Low-protein intake (LP).
838

839 Table 1. Milk composition at 7, 14 and 21 days of lactation.

	Days	NP	LP
Total Protein (mg/dL)	7	21.2 ± 1.98	38.44 ± 3.79**
	14	25.4 ± 2.06	44 ± 2.55 ***
	21	42.6 ± 4.75	25.67 ± 1.92 **
Glucose (mg/dL)	7	112 ± 14.63	271 ± 39.51**
	14	114 ± 18.87	226.5 ± 23.57*
	21	220 ± 32.71	126.5 ± 15.46*
Total Cholesterol (mg/dL)	7	146 ± 28.02	356.8 ± 49.24**
	14	125.8 ±14.61	283.6 ± 33.84*
	21	262.9 ± 50.98	110.4 ± 19.10*
Triglycerides (mg/dL)	7	3638 ± 418.4	5061 ± 651.0
	14	4308 ± 613.2	5687 ± 634.1
	21	6356 ± 744.7	4160 ± 744.7

840 n = NP: 5 litters and LP: 4 litters. * = $p<0.05$, ** = $p<0.01$ and *** $p<0.001$.Values are expressed as the mean ±
841 S.E.M. Abbreviations: Normal protein intake (NP); Low-protein intake.
842

Table 2. Body weight, absolute and relative of reproductive organs weight from male and female offspring at 21 days old.

		NP	LP
Male	Body weight (g)	47.47 ± 1.46	34.28 ± 2.04 ***
	Testis weight (g)	0.236 ± 0.01	0.156 ± 0.01 ***
	Relative testis weight (g/100g)	0.497 ± 0.01	0.466 ± 0.04
	Epididymis (g)	0.041 ± 0.001	0.031 ± 0.002 **
	Relative epididymis weight (g/100g)	0.087 ± 0.003	0.094 ± 0.008
Female	Body weight (g)	45.74 ± 2.84	32.08 ± 2.20 **
	Ovaries weight (g)	0.024 ± 0.001	0.023 ± 0.002
	Relative ovaries weight (g/100g)	0.056 ± 0.002	0.070 ± 0.003*
	Uterus weight (g)	0.026 ± 0.002	0.022 ± 0.002
	Relative uterus weight (g/100g)	0.059 ± 0.003	0.064 ± 0.005

Males, n = NP: 7/ 6 litters and LP: 7/9 litters. Females, n = NP: 8/8 litters and LP: 7-8/7-8 litters. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal protein intake (NP); Low-protein intake (LP).

Table 3. Body weight, absolute and relative of reproductive organs weight from male and female offspring at 90 days old

		NP/NF	NP/HF	LP/NF	LP/HF	LP	HF	I
Male	Body weight (g)	367.1 ± 10.23	415.2 ± 7.45$^{\#\#}$	324.2 ± 7.29$^{\Omega\Omega}$	358.1 ± 6.41$^{\Phi aaaa}$	****	****	ns
	Testis weight (g)	1.541 ± 0.035	1.528 ± 0.041	1.289 ± 0.027$^{\Omega\Omega\Omega\Omega}$	1.278 ± 0.023$^{\delta\delta\delta\delta aaaa}$	****	ns	ns
	Relative testis weight (g/100g)	0.430 ± 0.015	0.369 ± 0.013$^{\#\#}$	0.392 ± 0.006	0.358 ± 0.008$^{\delta\delta\delta}$	*	***	ns
	Epididymis (g)	0.505 ± 0.020	0.521 ± 0.014	0.432 ± 0.023$^{\Omega}$	0.448 ± 0.008$^{\alpha}$	***	ns	ns
	Relative epididymis weight (g/100g)	0.140 ± 0.006	0.126 ± 0.004	0.131 ± 0.007	0.125 ± 0.002	ns	*	ns
Female	Body weight (g)	256.6 ± 7.12	285.2 ± 5.42$^{\#}$	228.3 ± 4.87$^{\Omega}$	250.3 ± 6.98$^{\alpha}$	***	**	ns
	Ovaries weight (g)	0.108 ± 0.007	0.102 ± 0.004	0.102 ± 0.004	0.103 ± 0.004	ns	ns	ns
	Relative ovaries weight (g/100g)	0.042 ± 0.002	0.037 ± 0.001	0.045 ± 0.001	0.041 ± 0.001	*	*	ns
	Uterus weight (g)	0.499 ± 0.032	0.481 ± 0.019	0.408 ± 0.019 $^{\Omega}$	0.440 ± 0.029	*	ns	ns
	Relative uterus weight (g/100g)	0.196 ± 0.015	0.173 ± 0.008	0.180 ± 0.011	0.178 ± 0.013	ns	ns	ns

Male n= NP/NF: 9/9 litters; NP/HF: 9/9 litters; LP/NF: 9/9 litters; LP/HF: 10/9 litters. Females n= NP/NF: 9/9 litters; NP/HF: 9/9 litters; LP/NF: 9/9 litters; LP/HF: 9/9. $^{\#}$significant difference between NP/NF and NP/HF, $^{\Omega}$significant difference between NP/NF and LP/NF, $^{\delta}$significant difference between NP/NF and LP/HF, $^{\alpha}$significant difference between NP/HF and LP/HF, $^{\Phi}$significant difference between LP/NF and LP/HF. * = p<0.05, ** = p<0.01, *** = p<0.001, and **** = p<0.0001. Values are expressed as the mean ± S.E.M. Abbreviations: not significant (ns); Normal protein intake (NP); Low-protein intake (LP); Normal fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP); High-fat diet (HF); and Interaction (I).

Figure legends

Figure S1. Dimorphism of absolute and relative hypothalamus weight at 21 and 90 days old of male and female offspring. Hypothalamus weight at 21 days old A. Absolute B. Relative. Hypothalamus weight at 90 days old C. Absolute .D. Relative. Male n= NP/NF: 9/9 litters; NP/HF: 9/9 litters; LP/NF: 9/9 litters; LP/HF: 10/9 litters. Females n= NP/NF: 9/9 litters; NP/HF: 9/9 litters; LP/NF: 9/9 litters; LP/HF: 9/9.* = $p<0.05$, ** = $p<0.01$ and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal protein intake (NP); Low-protein intake (LP); Normal fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP); S (Sex); and Interaction (I).

Figure 1. Evaluation through the lactational period . A. mother weight. B. mother food intake during. C Litter body weight and area under curve (AUC). n = NP: 9 litters and LP:10. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal protein intake (NP); Low-protein intake (LP).

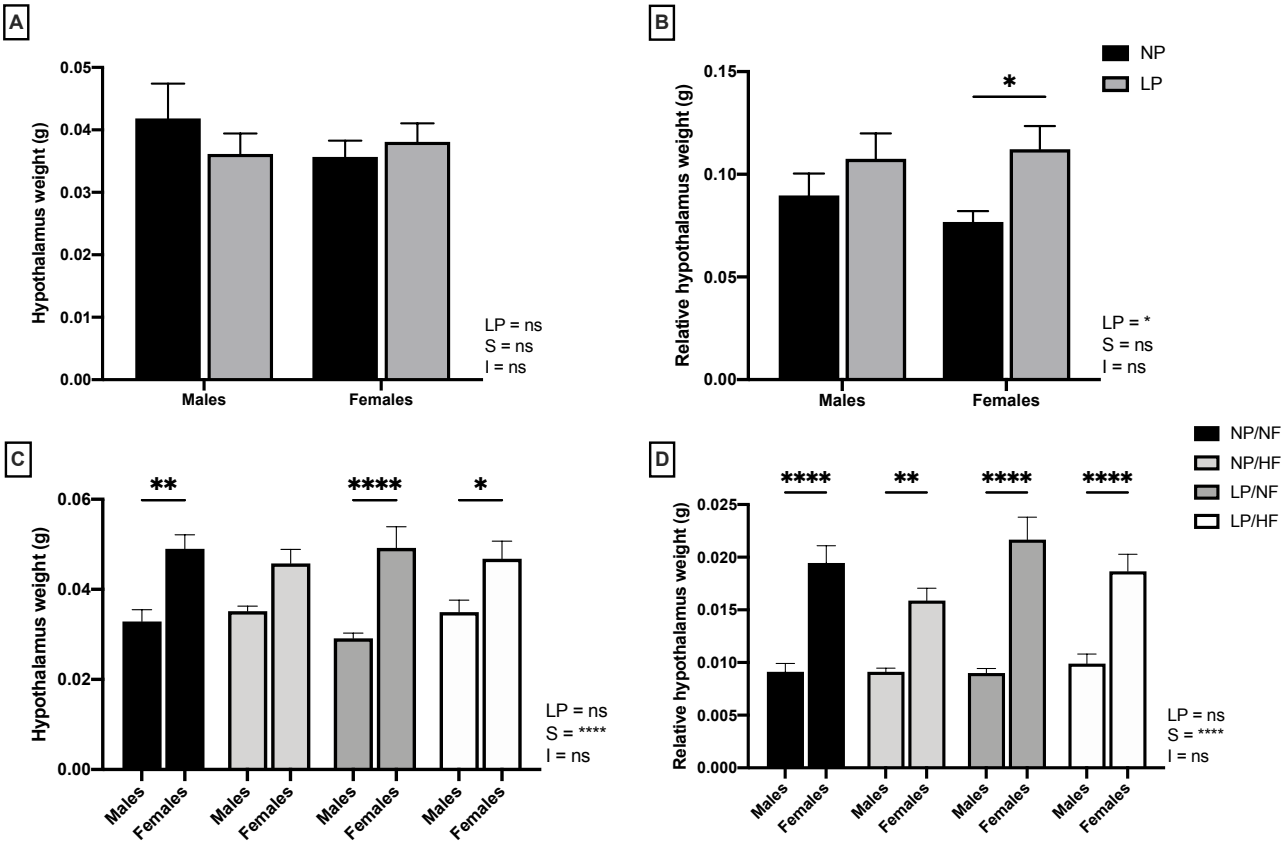
Figure 2. Milk intake and production. A. Average milk intake by pups in the litter. B. Average milk intake by individual pup in the litter per body weight. C. Average milk production by individual mother. n = NP: 8 litters and LP: 8 litters. * = $p<0.05$ and ** = $p<0.01$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal protein intake (NP); Low-protein intake (LP); BW (body weight).

Figure 3. Monitoring puberty onset. A. Preputial separation of male offspring. B. Vaginal opening of female offspring. C. First estrus in female offspring. Body weight on day of: D. Preputial separation, E. Vaginal opening, F. First estrus. Preputial separation n = NP: 30/9 litters and LP: 30/10 litters. Vaginal opening n = NP: 28/9 litters and LP: 30/10 litters. First estrus n = NP: 14/5 litters and LP: 17/6 litters. ** = $p<0.01$) and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal protein intake (NP); Low-protein intake (LP).

Figure 4. *Kiss1* mRNA and *Gnrh1* mRNA relative expression at 21 and 90 days old from male and female offspring. A. *Kiss1* mRNA relative expression of males and females at 21 days old. B. *Gnrh1* mRNA relative expression of males and females at 21 days old. C. *Kiss1* mRNA relative expression of males at 90 days old. D. *Gnrh1* mRNA relative expression of males at 90 days old. E. *Kiss1* mRNA relative expression of females at 90 days old. F. *Gnrh1* mRNA

888 relative expression of females at 90 days old. Male n= NP:6-7/6-7 litters; LP:5-7/5-7 litters;
889 NP/NF: 5/5 litters; NP/HF: 7/7 litters; LP/NF: 5-6/5-6 litters; LP/HF: 6-7/6-7 litters. Females
890 n= NP:7/7 litters; LP: 7/7 litters; NP/NF: 5-7/5-7 litters; NP/HF: 5-6/5-6 litters; LP/NF: 6-7/6-
891 7 litters; LP/HF: 5-7/5-7. ** = $p<0.01$ and **** = $p<0.0001$. Values are expressed as the mean
892 ± S.E.M. Abbreviations: Abbreviations: Post-natal day (PND); Normal protein intake (NP);
893 Low-protein intake (LP); Normal fat intake (NF); High-fat intake (HF). Factors: S (Sex); Low-
894 protein diet (LP); High-fat diet (HF) and Interaction (I).

895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0.02
0.01
0.00
Males
Females
LP = ns
S = ns
I = ns
B
NP
LP
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0.10
0.05
0.00
*
Males
Females
LP = *
S = ns
I = ns
C
Hypothalamus weight (g)
0.06
0.04
0.02
0.00
**
****
*
Males
Females
LP = ns
S = ****
I = ns
D
NP/NF
NP/HF
LP/NF
LP/HF
Relative hypothalamus weight (g)
0.025
0.020
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0.000
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Males
Females
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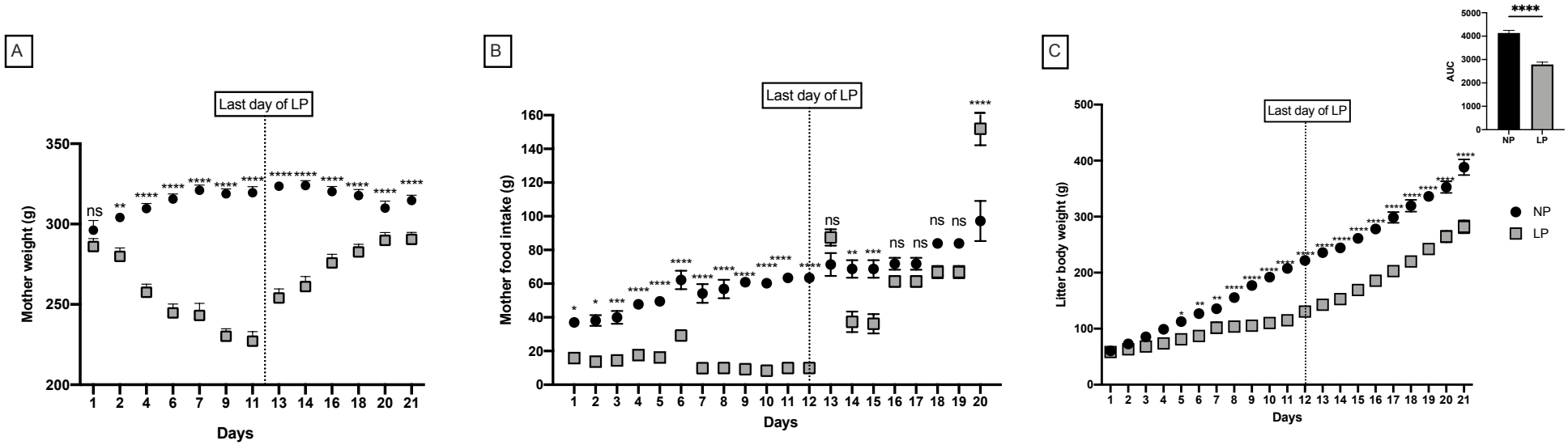
S = ****
I = ns

896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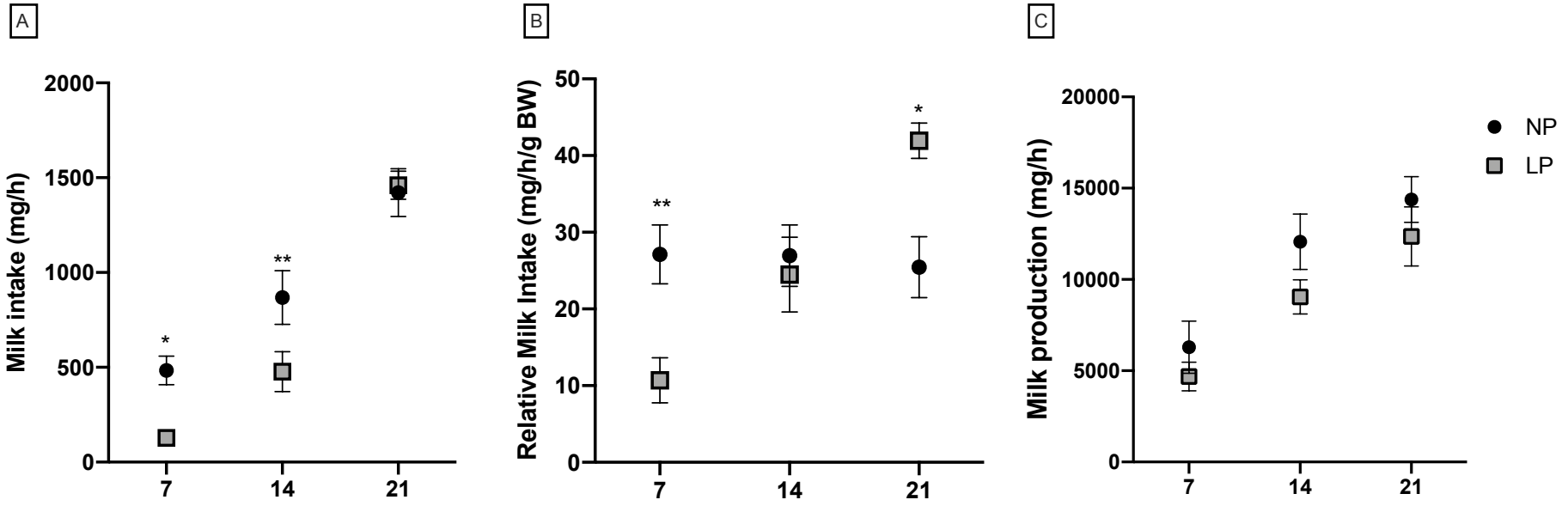
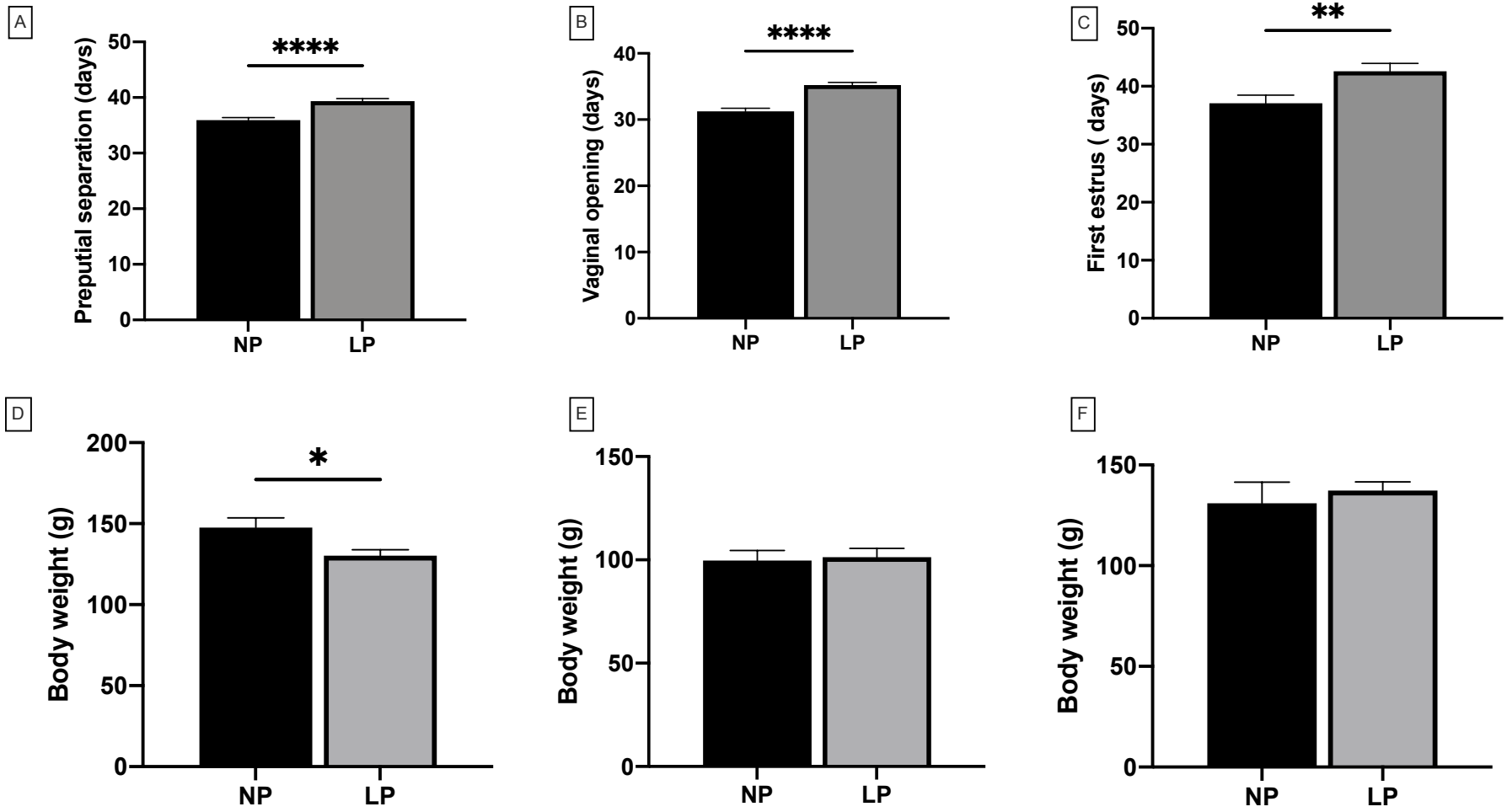
Last day of LP
Mother weight (g)
Days
B
Last day of LP
Mother food intake (g)
Days
C
Last day of LP
Litter body weight (g)
Days
AUC
NP
LP

897


A
B
C
Milk intake (mg/h)
Relative Milk Intake (mg/h/g BW)
Milk production (mg/h)
NP
LP
7
14
21
*
**

898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Preputial separation (days)
50
40
30
20
10
0
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NP
LP
B
Vaginal opening (days)
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30
20
10
0
****
NP
LP
C
First estrus (days)
50
40
30
20
10
0
**
NP
LP
D
Body weight (g)
200
150
100
50
0
*
NP
LP
E
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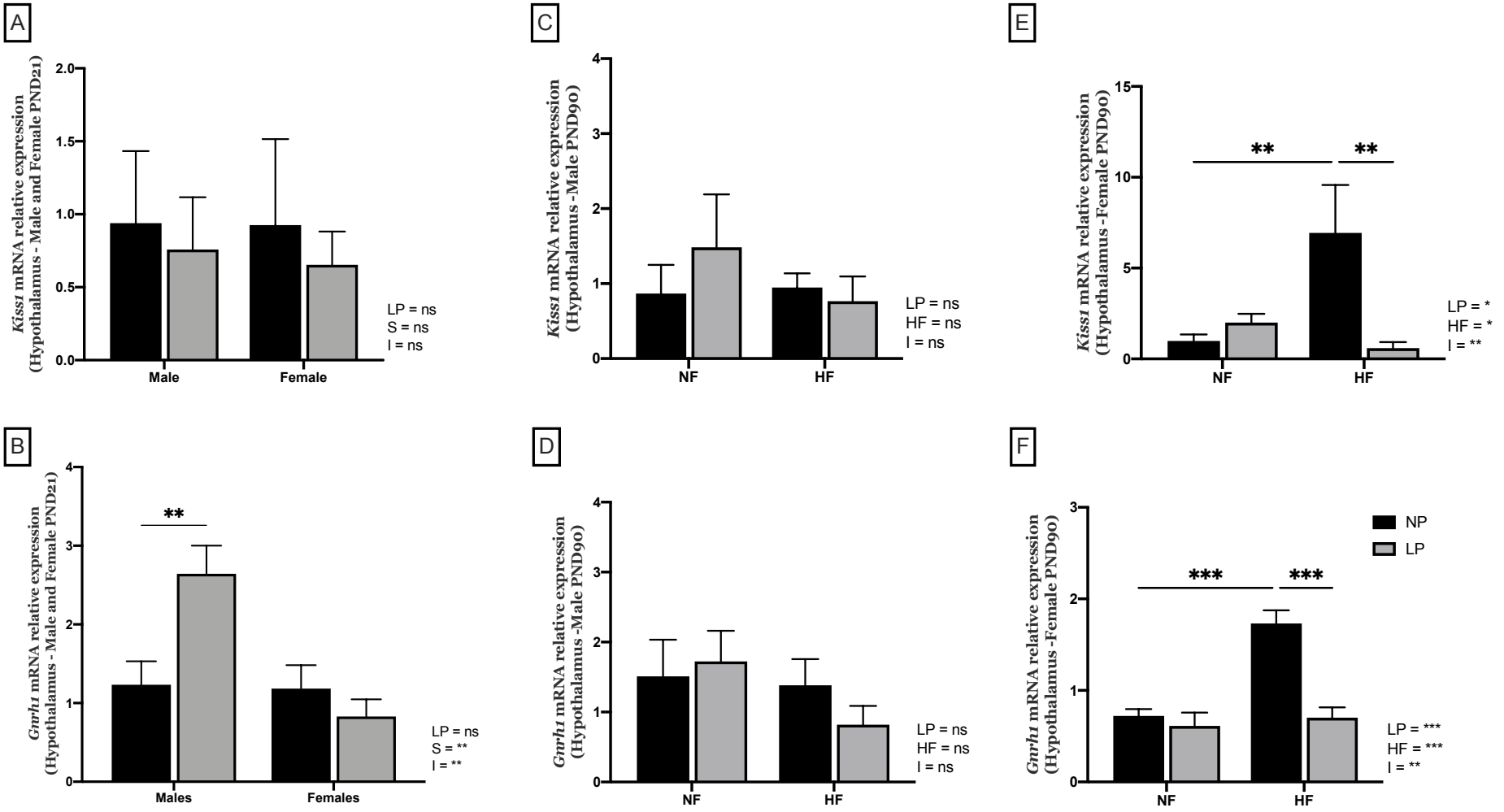
LP

899


A
Kiss1 mRNA relative expression (Hypothalamus - Male and Female PND21)
2.0
1.5
1.0
0.5
0.0
Male
Female
LP = ns
S = ns
I = ns
C
Kiss1 mRNA relative expression (Hypothalamus -Male PND90)
4
3
2
1
0
NF
HF
LP = ns
HF = ns
I = ns
E
Kiss1 mRNA relative expression (Hypothalamus -Female PND90)
15
10
5
0
**
**
NF
HF
LP = *
HF = *
I = **
B
Gnrh1 mRNA relative expression (Hypothalamus - Male and Female PND21)
4
3
2
1
0
**
Males
Females
LP = ns
S = **
I = **
D
Gnrh1 mRNA relative expression (Hypothalamus -Male PND90)
4
3
2
1
0
NF
HF
LP = ns
HF = ns
I = ns
F
Gnrh1 mRNA relative expression (Hypothalamus -Female PND90)
3
2
1
0
***
***
NF
HF
NP
LP
LP = ***
HF = ***
I = **

900

MANUSCRITO 2

Shortly postnatal malnutrition by low-protein diet alters spermatic function in the male offspring when or not overfed to a high-fat diet in adulthood

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Abstract

In 2010 were estimated that around 50 million couples have difficulty getting pregnant. In half of the cases, men showed some fertility deficiency. The causes of infertility in men can include poor nutrition, but some reasons for infertility are still unknown. The change in early life has been associated with metabolic syndrome in adults, but its effects on the reproductive system are little approach in literature. That way, we aimed that a postnatal low-protein during lactation would affects the male reproductive function, exacerbating its effects when exposed to a 'second hit' in adults. For that, the first twelve days of lactation rat mothers were fed with a low-protein (LP; 4% protein) diet or a normal-protein (NP; 20% protein) diet throughout lactation. At 60 days the males from both groups were subdivided and fed a high-fat (35% fat) diet or a normal-fat (NF; 4% fat) diet, until 90 days old. The data obtained in this current study demonstrated a susceptibility in the LP groups to develop alterations in the sperm parameters as a reduction in the sperm count and sperm motility. And the intake of HF diet caused an increase in the abnormal sperm, immobile sperm as well as disbalance in oxidative stress parameters in both testis and epididymis. The lactational period seems to be an important phase of predictive adaptive response and the insult caused by LP in this period may induce modulation of reproductive system physiology throughout the animal life.

1. Introduction

The inability of a couple to become pregnant after 12 months of frequent sexual unprotected intercourse is classified as infertility, affecting around 15% of couples worldwide [1]. Besides, 50% of the infertility cases in couples correspond to some alteration in the men, disturbing 1 in 20 men in the population [1, 2]. Being medical history, physical examination, and semen analysis used for the male infertility diagnosis [3]. Males age, health status, lifestyle, and environment are among the factors for the occurrence of male infertility, however, in many cases the cause is unknown [4]. Interesting, a high prevalence of infertility cases in the world regions as South Asia, sub-Saharan Africa, and Eastern Europe where, the diet patterns are nutritionally deficient [4].

With the expansion of the Development origins of health and disease (DOHaD) concept, a range of studies has been showing an association with the incidence of non-communicable chronic disease in adults with insults in early life [5]. Thus, disease programming can be caused by injuries in life phases, such as pre-conception, gestation, and lactational period [6]. Still, gestational nutrition seems to have an important role in the proper offspring development, since a poor nutrition in this period was related to high mortality and sexual maturation disturbs [5]. In the same way, a maternal dietary status during the lactational period can be involved with alteration in the offspring nutrition, mainly by modification in the amount and type of essential proteins present in breastmilk [6].

Metabolic syndrome is caused by a complex of factors that are associated with cardiovascular disease, diabetes, and obesity [7]. The thrifty phenotype hypothesis proposes that the incidence of metabolic syndrome in the population can be associated with poor nutrition in early life and low birth weight. Interestingly, in children exposed to intensive food deprivation in utero during the 'Dutch famine', an elevated weight gain was observed after the food intake restoration [8]. Because the postnatal malnutrition seems to induce anatomical, hormonal, and physiological changes in the individual to allow survival in a "low resource", exposure to high-calorie food in late life is related with obesity risk [8, 9].

Thereby, the protein seems to have an essential role in offspring development and some studies showed the relationship between poor protein nutrition in postnatal life and its relation to the prevalence of metabolic syndrome and changes in the reproductive system in adults [8, 10]. Also, the association between adult obesity and male reproductive system

injuries leading to infertility is highlighted in the literature [11]. However, a little or nothing is known about how the maternal low protein diet during breastfeeding would play a key role in the male reproductive system and how it can be susceptible to a second insult in later life. Thus, in the current study, we hypothesized that protein restriction during lactation would negatively affect male rat reproductive function, which may be associated with alterations in the sperm parameters and the oxidative stress parameters after a high-fat diet intake in adulthood.

2. Materials and methods

2.1 Experimental design

Males and females Wistar rats (85 and 75 days of age, respectively) from the central animal house of State University of Maringa (UEM) were adapted during one week in the animal house of Secretion Biology Laboratory and received water and food ad libitum. After the period of adaptation, the animals were mated in a ratio of two females and one male per cage. Pregnant rats were transferred to individual cages and fed a standard diet (Nuvital®; Curitiba/PR, Brazil). The litter was standardized on the post-natal day (PND) 1 in eight-nine pups per littler and maintaining as close to a 1:1 sex ratio, as possible, being the day of birth considered PND 0. Two experimental groups (n=9/group) were formed, and the dams received during the first 12 days of breastfeeding a normal-protein diet (NP, 20% protein; 4128 kcal/Kg) or a low-protein diet (LP, 4% protein; 4128 kcal/Kg) [11, 12]. After this period, in both groups, the dams were fed a standard diet (3810 kcal/Kg, Nuvital®; Curitiba/PR, Brazil). The litters were separated from the mothers at PND 21 and males were allocated four per cage. From PND 21 to 60, the animals had body weight (BW) measured once a week. During PND 60 to 90, male offspring from NP and LP dams were subdivided and fed at a normal-fat diet (NF, 4% fat; 3810 kcal/Kg) or a high-fat diet (HF; 35% fat; 5370 kcal/Kg) [13] and weighed once a week. The groups were composed by NP/NF, control offspring fed a normal-fat diet (n=15/9 litters), NP/HF, control offspring fed a high-fat diet (n=15/9 litters), LP/NF, low-protein offspring fed a normal-fat diet (n=15/9 litters), and LP/HF, low-protein offspring fed a high-fat diet (n=15/9 litters). All animals throughout the experimental procedures were kept under controlled temperature (22°C ± 2°C) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle) conditions.

2.2 Collection of organs

From 21 days old to 90 days old one or two males per litter/group were anesthetized (between 8:00-12:00 a.m.) with inhalation of isoflurane ® (Cristália, Itapira, São Paulo, Brazil) inside of laminar flow chamber. The anogenital distance and BW was assessed and the animals were decapitated using a sharp guillotine. The blood was collected, centrifuged and serum stored in a freezer -20ºC . The testis and epididymis were weighted (absolute and relative weights) and used by histological or oxidative stress analysis. The vas deferens, seminal vesicle (full and empty), prostate and perigonadal, mesenteric, and retroperitoneal fat were dissected and weighed.

2.3 Biochemical analysis

A colorimetric method using commercial kits (Gold Analisa; Belo Horizonte, MG, Brazil) [12] was used by the total glucose, cholesterol, and protein content in serum samples, according to the manufacturer's recommendations. All data were expressed in mg/dL.

2.4 Histological processing

The left testes and epididymis (5 per group), in both PND 21 and 90, were removed, fixed in Metacarn (60% methanol, 30% chloroform, and 10% acetic acid) for 6 -8 hours, and kept at 70% ethanol. The samples were embedded in paraffin and sectioned using microtome into semi-serial sections (interval of 50 μm) at 5 μm. The slides with 3 different sections were stained with hematoxylin and eosin (HE) and used for histological analysis using a photomicroscope at a magnification of 100x and 400x and Fiji-ImageJ software.

2.5 Morphometric, spermatogenic kinetics and stereological analysis

To avoid variables for morphometric analysis, 10 random seminiferous tubules, per animal, with the presence of lumen (for 21 days old animals) or in stage IX of the seminiferous epithelium cycle (for 90 days old animals), were used to measurement of seminiferous tubular diameter (2 different regions per tubule) and seminiferous epithelium height (4 different regions per tubule). Together, one-hundred random seminiferous tubular sections per rat were classified into one of the four categories of the seminiferous epithelium cycles (stages I–VI, VII–VIII, IX–XIII, and XIV), under a light microscope at magnifications of 100× and 400× [13].

In the stereological analysis, 10 random cross-sections per animal of caput and cauda of the epididymis (both, 21 and 90 days old) were captured at a magnification of 100x. This analysis was performed using Weibel's multipurpose graticule with 168 points to compare

relative proportions among the epididymal components (epithelium, stroma, and lumen) in the experimental groups [14]. For each animal, the mean of values was calculated and used in the statistical analysis.

2.6 Sperm counting

The left testis (decapsulated) and epididymis (sectioned in caput + corpus and cauda) from 90 days old animals, were weighed and homogenized as described previously by Robb et al., 1978 [15], with the adaptations described by Viera et al., 2020 [14]. After dilution of the homogenate, a small sample was transferred to the Neubauer chamber (4 fields per animal) for counting of spermatids heads using a light microscope (Leica Microsystems, Wetzlar, Germany), and the average of 4 fields was used to concentrate of spermatids per testis. To calculate the daily production of sperm (DPS), the concentration of spermatids per testis was divided by 6.03 (number of days in which mature spermatids are present in the seminiferous epithelium). To calculate sperm transit time in days, the sperm concentration in caput or cauda was divided by DPS.

2.7 Sperm morphology and motility

In the right vas deferens, the sperm were removed by internal rinsing with 1.0 mL of saline formol 10% and stored in a refrigerator at 8ºC. Histological slides were prepared and observed in a light microscope at 400× magnification. Two hundred spermatozoa were analyzed per animal. Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached), and tail abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached) [16].

The spermatozoa present in the left vas deferens was removed and kept at 37 ºC in physiological solution. At the same temperature, a Neubauer chamber was filled with 10µL aliquot of the sperm solution. Sperm motility was assessed by visual estimation (100 spermatozoa per animal) under a light microscope at 100x magnification and was performed by the same person (G.D.G.) throughout the study. Spermatozoa were classified as mobile or immobile [16].

2.8 Oxidative stress parameters

2.8.1 Sample preparation

Both right testis, epididymis caput, and epididymis cauda (kept stored at -80 ºC) were used to the obtention of homogenate. For that, the samples were immersed in potassium phosphate buffer (200 mM, pH 6.5) in a 3:1 ratio and homogenized. The homogenate was divided, one part was used to evaluate reduced glutathione (GSH) levels, and the other half was centrifuged at 9000 rotations per minute for 20 min. The resulting supernatant was used to detect total proteins, glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and lipid hydroperoxide (LOOH). The protocol used in this current study was been cited by Borges et al., 2018 [17] and da Silva de Souza et al., 2015[18].

2.8.2 Total proteins

BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was used to measure protein levels in the supernatant, according to manufacture instructions. 96-well plates were read using a spectrophotometer at 462 nm.

2.8.3 Reduced glutathione and glutathione s-transferase enzymatic activity

To GSH dosage levels, the homogenate was mixed with 5,5′-dithiobis-2-nitrobenzoic acid. The reaction was read at 412 nm. Individual values were interpolated based on a GSH standard curve and expressed as μg of GSH/g of tissue. To determine the enzymatic activity of GST, the sample was diluted in a solution reaction that contained CDNB (1-chloro-2,4-dinitrobenzene- Sigma-Aldrich, São Paulo, São Paulo, Brazil), GSH, and 0.1 M potassium phosphate buffer (pH 6.5). The formation of a conjugate of glutathione and CDNB was performed in a spectrophotometer at 340 nm. Calculations were performed using the extinction coefficient of 9.6 mmolar 1/cm. The results were expressed as μmol/min/mg of protein.

2.8.4 Superoxide dismutase, catalase, and lipid hydroperoxide levels

The ability of SOD to inhibit the autooxidation of pyrogallol was used as a base for its measurement. The results were obtained at 405 nm using a spectrophotometer and expressed as U of SOD/mg of protein. The addition of H_2O_2 subtract in the centrifuged samples, was used to CAT activity measures. Readings were performed at 240nm of absorbance over 5 min and data expressed as μmol/min/mg protein. LOOH measurement in the samples was performed using a spectrophotometer at 560 nm, according to the reaction of the iron II oxidation assay in the presence of xylenol orange (Sigma-Aldrich, São Paulo, São Paulo, Brazil). LOOH concentration was calculated by an extinction coefficient of 4.3 mmolar 1/cm expressed as mmol/mg tissue.

2.9 Ethical Approval

All experimental animals and experiments were approved by the Ethics Committee on Animal Use (CEUA number: 6328301019) of State University of Maringa (Maringa-PR, Brazil) following the Brazilian College of Animal Experimentation. The female data were not used in this current study.

2.10 Statical Analysis

GraphPad Prism version 9.0 for IOS (GraphPad Software, Inc. San Diego, CA, USA) was used for statistical analysis, and $p<0.05$ was considered statistically significant. All data were subjected to a normality test and expressed as means ± standard errors (S.E.M). According to the groups, was used Student's t-tests (two-tailed) or two-way ANOVA test, followed by multiple comparisons Bonferroni's post hoc analyses.

3. Results

3.1 Postnatal low protein diet affected body weight and organs weight but did not alter the testis and epididymis structure in 21 days old offspring,

As showed in Table1, the LP males presented a BW and anogenital distance reduction of 29% and 12%, respectively compared with NP, maintaining the body reduction until 60 days old (Fig. 1A). Also, absolute testis and epididymis weight, perigonadal fat, and retroperitoneal fat were reduced in the LP group. Otherwise, was not observed differences in the relative testis and epididymis weight, vas deferens, seminal vesicle, and prostate, as well as mesenteric fat. In biochemical parameters, only total cholesterol presented augmented concentration in the LP groups compared with NP, while total protein and total glucose did not differ between the groups.

The seminiferous tubular diameter and seminiferous epithelium height showed a similar measurement between the groups. Together, both epididymal caput and cauda did not show differences in the structural components for lumen, epithelium, and stroma (Table 2).

3.2 The high-fat diet caused alteration in the body and organs weight, as well in the testicular structure in animals malnourished during breastfeeding by a low protein diet

The BW was kept down throughout the experimental period in the LP/NF group (Fig. 1B) when compared with NP/NF ($p<0.05$) (Table 3). At PND 90, a high-fat diet increased the BW from both groups, however, the LP/HF group presented a similar BW to NP/NF, while the NP/HF had the BW increased in relation to NP/NF and LP/HF (both, $p<0.0001$). Concerning reproductive organs, both absolute testis and epididymis were not altered by HF diet, despite both LP group maintained a reduction in the testis and epididymis weight (Table 3). On other hand, the HF diet, in both NP/NF and LP/HF, decreased the relative epididymis weight compared with NP/NF group (Table 3). The prostate, vas deferens, and full seminal vesicle were not altered by HF or LP diet, though LP/HF group presented a reduction in the empty vesicle compared with the NP/NF, NP/HF, and LP/NF (both, $p<0.005$). As expected, the HF diet increased the fat pad in both groups, but the LP/HF showed a less fat gain than NP/HF when compared with NP/NF. Moreover, only LP/HF presented alteration in the biochemical serum analysis, with augment of total cholesterol (LP/NF, $p<0.01$) and total protein (NP/NF and LP/HF, $p<0.01$).

At 90 days old, the seminiferous tubular diameter showed reduced in the LP/HF compared with NP/NF and LP/NF (both, $p<0.05$), otherwise seminiferous epithelium height was similar among the groups, but the factor HF was significant in this parameter (Table 4). Was not observed significant difference in the number of tubes in the spermatogenic stages, despite the stage I-VI reveled a significance in the factor HF ($p<0.05$) (Table 4). Likewise, in the epididymis, the percentage of the components was similar percentage among the groups, in both epididymal caput and cauda (Table 4).

3.3 Altered postnatal in early life and adulthood environment by different diets affect sperm parameters in male 90 days old

The sperm counting in both testis and epididymis was altered by the LP diet (Table 5). The sperm number in testis and the daily production of sperm had a decrease in LP/NF and LP/HF groups compared with NP/NF ($p<0.05$ and $p<0.01$, respectively in both parameters). In the epididymis, sperm number in caput/corpus was diminished in LP/NF in relation to NP/NF ($P<0.05$), while the sperm transit time in caput/corpus had an augment in LP/HF compared with NP/NF and NP/HF (both, $p<0.05$). While in the epididymis cauda, the sperm number was reduced in both LP group, compared with NP/NF (both, $p<0.05$), but sperm transit time in cauda was similar among the groups, despite the I factor presented a significance in this parameter.

The spermatozoa morphology analysis showed effects of HF diet in both NP/HF and LP/HF groups with a decrease in the normal sperm and increase of abnormal sperm in both groups, compared with NP/NF (Fig 2A and B). Alteration in spermatozoa head was increased in NP/HF and LP/HF groups (NP/NF: 13.56 ± 0.82; NP/HF: 24.29 ± 0.94; LP/NF:17.68 ± 1.43; LP/HF: 20.74 ± 0.91) compared with NP/NF ($p<0.0001$ and $p<0.05$, respectively). While alteration in spermatozoa cauda did not differ among the groups (NP/NF: 9.49 ± 1.22; NP/HF: 11.94 ± 1.94; LP/NF:8.97 ± 1.28; LP/HF: 10.12 ± 1.41).

Similar to morphology analysis, both NP/HF and LP/HF groups demonstrated an increase in the percentage of immobile spermatozoa, as well as a decrease in mobile spermatozoa (Figure 2C and D). In addition, the number of immobile sperm in the NP/HF group was 27% higher than NP/NF group, and in LP/HF was 33% and 21 % higher than NP/NF and LP/NF groups, respectively.

3.4 Postnatal low-protein in breastfeeding and adulthood high-fat diets induce a disbalance in the oxidative stress parameters of testis and epididymis at 90 days old

In testis, the LP/NF group had an increase in GST and SOD activity compared with NP/NF ($p<0.05$ and $p<0.001$, respectively) and LP/HF ($p<0.05$ and $p<0.01$, respectively). Otherwise, LP/NF presented a decrease of GSH in relation to NP/NF ($p<0.05$) and total protein in comparison to NP/NF ($p<0.05$) and LP/HF ($p<0.01$). Also, the group NP/HF seems to have an augment of SOD and reduction of total protein when compared with NP/NF and LP/HF in both parameters (Fig 3C and F). Interestingly, LP/HF group showed a similarity in almost all parameters to NP/NF group, except for a decrease found in the GSH compared with NP/NF($p<0.05$) and NP/HF ($p<0.01$). The CAT activity and LOOH were similar among the groups (Fig. 3D and E).

Figure 4, shows the epididymis caput oxidative stress parameters. The isolate LP diet caused a decrease in the activity of GST compared with NP/HF ($p<0.05$) and an increase in the LOOH in relation to NP/NF and LP/HF (both, $p<0.01$). Also, NP/HF group showed a decrease in the SOD with augment of LOOH compared with NP/NF ($p<0.05$, in both parameters). LP/HF group was similar to the NP/NF group, together with the I factor was significant in all parameters analyzed. Alterations in GSH, CAT, and total protein was not observed in this tissue.

The epididymis cauda oxidative stress parameters was altered in GST, SOD, CAT, and total protein. The GST and CAT activity was reduced in NP/HF, LP/NF, and LP/HF groups when compared with NP/NF (Fig. 5A and D). Controversially, SOD showed a reduction while

total protein was augmented in both NP/HF (compared with NP/NF) and LP/NF group (compared with NP/NF and LP/HF), as presented in Figure 5C and D. Similar to epididymis caput the I factor was significative in all parameters analyzed. Together, GSH and LOOH did not differ among the groups (Fig. 5B and E).

4. Discussion

The brain development in rats starts during gestation but ends around the PND 10, also the perinatal life in rats corresponds to be a critical period for sexual physiology and behavior [19, 20]. This way, the lactational period represents a sensitive period to insults for male sexual development. The current work demonstrated that the LP diet affected the sperm number in testis and epididymis and the daily production of spermatozoa. In addition, changes in the oxidative stress parameters, in both testis and epididymis, was observed in the LP/NF group, however the HF intake may induce a disbalance in the 'normal homeostasis' in this group, causing alteration in testis structure, sperm morphology and motility.

Both body and organs weight are an indicator of nutritional status and reproductive potential in animals [21]. At 21 and 90 days old the body, testis, and epididymis weight presented a reduction in LP animals, despite the LP/HF presented a similar weight to the control group, probably due to the increase of fat in these animals. Previously, our lab showed similar results regarding the body weight, demonstrating protection by LP against the obesity caused by HF diet, although impairment in the glucose homeostasis was observed [12]. Interesting, a 5% protein diet in 6 weeks rats for 14 days caused a decrease in the BW by reduction of lean mass, also after a refeeding, they kept a low body weight while the animals fed with 10% protein diet showed an increase of BW with a fat pad stoke [22]. This way, a very low-protein diet seems to increase the energy expenditure and increase of substrate oxidation from fat to carbohydrates in the LP animals [22], which can explain a minor increase of body weight and fat stokes by the LP/HF group, as well as the increase of total serum cholesterol and protein.

Absolute and relative testis and epididymis weight can be associated with organ atrophy, changes in the morphological structure, or compensation by the low body weight [21, 23]. At 21- and 90-days old no changes were observed in the testis and epididymis structure caused by the LP diet, however, the intake of high-fat diet in this group caused a decrease in the seminiferous tubular diameter. Similar to our findings, the 8% low protein diet in gestation and/or lactation caused a reduction in the BW and testis weight at PND25 and 70 [10], moreover, changes were not observed in the testis structure at 21-days old [23]. Also, in adult rats who submitted a low protein diet at 5% for 30 days the decrease in the testis, epididymis,

seminal vesicle, and prostate weight were related to alterations in sperm number and morphology by a decrease of testicular protein content [24]. In this current study, the reduction of testis in the LP/NF may be related with the alteration in the sperm counting in the testis, despite the spermatogenic kinetics was not affected. In relation to testicular structure the alteration found in the LP/HF group may be associated with modifications in the sperm morphology, showing a susceptibility to a 'second hit' in the LP group.

The epididymis plays a fundamental role in the maturation of sperm, leading to motility and fertilization capacity, through reactions in the epididymal lumen environment [25]. In adult rats a protein-deficient diet for 75 days caused a decrease in sperm concentration in epididymis cauda, and in the percentage of normal and mobile sperm, these alterations were related to a deficiency of essential amino-acid, as L-arginine and taurine [26]. On other hand, the lactational protein restriction of 8% did not affect the testicular sperm count at 270 days old [10]. Our study showed a reduction in the sperm number of the testis and epididymal cauda, as well as an alteration in the daily production of sperm and caput sperm transit. As the sperm motility acquires occurs during the transit in the epididymis, alteration in the transit time may affect the properly sperm maturation causing the increase of immobile sperm [25], as seen in this study after then HF intake by LP group. In addition, although we have not evaluated the ejaculated sperm, the reduction of the empty seminal vesicle may indicate changes in its morphology [21] and would contribute to the rise of sperm damage in the LP/HF group.

One of the most common factors related to infertility is a disbalance in the oxidative stress homeostasis due to its impact on sperm quality and function in human and rats [27]. The antioxidative enzymes has importance in the health status of the testes, playing a protective role in the correct function in the reproductive organs, since the presence of GST, CAT, and, SOD seems to be in a high amount in the seminal plasma, as a way to avoid sperm damages and kept the sperm normal function [28]. In old age, in offspring fed by obese mothers, the augment of oxidative stress in the testes tissue was related to sperm DNA damages and apoptosis together with altered sperm function [29]. Interestingly, an organ-specific response to the balance between antioxidants and oxidants is observed during the organs development [30], as observed different responses by testis and epididymis in this study.

The testes also present elevated protection by antioxidants, as SOD, GST, and glutathione peroxidase (GPx), once it is an organ that demands a high cellular metabolism and has an augmented presence of unsaturated fatty acids [27]. Here, we observed an augment of GST and SOD in the testes caused by LP diet during lactation, otherwise, it was not observed when these animals received HF diet. As known, malnutrition in early life can cause adaptation

in the individual to allow your postnatal life survival and in this case, keeping the transmission of your genetic through the next generations [31]. Zambrano et al., [10] did not observe changes in the fertility rate of 70 and 90 days-old male offspring fed by dams submitted to 8% of protein throughout lactation. The increase of GST and SOD found in the testicular tissue can be an adaptative response to lactational environments in the LP group. In contrast, the HF intake by LP group decreased the antioxidative enzymes causing a disbalance in the 'normal homeostasis', despite the LP/HF group showed similarities with the NP/NF. Thus, the disbalance of endogen antioxidants enzymes in the LP/HF in comparison to LP/NF may explain the alteration in sperm morphology found in this group.

As previously mentioned, the epididymis has a role in sperm maturation leading to its capacitation. Similar to testes, oxidative stress affects epididymal function, this way SOD and glutathione are the most related to protect the action of oxidants in the epididymis, also it is correlated to sperm maturation by the protection of sperm DNA during compaction and sperm cauda storage, as well as sperm motility through the regulation of tyrosine phosphorylation events [32]. Also, recently was observed different testicular and epididymal responses to the same insult, with small or no alteration in the oxidative stress status in the testis while epididymis presented an imbalanced rate of antioxidants and oxidants [33]. Likewise, we observed a major susceptibility to oxidative damages in epididymis than in testes for both the postnatal LP diet and adult HF diet, showed by an increase of lipid peroxidation in epididymis caput, and decrease of antioxidants enzymes on epididymis cauda. Being, these alterations associated with disorders in the sperm motility found in the NP/HF and LP/HF groups.

5. Conclusion

In conclusion the postnatal undernutrition by a low-protein diet can interfere in the spermatic paraments reducing or difficulty the male reproduction in later life. Besides, the LP showed to be more susceptible in some cases for the second insult by high-fat diet in those animals, mainly by a disbalance in the oxidative stress parameters leading to sperm alteration observed. In addition, the breastfeeding period represents an important phase of plasticity and the insult caused by LP in this period could induce physiological programming of the reproductive system throughout the animal life, of which this programming was not able to protect against a 'second insult' did not expect by the organism.

Author Contributions: G.D.G and P.C.F.M. were responsible for the conception, design of the experiments, and drafting of the article. N.C.B, G.S.A.F, and P.C.F.M. had revised it critically for important intellectual content. G.D.G, L.P.J.S., C.Q.N., A.R.O.F., K.V.P, H.R.V, S.P., P.L.Z., L.F.B., and I.P.M. were responsible for the collection, experimental procedures and analysis and interpretation of the data.

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Table 1. Outcomes in 21 days old male offspring caused by maternal low-protein diet during breastfeeding

	NP	**LP**
Body weight (g)	48.05 ± 1.08	34.19 ± 1.43****
Anogenital distance (cm)	10.68 ± 0.21	9.33 ± 0.16***
Testis weight (g)	0.024 ± 0.008	0.016 ± 0.011****
Relative testis weight (g/100g)	0.50 ± 0.01	0.49 ± 0.03
Epididymis (g)	0.043 ± 0.001	0.033 ± 0.001**
Relative epididymis weight (g/100g)	0.089 ± 0.003	0.099 ± 0.006
Vas deferens (g)	0.020 ± 0.0008	0.019 ± 0.001
Seminal vesicle (g)	0.017 ± 0.001	0.015 ± 0.001
Prostate(g)	0.047 ± 0.003	0.041 ± 0.003
Perigonadal Fat (g)	0.094 ± 0.010	0.056 ± 0.006**
Mesenteric Fat (g)	0.154 ± 0.011	0.122 ± 0.010
Retroperitoneal Fat (g)	0.110 ± 0.006	0.067 ± 0.009**
Total Glucose (mg/dL)	240.0 ± 19.23	212.0 ± 22.57
Total Cholesterol (mg/dL)	103.1 ± 6.87	142.8 ± 14.53*
Total Proteina (mg/dL)	7.03 ± 0.40	6.48 ± 0.27

n = NP: 6/6 litters and LP: 7/7 litters. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP).

Table 2. Morphometric and stereological analysis in 21 days old male offspring malnourished during lactation

	NP	LP
Testicular morphometric (µm)		
Seminiferous tubular diameter	120.5 ± 2.18	120.6 ± 5.86
Seminiferous epithelium height	47.72 ± 0.85	48.66 ± 2.24
Epididymis steriology (%)		
Caput lumen	7.78 ± 0.61	9.38 ± 0.84
Caput epithelium	26.4 ± 1.47	26.04 ± 0.98
Caput stroma	65.52 ± 1.58	67.2 ± 2.85
Cauda lumen	8.69 ± 0.31	8.46 ± 0.46
Cauda epithelium	26.86 ± 1.37	24.34 ± 2.44
Cauda stroma	64.34 ± 1.19	67.19 ± 2.86

n = NP: 5/5 litters and LP: 5/5 litters. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP).

1424 Table 3. Consequences of a high-fat diet in adult male offspring malnourished by low-protein diet in lactational period

	NP/NF	NP/HF	LP/NF	LP/HF	LP	HF	I
Body weight (g)	363.86 ± 7.88	416.0 ± 8.88$^{\#\#\#\#}$	330.2 ± 7.30$^{\Omega}$	356.5 ± 6.83$^{\alpha\alpha\alpha\alpha}$	****	****	ns
Ano genital distance (cm)	22.11 ± 0.19	23.33 ± 0.20$^{\#\#\#}$	22.25 ± 0.17	22.35 ± 0.233$^{\alpha}$	ns	**	**
Testis weight (g)	1.52 ± 0.03	1.53 ± 0.03	1.29 ± 0.02$^{\Omega\Omega\Omega\Omega}$	1.28 ± 0.01$^{\delta\delta\delta\delta\alpha\alpha\alpha\alpha}$	****	ns	ns
Relative testis weight (g/100g)	0.39 ± 0.03	0.37 ± 0.01	0.39 ± 0.01	0.36 ± 0.01	ns	ns	ns
Epididymis (g)	0.51 ± 0.01	0.51 ± 0.01	0.45 ± 0.01$^{\Omega\Omega\Omega\Omega}$	0.45 ± 0.01$^{\delta\delta\delta\delta\alpha\alpha\alpha\alpha}$	****	ns	ns
Relative epididymis weight (g/100g)	0.14 ± 0.003	0.12 ± 0.003$^{\#\#\#}$	0.13 ± 0.003	0.12 ± 0.002$^{\delta\delta}$	ns	****	ns
Vas deferens (g)	0.09 ± 0.003	0.09 ± 0.005	0.08 ± 0.002	0.08 ± 0.004	ns	ns	ns
Full seminal vesicle (g)	1.01 ± 0.03	1.02 ± 0.08	0.90 ± 0.05	0.92 ± 0.04	ns	ns	ns
Empty seminal vesicle (g)	0.52 ± 0.02	0.52 ± 0.02	0.52 ± 0.02	0.43 ± 0.01$^{\delta\alpha\Phi}$	*	ns	*
Prostate(g)	0.73 ± 0.02	0.77 ± 0.03	0.63 ± 0.03	0.70 ± 0.02	**	ns	ns
Perigonadal Fat (g)	2.87 ± 0.16	6.47 ± 0.41$^{\#\#\#\#}$	2.40 ± 0.16	4.64 ± 0.55$^{\delta\delta\alpha\alpha\Phi\Phi\Phi}$	**`	****	ns
Mesenteric Fat (g)	2.02 ± 0.19	4.29 ± 0.31$^{\#\#\#\#}$	1.52 ± 0.09	3.42 ± 0.40$^{\delta\delta\Phi\Phi\Phi\Phi}$	*	****	ns
Retroperitoneal Fat (g)	3.69 ± 0.26	9.29 ± 0.44$^{\#\#\#\#}$	3.09 ± 0.18	6.23 ± 0.48$^{\delta\delta\delta\delta\alpha\alpha\alpha\alpha\Phi\Phi\Phi\Phi}$	****	****	**
Total Glucose (mg/dL)	207.2 ± 23.29	213.4 ± 21.16	183.5 ± 11.04	231.2 ± 20.63	ns	ns	ns
Total Cholesterol (mg/dL)	64.13 ± 5.45	64.80 ± 7.10	53.90 ± 3.42	86.92 ± 6.91$^{\Phi\Phi}$	ns	**	**
Total Proteina (mg/dL)	7.83 ± 0.22	8.32 ± 0.39	7.86 ± 0.14	9.94 ± 0.67$^{\delta\delta\Phi\Phi}$	ns	**	ns

1425 n= NP/NF: 15/9 litters; NP/HF: 15/9 litters; LP/NF: 15/9 litters; LP/HF: 15/9. $^{\#}$significant difference between NP/NF and NP/HF, $^{\Omega}$ significant difference between NP/NF and
1426 LP/NF, $^{\delta}$significant difference between NP/NF and LP/HF, $^{\alpha}$significant difference between NP/HF and LP/HF, $^{\Phi}$significant difference between LP/NF and LP/HF. * = p<0.05,
1427 ** = p<0.01 and **** = p<0.0001. Values are expressed as the mean ± S.E.M. Abbreviations: Not significant (ns), Normal-protein intake (NP); Low-protein intake (LP);
1428 Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).

Table 4. Influence on the testicular and epididymal structure by high-fat diet in adult male offspring malnourished by maternal low-protein diet during breastfeeding

	NP/NF	NP/HF	LP/NF	LP/HF	LP	HF	I
Testicular morphometric (µm)							
Seminiferous tubular diameter	284.4 ± 7.40	273.8 ± 5.22	282.2 ± 6.57	253.3 ± 7.53$^{\delta\Phi}$	ns	**	ns
Seminiferous epithelium height	86.51 ± 3.01	83.29 ± 1.24	87.65 ± 2.56	78.03 ± 2.25	ns	*	ns
Spermatogenesis kinetics (Absolute number)							
I-VI	18.2 ± 1.06	22.4 ± 1.12	16.4 ± 2.20	19.8 ± 1.93	ns	*	ns
VII-VIII	44.0 ± 2.12	45.2 ± 4.44	45.0 ± 2.96	45.4 ± 4.91	ns	ns	ns
IX-XIII	33.4 ± 1.88	27.8 ± 2.72	33.6 ± 2.78	29.4 ± 2.78	ns	ns	ns
XIV	4.40 ± 1.20	4.80 ± 1.24	4.80 ± 0.58	5.40 ± 1.12	ns	ns	ns
Epididymis steriology (%)							
Caput lumen	53.94 ± 1.97	57.84 ± 3.65	56.51 ± 0.71	55.392 ± 1.10	ns	ns	ns
Caput epithelium	22.04 ± 1.29	21.36 ± 0.82	21.28 ± 0.57	21.98 ± 0.78	ns	ns	ns
Caput stroma	24.01 ± 2.93	20.78 ± 3.34	22.22 ± 1.14	22.64 ± 0.81	ns	ns	ns
Cauda lumen	57.11 ± 1.93	58.98 ± 2.86	57.81 ± 2.92	55.64 ± 2.35	ns	ns	ns
Cauda epithelium	19.33 ± 1.84	18.09 ± 2.14	16.60 ± 2.58	16.24 ± 1.78	ns	ns	ns
Cauda stroma	23.56 ± 1.88	22.91 ± 0.93	25.58 ± 4.23	28.14 ± 2.00	ns	ns	ns

n=NP/NF: 15/9 litters; NP/HF: 15/9 litters; LP/NF: 15/9 litters; LP/HF: 15/9. $^{\delta}$significant difference between NP/NF and LP/HF; $^{\Phi}$significant difference between LP/NF and LP/HF. * = $p<0.05$ and ** = $p<0.01$. Values are expressed as the mean ± S.E.M. Abbreviations: Not significant (ns), Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).

Table 5. Sperm counting in male offspring fed by a low-protein mother diet and submitted a high-fat diet in adulthood

	NP/NF	NP/HF	LP/NF	LP/HF	LP	HF	I
Testis sperm count							
Sperm number in testis ($\times 10^6$)	211.3 ± 13.03	225.03 ± 17.08	146.50 ± 12.41$^{\Omega}$	129.51 ± 13.50$^{\delta\delta\alpha\alpha\alpha}$	****	ns	ns
Daily production of sperm ($\times 10^6$ /day)	36.64 ± 2.14	36.89 ± 2.80	24.01 ± 2.03$^{\Omega}$	21.23 ± 2.210$^{\delta\delta\alpha\alpha\alpha}$	****	ns	ns
Epididymis sperm count							
Sperm number in caput/corpus epididymal ($\times 10^6$)	166.6 ± 8.65	172.3 ± 9.39	132.4 ± 4.17$^{\Omega}$	149.2 ± 5.93	***	ns	ns
Sperm transit time in caput/copus epididymal ($\times 10^6$ /day)	5.19 ± 0.39	4.45 ± 0.24	5.62 ± 0.39	7.16 ± 0.78$^{\delta\alpha}$	**	ns	ns
Sperm number in cauda epididymal ($\times 10^6$/g)	245.1 ± 8.17	223.8 ± 14.63	186.7 ± 15.24$^{\Omega}$	189.9 ± 13.72$^{\delta}$	**	ns	ns
Sperm transit time in cauda epididymal ($\times 10^6$ /day)	7.73 ± 0.65	6.33 ± 0.57	7.46 ± 0.60	9.53 ± 1.39	ns	ns	*

n=NP/NF: 10/8 litters; NP/HF: 10/8 litters; LP/NF: 10/8 litters; LP/HF: 10/9. $^{\Omega}$ significant difference between NP/NF and LP/NF, $^{\delta}$significant difference between NP/NF and LP/HF, $^{\alpha}$significant difference between NP/HF and LP/HF. * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001. Values are expressed as the mean ± S.E.M. Abbreviations: Not significant (ns), Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).

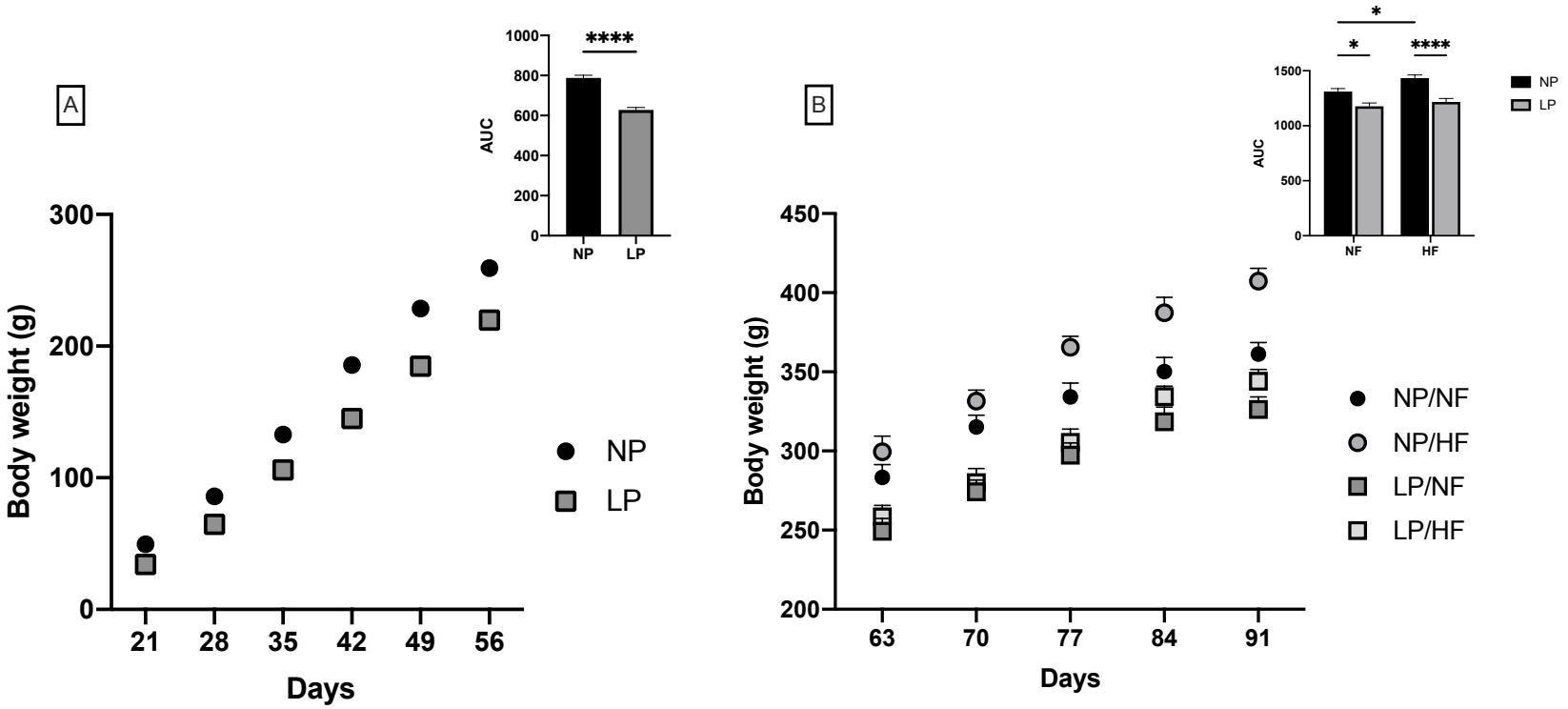
Figure 1. Males body weight monitoring. A. Body weight gain from 21 to 56 days old. B. Body weight gain from 63 to 91 days old. The inset represents the area under the curve (AUC). = NP:30/9 litters; LP: 30/9 litters ; NP/NF: 14/9 litters; NP/HF: 15/9 litters; LP/NF: 15/9 litters; LP/HF: 14/9 . * = $p<0.05$, ** = $p<0.01$, and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF).

Figure 2. Sperm morphology and motility at 90 days old male offspring. A. Percentage of normal sperm. B. Percentage of abnormal sperm. C. Percentage of mobile spermatozoa. D. Percentage of immobile spermatozoa. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).

Figure 3. Oxidative stress parameters in the testis of offspring malnourished by low-protein diet in lactational period and overfed at adulthood. A. Glutathione s-transferase (GST). B. Reduced glutathione (GSH). C. Superoxide dismutase (SOD). D. Catalase activity E. Lipid hydroperoxide (LOOH). F. Protein per mg of tissue. n= NP/NF: 5/5 litters; NP/HF: 5/5 litters; LP/NF: 5/5 litters; LP/HF: 5/5. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).

Figure 4. Oxidative stress parameters in the caput of offspring malnourished by low-protein diet in lactational period and overfed at adulthood. A. Glutathione s-transferase (GST). B. Reduced glutathione (GSH). C. Superoxide dismutase (SOD). D. Catalase activity E. Lipid hydroperoxide (LOOH). F. Protein per mg of tissue. n= NP/NF: 5/5 litters; NP/HF: 5/5 litters; LP/NF: 5/5 litters; LP/HF: 5/5. * = $p<0.05$ and ** = $p<0.01$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).

Figure 5. Oxidative stress parameters in the cauda of offspring malnourished by low-protein diet in lactational period and overfed at adulthood. A. Glutathione s-transferase (GST). B. Reduced glutathione (GSH). C. Superoxide dismutase (SOD). D. Catalase activity E. Lipid hydroperoxide (LOOH). F. Protein per mg of tissue. n= NP/NF: 5/5 litters; NP/HF: 5/5 litters; LP/NF: 5/5 litters; LP/HF: 5/5. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).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Body weight (g)
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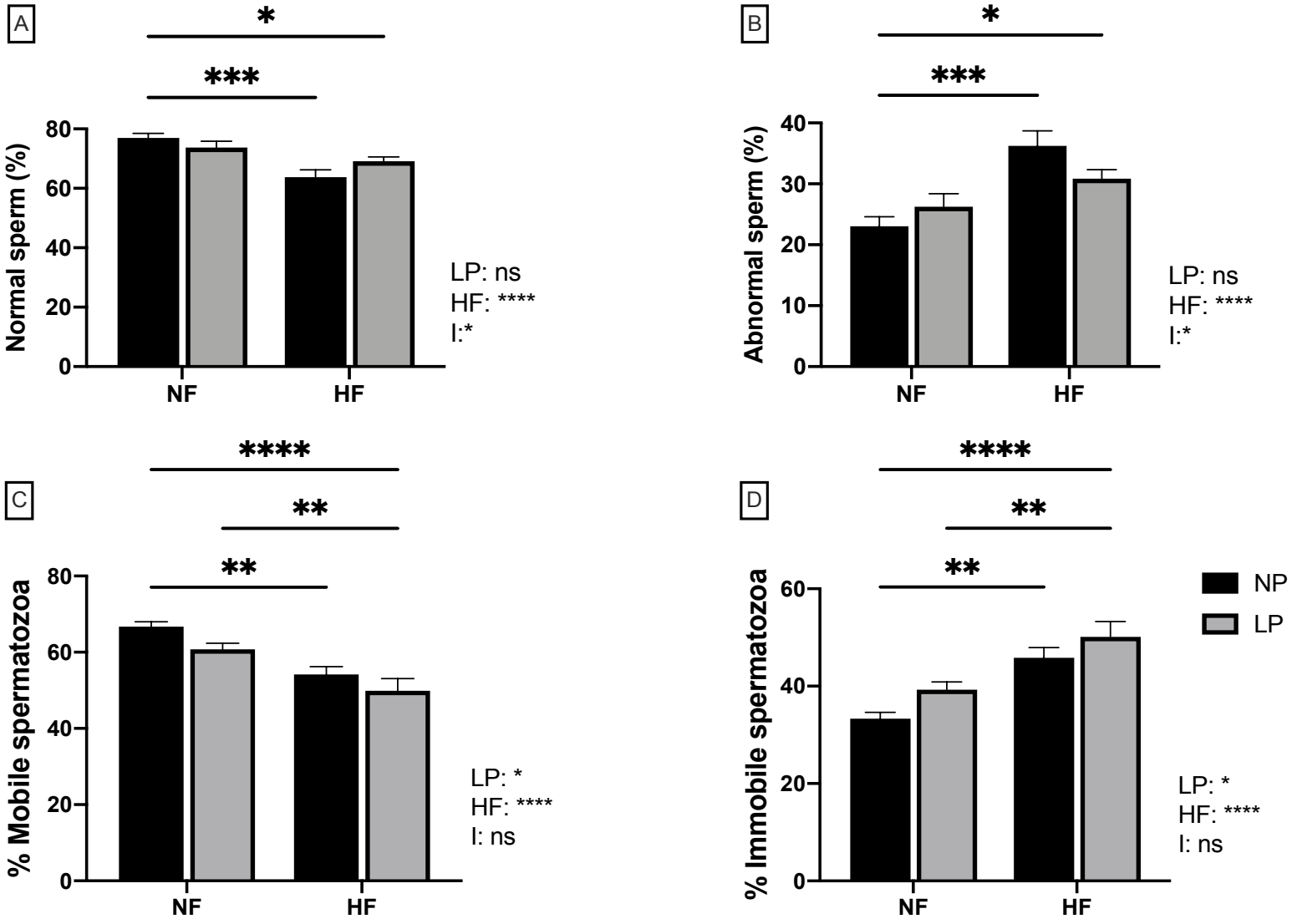
200
100
0
21
28
35
42
49
56
Days
NP
LP
AUC
1000
800
600
400
200
0
****
NP
LP
B
Body weight (g)
450
400
350
300
250
200
63
70
77
84
91
Days
NP/NF
NP/HF
LP/NF
LP/HF
AUC
1500
1000
500
0
*
*
****
NF
HF
NP
LP

1479


A
*
***
Normal sperm (%)
80
60
40
20
0
NF
HF
LP: ns
HF: ****
I:*
B
*
***
Abnormal sperm (%)
40
30
20
10
0
NF
HF
LP: ns
HF: ****
I:*
C
****
**
**
% Mobile spermatozoa
80
60
40
20
0
NF
HF
LP: *
HF: ****
I: ns
D
****
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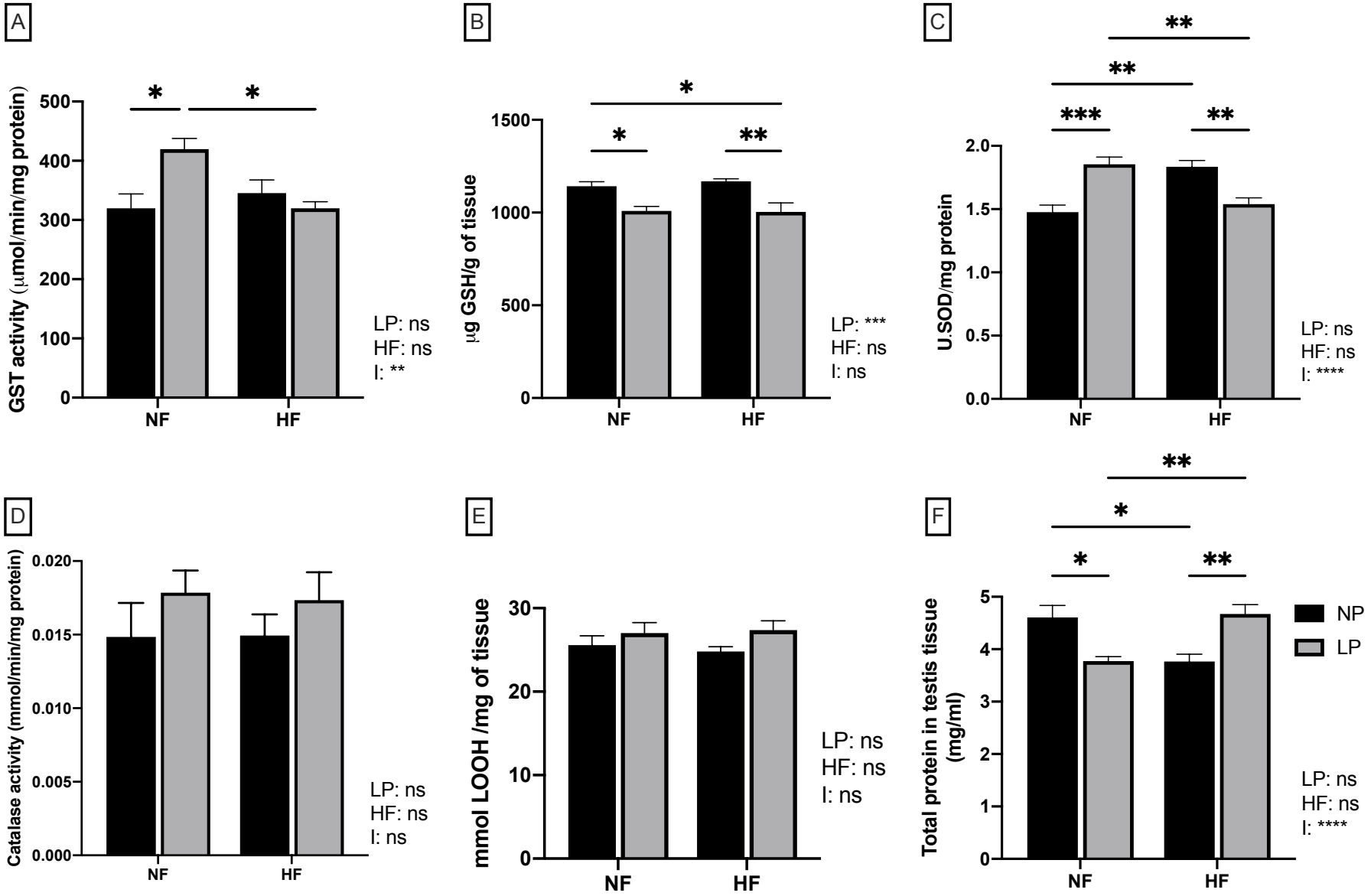
% Immobile spermatozoa
60
40
20
0
NF
HF
NP
LP
LP: *
HF: ****
I: ns

1480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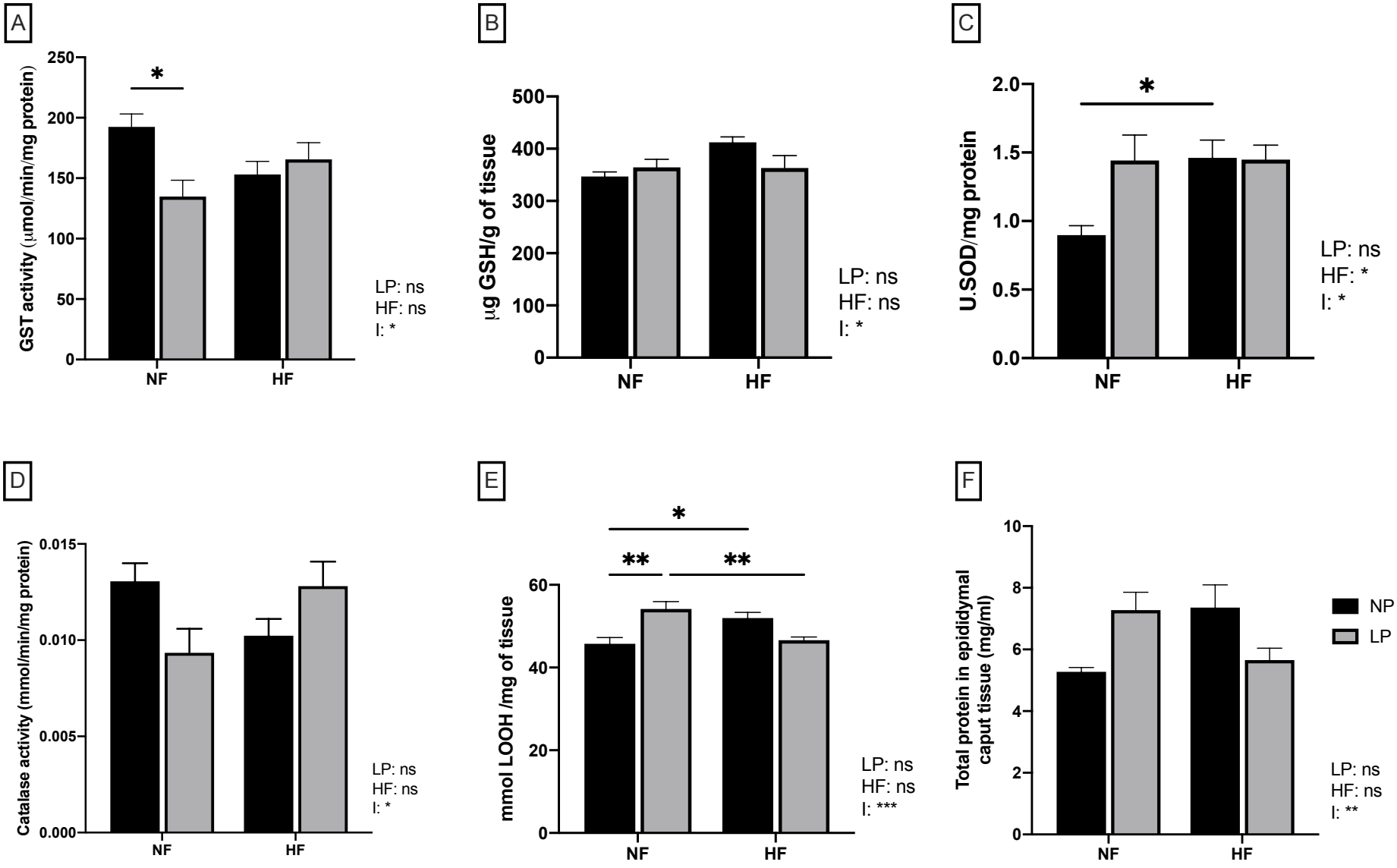
GST activity (µmol/min/mg protein)
500
400
300
200
100
0
*
*
NF
HF
LP: ns
HF: ns
I: **
B
µg GSH/g of tissue
1500
1000
500
0
*
*
**
NF
HF
LP: ***
HF: ns
I: ns
C
U.SOD/mg protein
2.0
1.5
1.0
0.5
0.0
**
**
***
**
NF
HF
LP: ns
HF: ns
I: ****
D
Catalase activity (mmol/min/mg protein)
0.020
0.015
0.010
0.005
0.000
NF
HF
LP: ns
HF: ns
I: ns
E
mmol LOOH /mg of tissue
30
20
10
0
NF
HF
LP: ns
HF: ns
I: ns
F
Total protein in testis tissue (mg/ml)
5
4
3
2
1
0
**
*
*
**
NF
HF
NP
LP
LP: ns
HF: ns
I: ****

1481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GST activity (µmol/min/mg protein)
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200
150
100
50
0
*
NF
HF
LP: ns
HF: ns
I: *
B
µg GSH/g of tissue
500
400
300
200
100
0
NF
HF
LP: ns
HF: ns
I: *
C
U.SOD/mg protein
2.0
1.5
1.0
0.5
0.0
*
NF
HF
LP: ns
HF: *
I: *
D
Catalase activity (mmol/min/mg protein)
0.015
0.010
0.005
0.000
NF
HF
LP: ns
HF: ns
I: *
E
mmol LOOH /mg of tissue
60
40
20
0
*
**
**
NF
HF
LP: ns
HF: ns
I: ***
F
Total protein in epididymal caput tissue (mg/ml)
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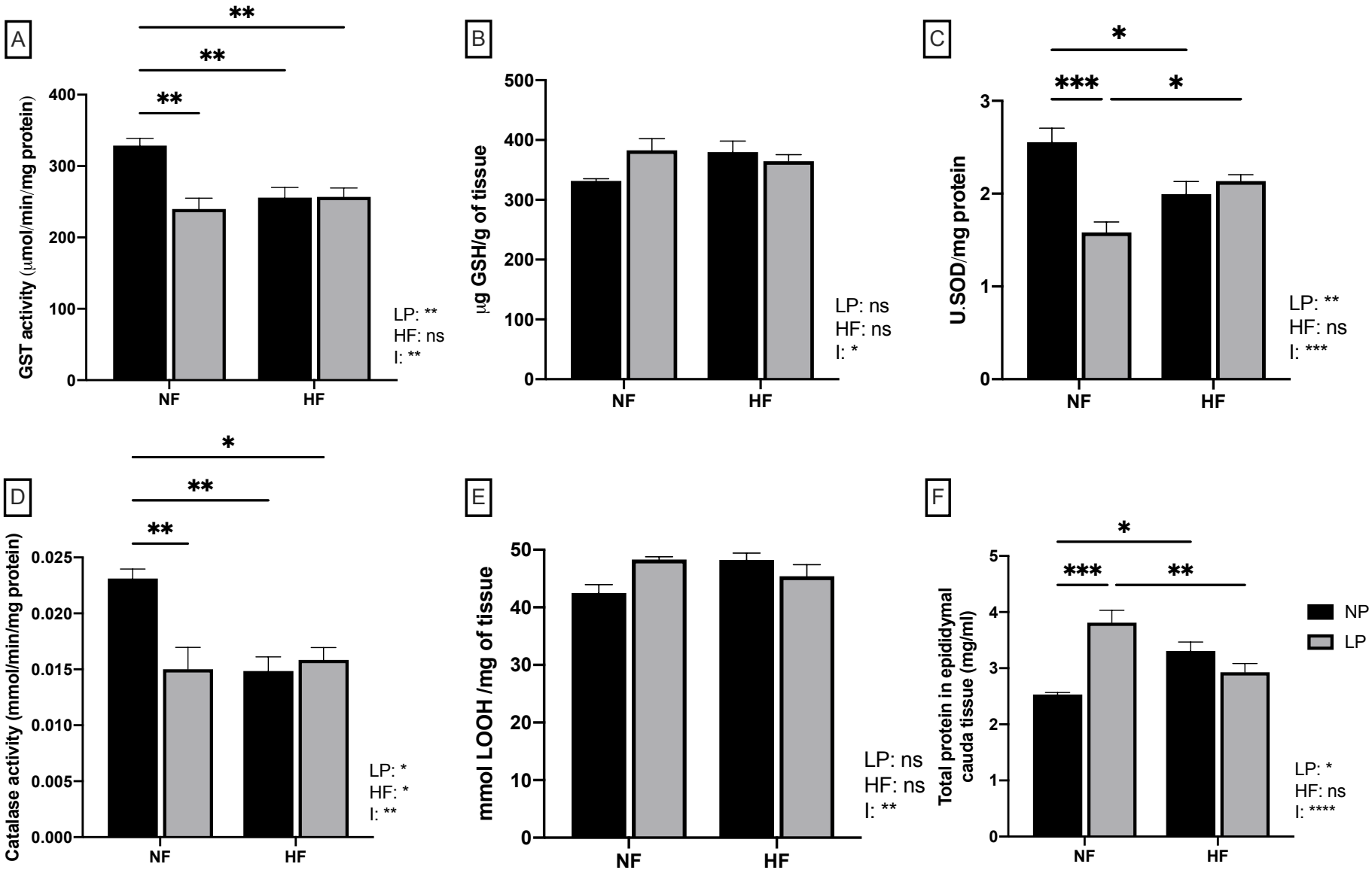
8
6
4
2
0
NF
HF
NP
LP
LP: ns
HF: ns
I: **

1482


A
GST activity (µmol/min/mg protein)
400
300
200
100
0
**
**
**
NF
HF
LP: **
HF: ns
I: **
B
µg GSH/g of tissue
500
400
300
200
100
0
NF
HF
LP: ns
HF: ns
I: *
C
U.SOD/mg protein
3
2
1
0
*
***
*
NF
HF
LP: **
HF: ns
I: ***
D
Catalase activity (mmol/min/mg protein)
0.025
0.020
0.015
0.010
0.005
0.000
*
**
**
NF
HF
LP: *
HF: *
I: **
E
mmol LOOH /mg of tissue
50
40
30
20
10
0
NF
HF
LP: ns
HF: ns
I: **
F
Total protein in epididymal cauda tissue (mg/ml)
5
4
3
2
1
0
*
***
**
NF
HF
LP: *
HF: ns
I: ****
NP
LP

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MANUSCRITO 3

Early exposition to low protein during breastfeeding caused reproductive programming in female offspring exposed to a high-fat diet challenge in adulthood

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Abstract

The infant undernutrition still a global health problem which is linked with a range of non-communicable disease at adulthood. Together, the lactational period is low approached in literature, and its importance for individual plasticity in the later life is little known. We hypothesize that low-protein intake during breastfeeding would affect female reproductive development in adulthood, exacerbating its effects when exposed to high-fat diet insult. For that, lactating rats dams were fed with a low-protein (LP; 4% protein) diet during the first twelve days of lactation or a normal-protein (NP; 20% protein) diet throughout lactation. At post-natal day (PND) 60 a batch of female offspring from both groups was fed a high-fat (35% fat) diet or a normal-fat (NF; 4% fat) diet, until PND 90. The LP diet decreased the body weight throughout the experimental period. Also, in weaned females the number of endometrial glands was reduced, however, it was not observed at PND 90. In adulthood, the LP caused an increase in the number of corpora lutea and oxidative stress parameters in the in ovaries. While the HF induced increase in the number of corpora lutea with a disbalance in oxidative stress parameters in the in both ovary and uteri. Interesting, the LP/HF group presented a reduction of estrus number, due a reduction in the ovary activity, and alterations in the ovary structure compared to LP/NF. Thus, female reproductive development is sensitive to modification during breastfeeding, exhibiting a 'mismatch' when exposed to high-fat intake in adulthood.

Introduction

Disease as obesity, hypertension, and diabetes, are the most common non-communicable diseases in adults, affecting people of reproductive age (Araújo et al., 2019). The first studies associating the gestational undernutrition to cardiac disease in adulthood (Barker, 2007) lead to Development Origins of Health and Disease (DOHaD) theory, which shows an association between insults in early life with an increase of disease in advanced age (Block and El-Osta, 2017). Moreover, periods of organs development due to system plasticity is linked with sensitive periods that can be affected by an environmental factor, as called critical periods (Barker, 2007) such as gestational (de Oliveira et al., 2016), lactational (Martins et al., 2018) and adolescence (de Oliveira et al., 2018) periods, being the latter two the least addressed by studies in this area.

In females the lactational period has a role in the reproductive system, in both nonhuman primates and rats, the ovary follicles and uterus structure continue the maturation shortly after the birth (Laffan et al., 2018). Despite, in human, the primordial follicles are formed until the last week of gestation (Mai and Ann, 2012). On other hand, both humans and rats present brain development continuing after birth (Maggi et al., 2016; Terasawa, 2018). During infant life in humans, there is an increase of pulsatile GnRH release together with an augment of luteinizing hormone (LH) which is associated with the maturation of female reproductive functions (Maggi et al., 2016). Not only factor hormonal can interfere in the female reproductive system, but the oxidant and antioxidant process also presents a regulatory role in the oocyte maturation and folliculogenesis, in which SOD expression is found in all follicular stages, as well as the oxide nitric seems to improve blood flow into the uterus (Agarwal et al., 2005).

Then, the lactational period seems to be a critical period for organism development. In addition, we showed that the undernutrition by a maternal low-protein diet, in the first two weeks of lactation, can cause in male rats decrease in the body weight throughout the life, a disbalance in the glucose and insulin release as well as altered autonomic nervous system (de Oliveira et al., 2011; de Oliveira et al., 2013). This way, breastfeeding is an important phase to correct progenies development and maternal lifestyle is responsible for milk composition (Black et al., 2008). Still, the undernutrition is a global problem health and affect thousands of people worldwide, including breastfeeding mothers. (Black et al., 2008).

The cases of overweight/obesity throughout the world doubled since 1980, affecting more than 1.9 billion people in 2016, of which in women aged over 18 years, 40% presented

overweight and 15% obesity (Popkin et al., 2012; Organization, 2020). Together, the lifestyle of the population changed in the last decades, mostly in developing countries, causing a "nutritional transition". The shift from an activity routine and a healthy diet intake to a sedentary lifestyle, in addiction with a high caloric food intake can lead to a disbalance of energy intake and a cumulative fat pad influencing metabolic activity such as reproduction (Popkin et al., 2012; Urlacher and Kramer, 2018).

The "thrifty phenotype hypothesis" placed by Hales and Barker (1992), demonstrated that metabolic alterations during early life, such as nutritional status, prepare the neonate for a similar condition in adult life, leading to a "matching" metabolism. However, when the individual is exposed to a different environment between early and later life it can cause a "mismatch" and results in potentially negative consequences (Van Eetvelde and Opsomer, 2017). Between 8-12% of the worldwide population presents infertility with a problem to get pregnancy, and sometimes the causes remain unknown, still the women seem to be more vulnerable and affected by this condition (Ozturk et al., 2017). In this current study, we aimed to evaluate the female reproductive outcomes due to the maternal low-protein diet, during the first 12 days of life, and how the female reproductive organs would respond to facing a "mismatch" in the environment by high-fat diet in adulthood.

Materials and methods

Experimental design

Females and males *Wistar* rats (75 and 85 days of age, respectively) were adapted during 7 days in the animal house of Secretion Biology Laboratory at State University of Maringa (UEM) and mated in a ratio of two female and one male per cage. After detected pregnancy, the females were transferred to individual cages and fed a standard diet (Nuvital®; Curitiba/PR, Brazil). The day of birth was considered postnatal day (PND) 0. On the PND 1, the litter was standardized to eight-nine pups per dam and maintaining as close to a 1:1 sex ratio. Also, on PND 1 the dams were divided into two experimental groups (n=9/group) and received, for the first 12 days of lactation, a normal-protein diet (NP, 20% protein; 4128 kcal/Kg), or a low-protein diet (LP, 4% protein; 4128 kcal/Kg). After PND 21, males and female's offspring were weaned, separated, and allocated four per cage. From PND 21 to 60, the animals were fed a standard diet (3810 kcal/Kg, Nuvital®; Curitiba/PR, Brazil). On PND 60, female offspring from NP and LP dams were subdivided and fed at a normal-fat diet (NF,

4% fat; 3810 kcal/Kg) or a high-fat diet (HF; 35% fat; 5370 kcal/Kg) until 90 days of age. Thus, composing four groups: NP/NF, control offspring fed a normal-fat diet (n=14/9 litters); NP/HF, control offspring fed a high-fat diet (n=14/9 litters); LP/NF, low-protein offspring fed a normal-fat diet (n=15/9 litters); and LP/HF, low-protein offspring fed a high-fat diet (n=15/9 litters). The diets used had been published by Almeida et al. (2019) and Barella et al. (2012).

All animal experiments were approved by the Ethics Committee on Animal Use (CEUA number: 6328301019) of State University of Maringa (Maringa-PR, Brazil) under the Brazilian College of Animal Experimentation. Throughout the experimental period, animals received water and food *ad libitum* and were kept under controlled temperature (23°C ± 2°C) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle) conditions. The male data were not used in this current study.

Body weight monitoring and collection of organs

The females from 21 days old to 90 days old were weighed once a week. At PND 21 and 90 (estrus phase) one or two females per litter/group were anesthetized (between 8:00-12:00 a.m.) with inhalation of isoflurane ® (Cristália, Itapira, São Paulo, Brazil), inside of laminar flow chamber, anogenital distance assessed and decapitated. The blood was collected, centrifuged and serum stored in a freezer -20ºC. The ovaries and uterus were weighed (absolute and relative weights) and used by histology analysis or oxidative stress analysis. The fat stoke (ovarian, uterine, mesenteric, and retroperitoneal fat) were weighed.

Biochemical analysis

The total glucose, cholesterol, and protein were measured in serum samples by a colorimetric method using commercial kits (Gold Analisa; Belo Horizonte, MG, Brazil)(Martins et al., 2018). The data were expressed in mg/dL.

Estrous cyclicity

The estrous cyclicity from the four groups of female rats was assessed daily starting on PND 60 until 75. The vaginal fluid collected for 15 days, as described above, was used to assess the estrous cycle phases by cytology: predominance of nucleated epithelial cells (proestrus), a predominance of cornified epithelial cells (estrus), presence of cornified and nucleated epithelial cells, and leukocytes (metestrus), and predominance of leukocytes (diestrus). Data collected over this period were used to calculate the frequency of each phase,

the estrous cycle length, and the number of cycles during the evaluated period (Guerra et al., 2017).

Histology and histological analyses

From both ages, uterus and ovaries structure were evaluated. For this, both organs were fixed in Methacarn solution (60% methanol, 30% chloroform, and 10% acetic acid glacial) for 6 or 12 hours (PND 21 and 90, respectively) and stored at 70% of ethanol. For inclusion, the organs were cut in half, dehydrated in ethanol and xylol series, and embedded in paraffin. The organs were sectioned in 5 μm (three sections per animal, each section was collected at a distance of 50 μm) and stained with hematoxylin and eosin. The images were documented using a photomicroscope with an objective of 10x.

In ovaries the follicles and corpora lutea were counted, using a light microscope, in the 3 sections per animal and group. The follicles and corpora lutea were expressed in the percentage of total follicles observed (Borges et al., 2017). The follicles were classified following: primordial and primary follicles, when present oocytes surrounded by a single layer of either squamous or cuboidal epithelial cells; pre-antral follicles when present two to four layers of granulosa cells with no antral space was considered; antral follicles when present three or more layers of granulosa cells and a defined antral space; atretic follicles when present pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte, and detachment from the basement membrane; and cystic follicles when present a diameter higher than 1.1 cm and consisted of a large antrum and atrophy and degeneration of the granulosa cell (Mendes et al., 2019). The presence of large pale-staining granulosa lutein cells was identified as corpora lutea (Zin et al., 2013).

In the 3 different and spaced sections of the uterus per animal, 5 different regions were analyzed, resulting in a total of 15 measurements per animal for lumen distance and epithelium, endometrium, myometrium, and perimetrium thickness. The Photomicrograph in 100x magnification and Fiji-ImageJ software was used to measurement

Oxidative stress parameters

Sample preparation

To obtain the homogenate, both ovaries and uterus (kept stored at -80 ºC) were homogenized in potassium phosphate buffer (200 mM, pH 6.5) in a 3:1 ratio. As cited by Borges et al. (2018) and da Silva de Souza et al. (2015), a portion of the homogenate was used to evaluate reduced glutathione (GSH) levels, and the remainder was centrifuged at 9000

rotations per minute for 20 min. The resulting supernatant was used to detect total proteins, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and lipid hydroperoxide (LOOH),

Total proteins

BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was used to measure protein levels in the supernatant, according to manufacture instructions. 96-well plates were read using a spectrophotometer at 462 nm.

Reduced glutathione and lipid hydroperoxide levels

To GSH dosage levels, samples were mixed with 5,5′-dithiobis-2-nitrobenzoic acid. The reaction was read at 412 nm. Individual values were interpolated based on a GSH standard curve and expressed as μg of GSH/g of tissue. LOOH measurement in the samples was performed using a spectrophotometer at 560 nm, according to the reaction of the iron II oxidation assay in the presence of xylenol orange (Sigma-Aldrich, São Paulo, São Paulo, Brasil). And, the LOOH concentration was calculated by an extinction coefficient of 4.3 mmolar 1/cm expressed as mmol/mg tissue.

Catalase, superoxide dismutase, and glutathione s-transferase enzymatic activity

The addition of H2O2 subtract in the centrifuged samples, was used to CAT activity measures. Readings were performed at 240nm of absorbance over 5 min and data expressed as μmol/min/mg protein. The ability of SOD to inhibit the autooxidation of pyrogallol was used as a base for its measurement. The results were obtained at 405 nm using a spectrophotometer and expressed as U of SOD/mg of protein. To determine the enzymatic activity of GST, the sample was diluted in a solution reaction that contained CDNB (1-chloro-2,4-dinitrobenzene-Sigma-Aldrich, São Paulo, São Paulo, Brasil), GSH, and 0.1 M potassium phosphate buffer (pH 6.5). The formation of the conjugate of glutathione and CDNB was performed in a spectrophotometer at 340 nm as described by Warholm et al. (1985). Calculations were performed using the extinction coefficient of 9.6 mmolar 1/cm. The results were expressed as μmol/min/mg of protein.

Statical Analysis

The analyses were performed using GraphPad Prism version 9.0 for IOS (GraphPad Software, Inc. San Diego, CA, USA). Data are presented as means with their standard errors

(S.E.M). All data were subjected to a normality test. Statistical analysis was performed using Student's t-tests or two-way ANOVA analysis of variance, followed by multiple comparisons of Bonferroni's post hoc analyses, according to the group number. $P<0.05$ was considered statistically significant.

Results

Effects of maternal LP in 21 days old female offspring

As showed in Table 1, the protein restriction during the suckling period caused a decrease of 27% in the body weight ($p<0.001$), such as a reduction in the anogenital distance ($p<0.001$). In the fat stoke, the ovarian and mesenteric fat was not affected by the maternal LP diet. On other hand, uterine and retroperitoneal fat presented a reduction in its weights ($p<0.05$, in both parameters). Also, the biochemical blood analyses were similar between the groups in serum samples to total glucose, cholesterol, and protein.

Structure of ovaries and uterus in the NP and LP offspring

The absolute ovaries and uterus weight did not present differences between the NP and LP groups. However, the LP relative ovaries were 18% higher than the NP group, while the relative uterus weight was similar. The percentage of follicles number where analogous between the groups, for all parameters analyzed (Table 2). In the same way, epithelium, endometrium, myometrium, and perimetrium layers did not present differences in thickness between NP and LP groups, as well as lumen distance. But the number of glands in the endometrial layers was decreased in the LP group when compared with NP ($p<0.01$), Fig. 1 C and D.

Influence of high-fat diet in adult female offspring malnourished by low-protein diet in the lactational period

During the food intake of HF the estrous cycling was assessed, the LP/HF group showed a diminution of 22% in the estrus day and an augment of 28% of metestrus day compared with NP/NF. The proestrus and diestrus did not show differences between the groups, but there was a significance in the Interaction (I) factor ($p>0.05$) and LP factor ($p<0.05$), respectively. In the same way, the estrous cycle length was similar among the groups, although the I factor presented $p<0.05$. As presented in Table 3.

The LP group maintained reduced body weight throughout the experimental period (Fig. 2A and B). As shown in Fig. 2B and Table 4, the intake of a high-fat diet for 30 days increased the body weight of the NP/HF group compared with NP/NF and LP/HF (10%, $p<0.05$ and 11%, $p<0.001$, respectively). The group LP/HF showed augmented body weight compared with the LP/NF ($p<0.05$), however, was similar to the NP/NF group (Table 4). LP/NF group presented an anogenital distance lower than NP/NF, while LP/HF was similar between the groups. Interesting, LP/NF did not differ in the fat stoke compared with NP/NF, despite the LP factor was significant in all fat paraments analyzed. As expected, the NP/HF group showed an increase of fat stoke compared with NP/NF for ovarian ($p<0.0001$), uterine ($p<0.001$), mesenteric ($p<0.0001$), and retroperitoneal ($p<0.0001$) fat pads. Also, LP/HF had an increase in all fat pads compared with LP/NF and an augment in ovarian, mesenteric, and retroperitoneal fat pads in relation to NP/NF. Surprisingly, the LP/HF fat pads were lower than NP/HF, in the ovarian ($p<0.001$), uterine ($p<0.05$), and retroperitoneal ($p<0.05$) fat pads. The biochemical serum parameters were similar between the groups, although the two-way ANOVA test was significant to the LP factor in total cholesterol.

Alterations of a high-fat diet in the structure of ovaries and uterus in malnourished or not female offspring

Concerning the reproductive system evaluation, the absolute ovaries weight was similar among the groups, also the uterus weight, despite a significant ($p<0.01$) in the LP factor. Though, the NP/HF relative ovaries weight was lower than NP/NF($p<0.05$) - Table 4. The percentage of primordial and primary follicles was decreased in the NP/HF compared with NP/NF ($p<0.05$) (Table 5). On other hand, the number of corpora lutea was increased in the NP/HF and LP/NF, compared with NP/NF ($\approx$ 55%, $p<0.01$, in both groups) and LP/HF (43%, $p<0.05$, in both groups). Pre-antral, antral and atretic follicles did not differ among the groups. In the same way, uterus thickness layers, lumen distance, and the number of glands presented similarity in the groups, besides the I factor was significant in the myometrium ($p<0.05$) and perimetrium ($p<0.01$) layers (Table 5). Figure 3 shows the structure of ovaries and uterus in the groups analyzed.

Oxidative stress parameters in ovaries and uterus of LP female offspring after a second insult by HF diet

In the ovary, the LP diet increased GST ($p<0.05$), SOD ($p<0.01$), and Catalase ($p<0.01$) activity in LP/NF group compared with NP/NF (Fig. 4 A, C, and D). Similarly, the

SOD activity was increased in NP/HF compared with NP/NF. While GSH activity was reduced in both HF groups ($p<0.05$) compared with NP/NF (Fig 4 B), also the HF factor was significant in this parameter. There was no damage to the cellular membrane, as showed by a normal LOOH activity (Fig 4D). The interaction factor (I) was significant in GST, SOD, CAT, and total protein ($p<0.01$, all parameters). The total protein in the ovary presented a reduction in the NP/HF ($p<0.05$) and LP/NF ($p<0.05$) compared with NP/HF.

Figure 5, shows the oxidative stress parameters in uterus. The HF diet caused a decrease in the activity of GST ($p<0.05$) and CAT ($p<0.01$) activity in NP/HF groups, and only CAT in LP/HF groups ($p<0.05$). Both, GST and CAT, presented a significance in the HF factor ($p<0.05$ and $p<0.001$, respectively). Interesting, LOOH showed augmented in LP or/and HF groups (Fig. 5 E). On other hand, GSH, SOD, and total protein in the uterus were similar among the groups, as well as the factors analyzed.

Discussion

During the lactational period, the body development in mammals continues, being the breastfeeding responsible for the delivery of all nutrients required by properly newborn development, representing an important period of life that requires attention (Pillay and Davis, 2020). The current study showed that a maternal protein restriction during breastfeeding induces a reduced body weight throughout the experimental period, an increase in the number of corpora lutea, and altered endogens antioxidants defense in the ovaries. While the high-fat intake in this group seems to cause a disbalance and alteration in the estrus cyclicity.

The food restriction of 50% in mothers caused a reduction in the body weight in female offspring at weaning, however, these animals presented a ‘catch up’ having a similar weight at adulthood (Bernal et al., 2010). Also, the non-adequate intake of protein-energy in the infant is a risk for stunting related to a reduced height by age (Black et al., 2008). We observed a reduction in the body weight and height at the LP females group after weaning, following a reduction in the retroperitoneal and uterine fat. Other studies have shown a diminished adipocyte size and endowment in rat offspring from mothers fed an LP diet linking a continuation of adipogenesis after birth (Claycombe et al., 2013; Lecoutre and Breton, 2015; Martins et al., 2018). The data observed here, can be associated to altered adipogenesis during female LP offspring, however a reduced lean mass in these animals, can be associated with the low body weight.

Interestingly, the increased intake of a high-fat diet after post-natal is highly related to a 'catch up' of individuals and as consequence an increased risk of obesity and altered glucose and leptin metabolism (Claycombe et al., 2013). The cumulative fat in adipocyte tissue is dependent on leptin action, however, female offspring from dams fed at 50% less food than control, presented a decreased leptin at weaning, as well as a decrease in the fat stokes (Léonhardt et al., 2003). In this current study at PND 90, we observed an increase of body weight with fat stokes in the NP/HF group, indeed the LP/HF presented a gain in the body weight and fat stokes less exacerbate, being similar with the control. Previously, we have demonstrated a similar phenotype in males, but presenting insulin sensitivity and hyperinsulinemia (Martins et al., 2018). On other hand, males exposed in the late third trimester of gestation at a 4% maternal protein restriction showed an augmented body weight and fat pads at adulthood and a rapid catch-up after birth (de Oliveira et al., 2016). This way, critical periods seem to have importance in the metabolism response to the same insult. In addition, the amount of protein present in the diets can affect the energy expenditure, being a very low-protein diet (5%) responsible by an increased thermogenesis activity, as an increase of FGF-21 and UCP-1, allowing this animal continues with low weight even when exposed to a HF diet, the same response was not observed in animals fed at 10% of protein diet (Pezeshki et al., 2016).

20 months old females malnourished at 8% of maternal protein diet during gestational and lactation period were not different in the fasting plasm glucose (Fernandez-Twinn et al., 2005). The animals used in this study did not go through fasting before the euthanasia, however, neither total serum glucose nor protein showed differences between the groups in both ages analyzed, despite the total cholesterol showed a reduction at 90 days old in females LP/NF. In males, using a similar experimental protocol, a maternal low-protein diet at 4% during the first 2 weeks of lactation, caused an increased plasma glycemia associated with damage in the pancreatic islets in adults (de Oliveira et al., 2013). In addition, the 10% protein in the gestational diet showed the action of UCP2 and higher lipid oxidation, decreasing the fat stoke in males at 7 months old (Jousse et al., 2014), being similar to our findings of total serum cholesterol decreasing in LP/NF group. Despite, in males the glucose showed altered, it is known that male and females respond differently to the same insults.

Malnutrition was associated with impairment in the human reproductive function affecting both women and men (Bongaarts, 1980). The effects of Dutch famine have been studied in the last decade, due to the high decrease of energy intake getting at less than 700 kilocalories per capita/day which affected the population of all ages (Elias et al., 2005; Painter

et al., 2008). The fertility status in female seems to depend not only on the insult but also the time in the life that it occurs, despite females that are exposed to the intrauterine environment at undernutrition by famine showed increased fertility at adulthood, with a major propensity to have children than females not exposed to famine (Painter et al., 2008). Controversially, women who went through famine after birth presented a declined fertility with low chances of first and second childbirth (Elias et al., 2005).

In women who went through famine during perinatal life showed an association with early menopause probably caused by a diminished ovarian follicle reserve (Yarde et al., 2013). It is important to emphasize that during the firsts days of life the neonate is more susceptible to changes by environment due to proliferating tissue and growth pathways, suffering a "programming" throughout life (Barker et al., 2002; He et al., 2017). The LP intake did not change the estrous cycling in adult females, however, for the females who suffered a 'second insult' by HF intake the frequency of estrus number was reduced representing possible decrease infertility of this animal. Thus, the LP showed some influence in the estrous cyclicity, that could lead to modification in the reproductive organs only in old age.

In the current study, we demonstrated that the low-protein diet can interfere in the number of corpora lutea at adulthood, demonstrated a later effect of lactational undernutrition, since a normal ovary structure was observed in weaned females. However, mother fed at 8% protein throughout pre-conception, gestation, and lactation induced in the 24 weeks old female offspring a decrease in the number of primordial follicles and corpora lutea, without change the ovary volume, these alterations were related with a interferes in the ovulation (Winship et al., 2018). Also, the undernutrition after birth in humans seems to affect the age of fertility time by the anticipation of menopause (Yarde et al., 2013). An increase of corpora lutea in our study by LP diet and HF diet can indicate a possible anticipation of menopause by a decrease of follicle stokes in these animals, probably due to the increase of ovulation in reproductive age. Similarly, 70% of high-fat intake in females adults caused an augment of corpora lutea and a decrease of primordial and primaries follicles accelerating an earlier maturity and declined fertility (Wang et al., 2014). Also, the LP/HF showed to be similar to NP/NF in the amount of follicles number, however the estrus cyclicity showed altered, with diminished number of estrus cycle. This way, the results found in the LP/HF group, regarding ovaries structure, should be by a dysregulation in the estrus cycle.

Similar to our findings, the protein restriction of 10% through the lactational period at 40 days old females presented a decrease in the uterus weight and atrophy of endometrial glands (Brasil et al., 2005). We observed a diminished number of glands in LP females at 21

days old, however, after puberty females maintained a normal structure in the uterus in all groups observed. Guzman et al. (2006) did not observe changes in the uterine weight at 21 days, 70 days, and 20 months old of females born from mother fed at 10% protein in lactation. In the present study, there was no difference in the uterus weight at 21 days old, but at 90 days old the absolute weight was reduced only in LP/NF group, however when corrected by body mass was similar to the groups, this way the low uterus weight was due to the lower body weight of this group.

Oxidative stress plays a role in the reproductive function of females and controls the proper function of folliculogenesis, oocyte maturation, luteolysis, and maintenance of the uterus (Agarwal et al., 2005). A balance between reactive oxygen species (ROS) and antioxidants are required by the correct ovarian and uterine function (Agarwal et al., 2005). We observed a high increase of antioxidant enzymes activity, in ovaries by LP diet in LP/NF, otherwise, the uterus showed an increase of LOOH and decrease of antioxidant enzymes activity caused by HF and LP diets. Interesting ovaries and uterus presented a difference in the oxidative stress parameters, demonstrating a greater ability of ovaries to avoid damages than observed by uterus at 90 days old. SOD, CAT, and GSH activity seem to play an important role in the control of the follicles cycle avoiding an imbalanced action of ROS, being SOD increased after ovulation and the main antioxidant enzyme in the corpus luteum function control (Talukder et al., 2017; Wang et al., 2017).

The increase of antioxidants enzymes in the ovary can be related to the increased of corpora lutea find in this study. Like, the reduction of GSH in the ovary by HFD diet, can affect the proper development of primary and primordial, once the GSH activity is present in growing follicles avoiding the apoptotic process (Wang et al., 2017). The high-fat diet did not affect the oxidative stress in female offspring malnourished by maternal food restriction in 50% at pregnancy or pregnancy and lactation, otherwise, the food restriction at lactation group presented an augment in the ovary oxidative stress (Bernal et al., 2010). Curiously, in the current study LP/HF group did not show alterations in the number of follicles or corpora lutea, and similarities in the Oxidative stress parameters with NP/NF, however, we observed a decrease in the estrus cyclicity, what can be related to a diminished number of corpora lutea compared with LP/NF and NP/HF, such as a SOD activity similar to NP/NF. While in the uterus, an increase of ROS is associated with a presence of endometrioses in females, being SOD responsible by reduce the damages in endometrial cell dysfunction (Scutiero et al., 2017). A high caloric diet in females adults caused a decrease in SOD activity (Sadowska et al., 2019), as we observed in females fed at 35% fat diet. Also, was not observed any alteration in the

uterus structure at 90 days old, however, in women, the risk of endometrial cancer was highly related to intake of fast food in individuals older than 59 years (Zhao et al., 2016), this way the evaluation in an old life of this animal can show other perspectives linked to the increase of oxidative stress in uteri.

Taken together, our results suggest that a maternal low-protein diet can cause insults in ovaries and in reproductive age, and probably induce anticipation of menopause in the offspring. Apparently, the high-fat diet intake in the LP group, did not exacerbate the alterations observed in the ovary structure, however, the reduced number of estrus, could represent a diminished ovary activity, explaining the data found here. As known, the lactational period correspond an important phase of plasticity. The results found in the LP group after the intake of HF, may demonstrate a deregulation in the homeostasis balance, inducing low number of estrus and as consequence a different result when compared to LP/NF group. This way, the lactational period, represents an important phase of development, which occasioned in modifications of female reproductive development, resulting a 'mismatch' when facing a high-fat diet in adulthood.

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2081

2082 Table 1. Effect of a maternal low-protein diet in the lactational period in female offspring at
2083 21 days old.

	NP	**LP**
Body weight (g)	44.17 ± 1.90	32.14 ± 2.19**
Anogenital distance (cm)	10.23 ± 0.13	9.50 ± 0.14**
Ovaries (g)	0.025 ± 0.002	0.022 ± 0.002
Relative Ovaries (g/100g)	0.056 ± 0.003	0.068 ± 0.002*
Uterus (g)	0.025 ± 0.002	0.020 ± 0.002
Relative Uterus (g/100g)	0.057 ± 0.002	0.064 ± 0.004
Ovarian Fat (g)	0.031 ± 0.003	0.025 ± 0.002
Uterine Fat (g)	0.057 ± 0.006	0.037 ± 0.006*
Mesenteric Fat (g)	0.121 ± 0.014	0.116 ± 0.007
Retroperitoneal Fat (g)	0.089 ± 0.010	0.056 ± 0.008*
Total Glucose (mg/dL)	212.2 ± 22.57	222.0 ± 34.88
Total Cholesterol (mg/dL)	112.4 ± 8.01	128.5 ± 9.77
Total Proteina (mg/dL)	6.10 ± 0.17	5.88 ± 0.13

2084 n = NP: 7/7 litters and LP: 7/7 litters. * = $p<0.05$ and ** = $p<0.01$. Values are expressed as the mean ± S.E.M.
2085 Abbreviations: Normal-protein intake (NP); Low-protein intake (LP).

Table 2. Low-protein intake outcomes in follicles stoke, uterus thickness and number of uterus glands at 21 days old females.

	NP	**LP**
Number of Follicules (%)		
Primordial and primary follicles	23.366 ± 3.06	23.77 ± 2.14
Pre-antral follicles	33.264 ± 2.90	33.38 ± 1.03
Antral follicles	31.92 ± 3.30	33.38 ± 1.48
Cystic follicles	11.44 ± 1.85	9.46 ± 1.25
Uterus thickness layers (μm)		
Epithelium	10.04 ± 0.70	11.66 ± 1.06
Endometrium	109.52 ± 17.18	114.00 ± 10.12
Myometrium	38.96 ± 4.55	36.36 ± 3.42
Perimetrium	42.86 ± 5.32	39.56 ± 3.38
Lumen distancie (μm)	35.04 ± 9.32	30.02 ± 5.15
Number of endometrial glands	12.25 ± 1.31	7.34 ± 0.62**

n = NP: 5/5 litters and LP: 5/5 litters. ** = $p<0.01$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP)

2091 Table 3. Estrous cycling from female offspring during 60 to 75 days old.

Estrous cycling	NP/NF	NP/HF	LP/NF	LP/HF	LP	HF	I
Number of females evaluated/litters	12/9	11/9	12/9	12/9			
Estrous cycle length (days)	4.50 ± 0.23	7.33 ± 1.14	7.46 ± 1.06	6.45 ± 0.95	ns	ns	*
Frequency of proestrus (days)	3.50 ± 0.37	2.36 ± 0.20	2.83 ± 0.32	3.58 ± 0.54	ns	ns	*
Frequency of estrus (days)	3.63 ± 0.24	3.40 ± 0.40	2.81 ± 0.32	2.25 ± 0.30$^{\delta\delta}$	**	ns	ns
Frequency of metestrus (days)	3.44 ± 0.33	4.54 ± 0.47	4.54 ± 0.31	4.83 ± 0.27$^{\delta}$	*	*	ns
Frequency of diestrus (days)	3.81 ± 0.26	4.33 ± 0.43	5.00 ± 0.46	4.83 ± 0.38	*	ns	ns

2092 n= NP: ;LP: ; NP/NF: 14/9 litters; NP/HF: 14/9 litters; LP/NF: 15/9 litters; LP/HF: 15/9. $^{\delta}$ significant difference between NP/NF and LP/HF. * = $p<0.05$ and ** = $p<0.01$.
2093 Values are expressed as the mean ± S.E.M. Abbreviations: Not significant (ns); Normal protein intake (NP); Low-protein intake (LP); Normal fat intake (NF); High-fat intake
2094 (HF). Factors: Low-protein diet (LP); High-fat diet (HF); and Interaction (I).

Table 4. Influence of high-fat diet in adult female offspring malnourished by low-protein diet in the lactational period.

	NP/NF	NP/HF	LP/NF	LP/HF	LP	HF	I
Body weight (g)	256.1 ± 5.01	282.9 ± 6.43$^{\#\#}$	230.5 ± 3.89$^{\Omega\Omega}$	250.6 ± 4.83$^{\alpha\alpha\alpha\Phi}$	****	****	ns
Anogenital distance (cm)	19.76 ± 0.17	19.94 ± 0.21	18.79 ± 0.17$^{\Omega\Omega}$	19.39 ± 0.13	****	*	ns
Ovaries (g)	0.109 ± 0.005	0.101 ± 0.003	0.102 ± 0.004	0.102 ± 0.003	ns	ns	ns
Relative Ovaries (g)	0.042 ± 0.001	0.036 ± 0.001$^{\#}$	0.044 ± 0.001	0.040 ± 0.001	ns	**	ns
Uterus (g)	0.506 ± 0.03	0.482 ± 0.02	0.412 ± 0.01$^{\Omega}$	0.438 ± 0.02	**	ns	ns
Relative Uterus (g)	0.199 ± 0.01	0.172 ± 0.007	0.180 ± 0.008	0.164 ± 0.015	ns	ns	ns
Ovarian Fat (g)	1.830 ± 0.11	4.468 ± 0.32$^{\#\#\#\#}$	1.730 ± 0.12	3.286 ± 0.24$^{\delta\delta\delta\delta\alpha\alpha\Phi\Phi\Phi\Phi}$	**	****	*
Uterine Fat (g)	1.980 ± 0.12	3.450 ± 0.38$^{\#\#\#}$	1.530 ± 0.10	2.505 ± 0.17$^{\alpha\Phi}$	**	****	ns
Mesenteric Fat (g)	1.890 ± 0.06	3.760 ± 0.30$^{\#\#\#\#}$	1.720 ± 0.07	3.073 ± 0.18$^{\delta\delta\delta\Phi\Phi\Phi\Phi}$	*	****	ns
Retroperitoneal Fat (g)	2.582 ± 0.08	5.541 ± 0.46$^{\#\#\#\#}$	2.071 ± 0.12	3.944 ± 0.29$^{\delta\delta\alpha\alpha\Phi\Phi\Phi\Phi}$	***	****	ns
Total Glucose (mg/dL)	254.5 ± 30.32	224.8 ± 20.68	226.1 ± 29.70	208.9 ± 10.80	ns	ns	ns
Total Cholesterol (mg/dL)	62.42 ± 3.31	61.68 ± 3.51	45.14 ± 2.82$^{\Omega}$	58.17 ± 5.18	*	ns	ns
Total Proteina (mg/dL)	7.61 ± 0.09	8.10 ± 0.17	7.87 ± 0.21	7.71 ± 0.15	ns	ns	ns

n= NP/NF: 14/9 litters; NP/HF: 14/9 litters; LP/NF: 15/9 litters; LP/HF: 15/9. $^{\#}$significant difference between NP/NF and NP/HF, $^{\Omega}$ significant difference between NP/NF and LP/NF, $^{\delta}$significant difference between NP/NF and LP/HF, $^{\alpha}$significant difference between NP/HF and LP/HF, $^{\Phi}$significant difference between LP/NF and LP/HF. * = p<0.05, ** = p<0.01, *** = p<0.001, and **** = p<0.0001. Values are expressed as the mean ± S.E.M. Abbreviations: Not significant (ns); Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF) and Interaction (I).

Table 5. Effects of a high-fat diet in follicles stoke, uterus thickness and number of uterus glands in female offspring malnourished by low-protein diet in lactational period at 90 days old females

	NP/NF	NP/HF	LP/NF	LP/HF	LP	HF	I
Number of Follicules (%)							
Primordial and primary follicles	38.96 ± 2.65	24.90 ± 1.31$^{\#}$	30.08 ± 3.55	29.18 ± 3.00	ns	*	*
Pre-antral follicles	21.12 ± 0.73	18.00 ± 1.42	25.29 ± 1.86	22.84 ± 2.80	*	ns	ns
Antral follicles	23.27 ± 2.08	28.28 ± 1.51	24.91 ± 3.10	30.31 ± 3.95	ns	ns	ns
Atretic follicles	7.29 ± 2.36	4.73 ± 0.87	4.29 ± 0.21	7.70 ± 1.70	ns	ns	ns
Corpora lutea	9.47 ± 0.93	21.49 ± 1.49$^{\#\#}$	21.56 ± 3.37$^{\Omega\Omega}$	12.17 ± 0.71$^{\alpha\Phi}$	ns	ns	****
Uterus thickness layers (µm)							
Epithelium	33.98 ± 3.08	37.34 ±4.10	37.12 ± 1.31	37.92 ± 1.85	ns	ns	ns
Endometrium	519.4 ± 34.39	570.44 ± 41.67	488.54 ± 25.78	541.37 ± 53.09	ns	ns	ns
Myometrium	187.02 ± 11.21	171.18 ± 15.52	163.46 ± 12.30	222.18 ± 18.57	ns	ns	*
Perimetrium	187.14 ± 12.07	145.82 ± 8.43	159.4 ± 11.00	191.26 ± 15.50	ns	ns	**
Lumen distancie (µm)	38.98 ± 5.60	49.56 ± 5.42	47.04 ± 5.98	64.44 ± 13.20	ns	ns	ns
Number of endometrial glands	33.60 ± 2.57	43.62 ± 5.38	37.17 ± 3.45	31.50 ± 2.34	ns	ns	ns

n= NP/NF: 5/5 litters; NP/HF: 5/5 litters; LP/NF: 5/5 litters; LP/HF: 5/5 .$^{\#}$significant difference between NP/NF and NP/HF, $^{\Omega}$ significant difference between NP/NF and LP/NF, $^{\delta}$significant difference between NP/NF and LP/HF, $^{\alpha}$significant difference between NP/HF and LP/HF, $^{\Phi}$significant difference between LP/NF and LP/HF. * = p<0.05, ** = p<0.01and **** = p<0.0001. Values are expressed as the mean ± S.E.M. Abbreviations: Not significant (ns); Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF) and, Interaction (I).

Figures

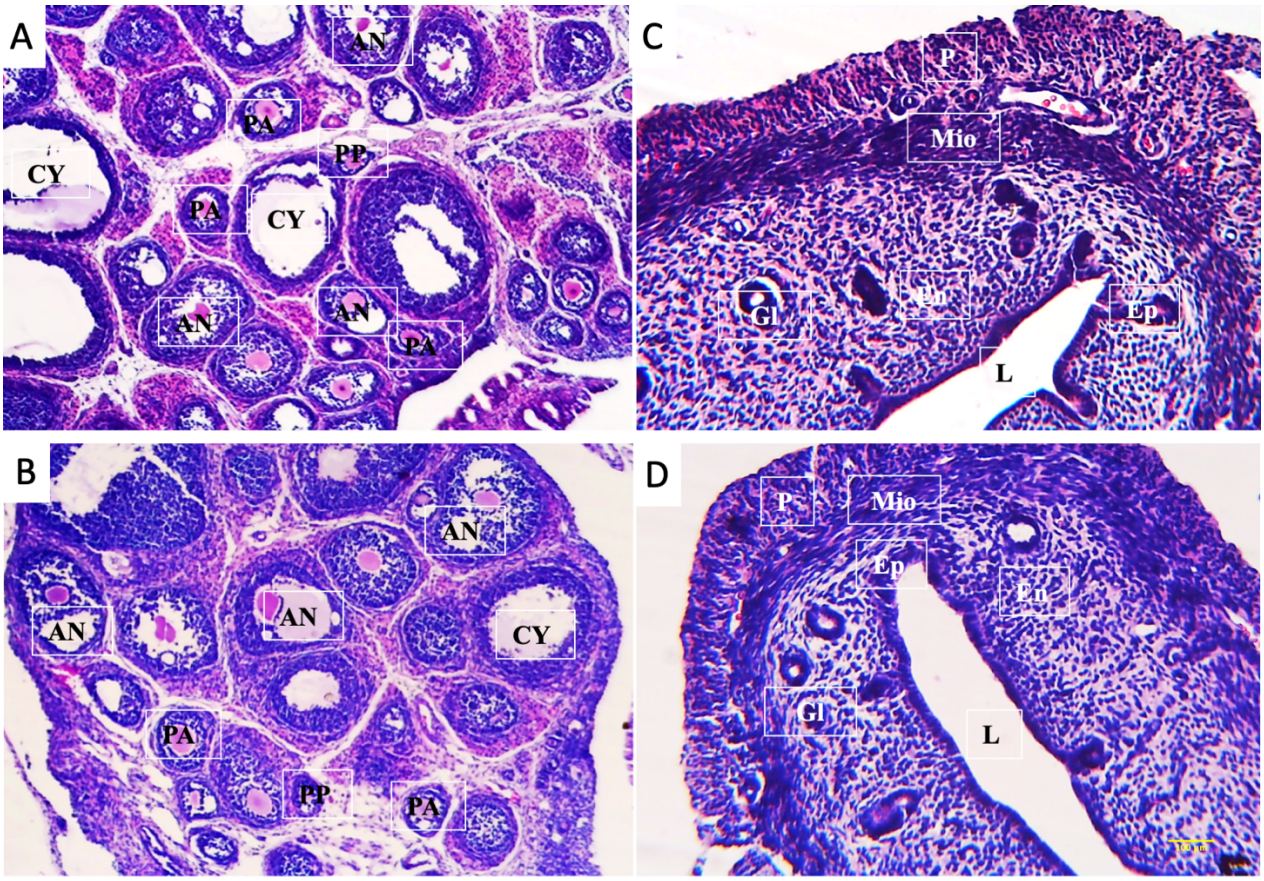
Figure 1. Ovaries and uterus histology from NP females (A and C) and LP females (B and D) at 21 days old. Abbreviations: primordial and primary follicles (PP), preantral follicles (PA), antral follicles (AN), cystic follicles (CY), the epithelium (Ep), endometrium (En), myometrium (Mio), perimetrium (P), lumen (L) and gland (Gl).

Figure 2. Females body weight gain. A. Body weight gain from 21 to 56 days old. B. Body weight gain from 63 to 91 days old. The inset represents the area under curve (AUC). = NP:28/9 litters; LP: 30/9 litters ; NP/NF: 14/9 litters; NP/HF: 14/9 litters; LP/NF: 15/9 litters; LP/HF: 15/9 . * = $p<0.05$, *** = $p<0.001$, and **** = $p<0.0001$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF).

Figure 3. Ovaries and uterus histology from NP/NF (A and E), NP/HF (B and F), LP/NF (C and G), and LP/HF (D and H) at 90 days old. Abbreviations: primordial and primary follicles (PP), preantral follicles (PA), antral follicles (AN), atretic follicles (AT), corpora lutea (CL), the epithelium (Ep), endometrium (En), myometrium (Mio), perimetrium (P), lumen (L) and gland (Gl).

Figure 4. Oxidative stress parameters in the ovaries of offspring malnourished by low-protein diet in lactational period and overfed at adulthood. A. Glutathione s-transferase (GST). B. Reduced glutathione (GSH). C. Superoxide dismutase (SOD). D. Catalase activity E. Lipid hydroperoxide (LOOH). F. Protein per mg of tissue. n= NP/NF: 5/5 litters; NP/HF: 5/5 litters; LP/NF: 5/5 litters; LP/HF: 5/5. * $p<0.05$ and ** $p<0.01$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).

Figure 5. Oxidative stress parameters in the uterus of offspring malnourished by low-protein diet in lactational period and overfed at adulthood. A. Glutathione s-transferase (GST). B. Reduced glutathione (GSH). C. Superoxide dismutase (SOD). D. Catalase activity E. Lipid hydroperoxide (LOOH). F. Protein per mg of tissue. n= NP/NF: 5/5 litters; NP/HF: 5/5 litters; LP/NF: 5/5 litters; LP/HF: 5/5. * $p<0.05$ and ** $p<0.01$. Values are expressed as the mean ± S.E.M. Abbreviations: Normal-protein intake (NP); Low-protein intake (LP); Normal-fat intake (NF); High-fat intake (HF). Factors: Low-protein diet (LP), High-fat diet (HF), and Interaction (I).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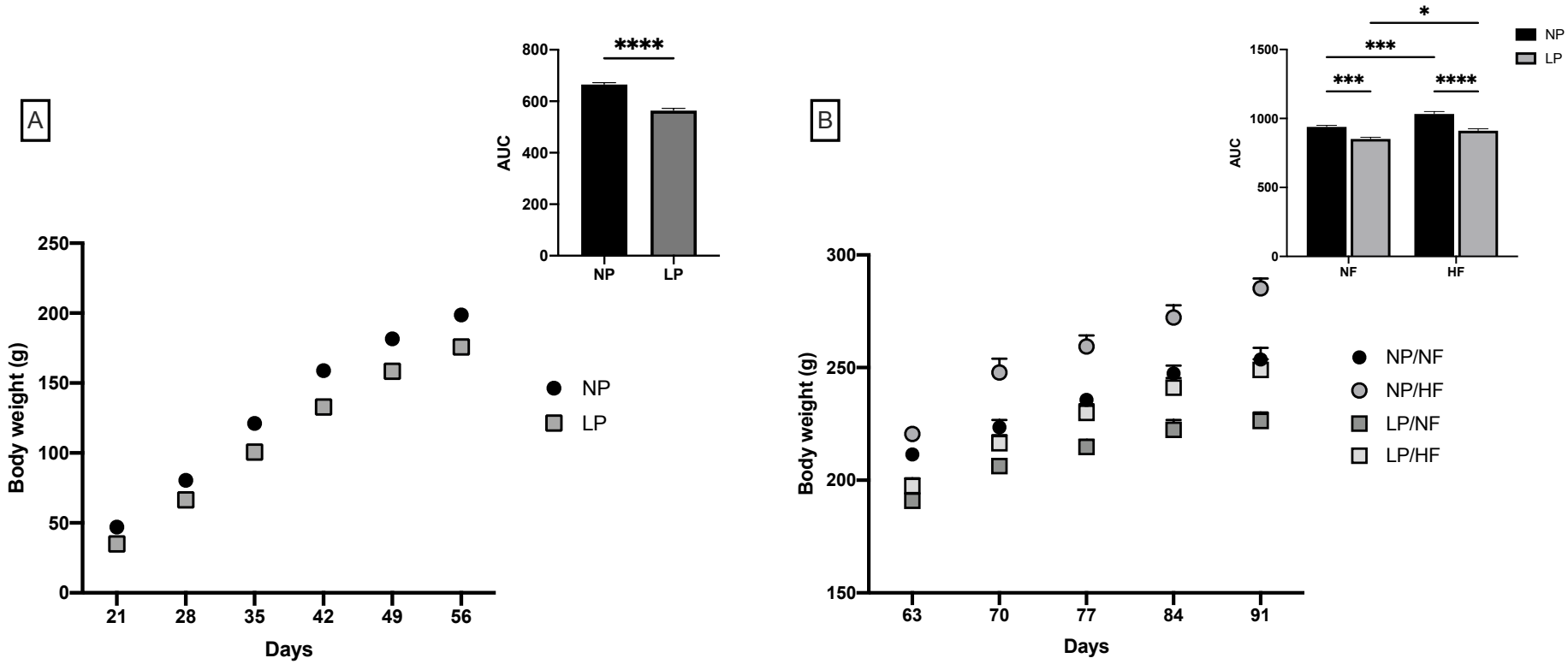

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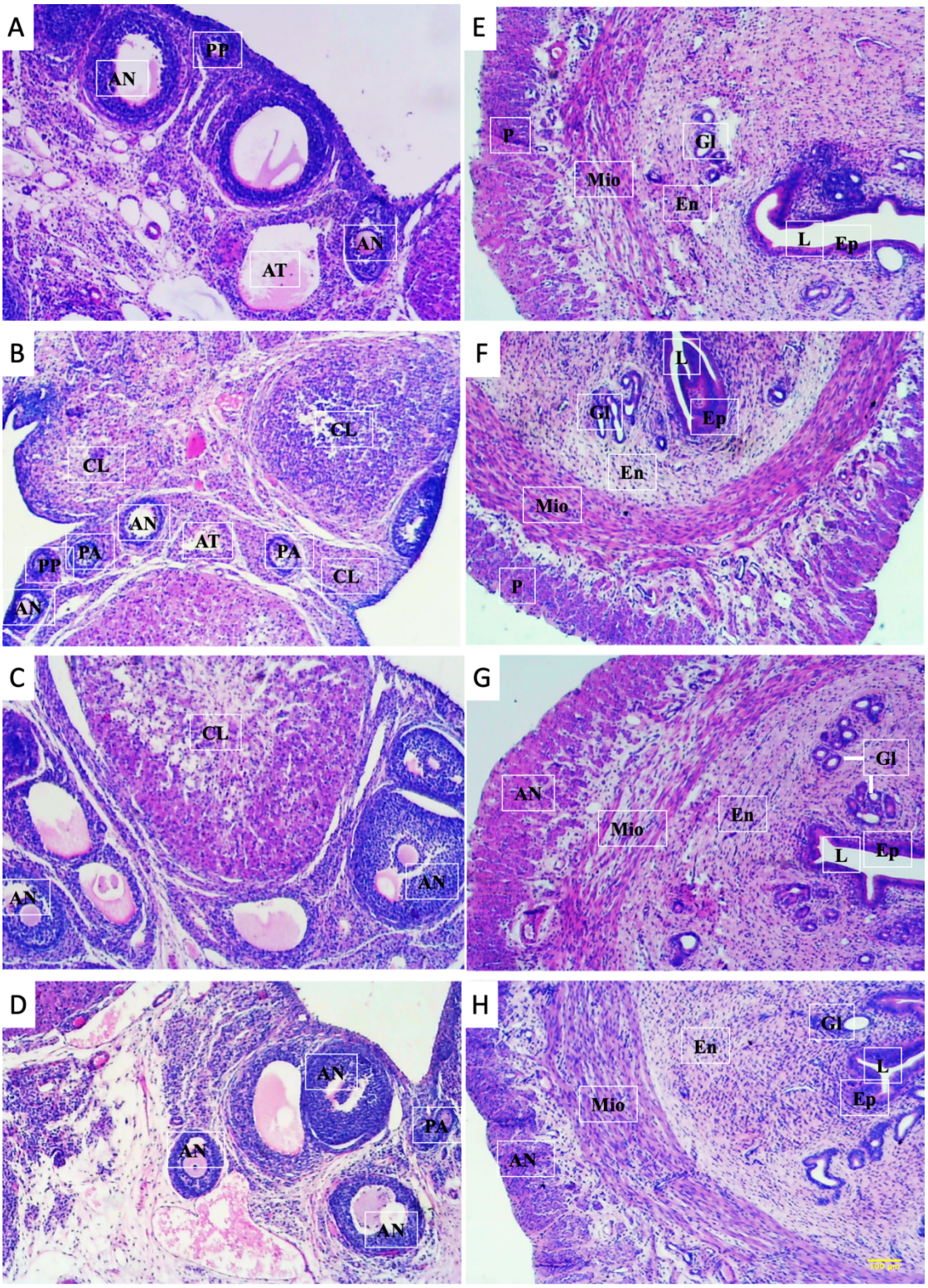
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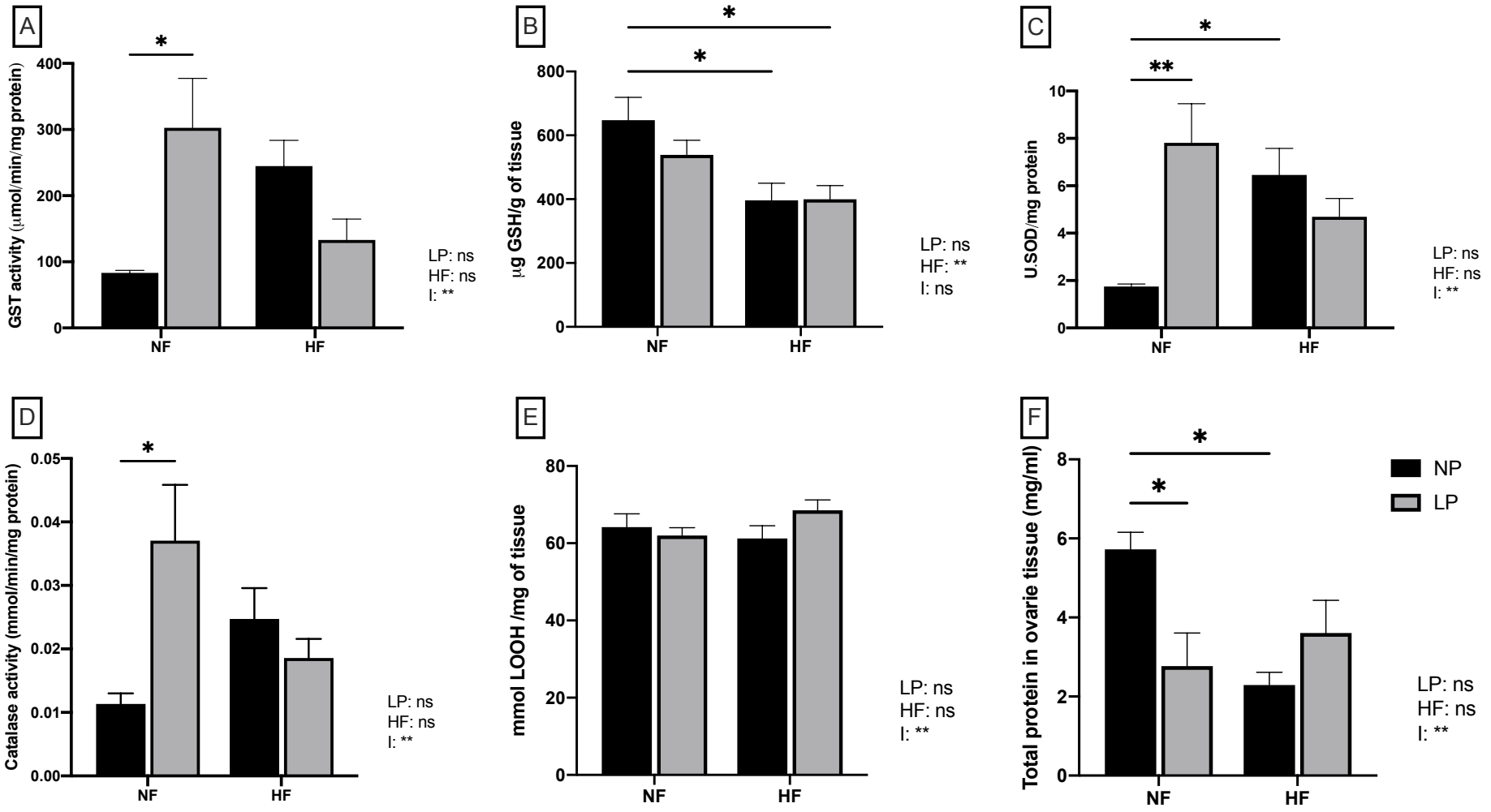
A
GST activity (µmol/min/mg protein)
400
300
200
100
0
NF
HF
*
LP: ns
HF: ns
I: **
B
µg GSH/g of tissue
800
600
400
200
0
NF
HF
*
*
LP: ns
HF: **
I: ns
C
U.SOD/mg protein
10
8
6
4
2
0
NF
HF
*
**
LP: ns
HF: ns
I: **
D
Catalase activity (mmol/min/mg protein)
0.05
0.04
0.03
0.02
0.01
0.00
NF
HF
*
LP: ns
HF: ns
I: **
E
mmol LOOH /mg of tissue
80
60
40
20
0
NF
HF
LP: ns
HF: ns
I: **
F
Total protein in ovarie tissue (mg/ml)
8
6
4
2
0
NF
HF
*
*
NP
LP
LP: ns
HF: ns
I: **

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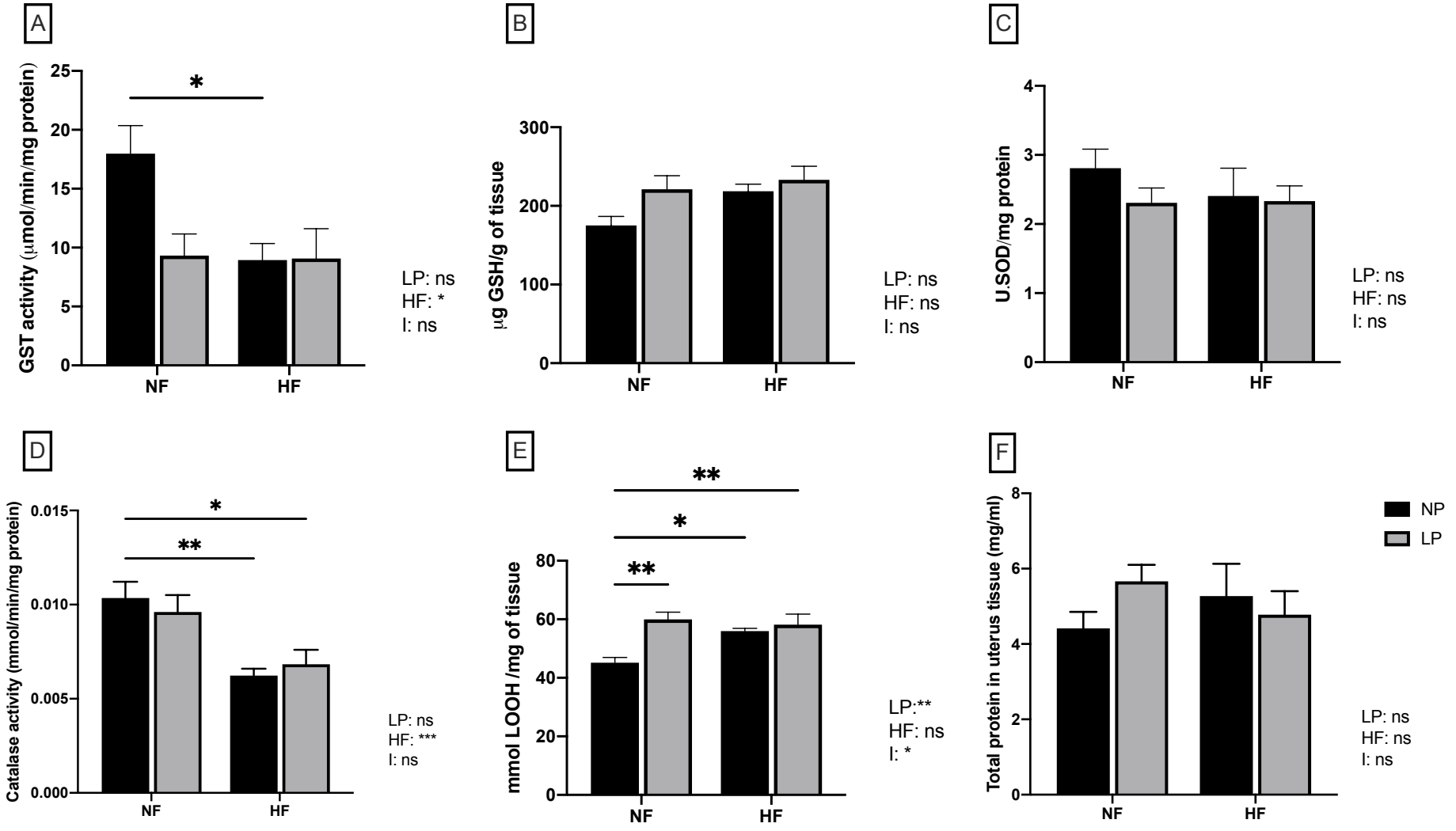
A
GST activity (µmol/min/mg protein)
25
20
15
10
5
0
*
NF
HF
LP: ns
HF: *
I: ns
B
µg GSH/g of tissue
300
200
100
0
NF
HF
LP: ns
HF: ns
I: ns
C
U.SOD/mg protein
4
3
2
1
0
NF
HF
LP: ns
HF: ns
I: ns
D
Catalase activity (mmol/min/mg protein)
0.015
0.010
0.005
0.000
*
**
NF
HF
LP: ns
HF: ***
I: ns
E
mmol LOOH /mg of tissue
80
60
40
20
0
**
*
**
NF
HF
LP:**
HF: ns
I: *
F
Total protein in uterus tissue (mg/ml)
8
6
4
2
0
NF
HF
LP: ns
HF: ns
I: ns
NP
LP

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Anexo- Aprovação Comitê de Ética

Comissão de Ética no Uso de Animais
da
Universidade Estadual de Maringá

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação das alterações do sistema reprodutor masculino e feminino de ratos Wistar submetidos a restrição proteica durante a lactação e a uma dieta hiperlipidica na vida adulta", protocolada sob o CEUA nº 6328301019 (ID 002497), sob a responsabilidade de **Paulo Cezar de Freitas Mathias** *e equipe; Maroly Valentin Alves Pinto; Gessica Dutra Gonçalves* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA/UEM) na reunião de 31/01/2020.

We certify that the proposal "Male and female reproductive system evaluation of Wistar rats submitted at a protein restriction diet during lactation and at a high fat diet in the adulthood", utilizing 190 Heterogenics rats (90 males and 100 females), protocol number CEUA 6328301019 (ID 002497), under the responsibility of **Paulo Cezar de Freitas Mathias** *and team; Maroly Valentin Alves Pinto; Gessica Dutra Gonçalves* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the State University of Maringá (CEUA/UEM) in the meeting of 01/31/2020.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 02/2020 a 02/2021 Área: Dbc-Biotecnologia, Genética E Biologia Celular

Origem:	Biotério Central da UEM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	80 a 85 dias	N:	10
Linhagem:	Wistar			Peso:	300 a 350 g		
Origem:	Biotério Central da UEM						
Espécie:	Ratos heterogênicos	sexo:	Fêmeas	idade:	70 a 75 dias	N:	20
Linhagem:	Wistar			Peso:	200 a 250 g		
Origem:	Biotério Setorial do Laboratório de Biologia Celular da Secreção - (LBCS, PRONEDO)						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	85 a 95 dias	N:	80
Linhagem:	Wistar			Peso:	300 a 400 g		
Origem:	Biotério Setorial do Laboratório de Biologia Celular da Secreção - (LBCS, PRONEDO)						
Espécie:	Ratos heterogênicos	sexo:	Fêmeas	idade:	85 a 95 dias	N:	80
Linhagem:	Wistar			Peso:	250 a 350 g		

Local do experimento: Laboratório de Biologia Celular da Secreção - (LBCS, PRONEDO).

Maringá, 10 de março de 2021

Profa. Dra. Tatiana Carlesso dos Santos
Coordenadora da CEUA/UEM
Universidade Estadual de Maringá

Profa. Dra. Erika Seki Kioshima Cótica
Coordenadora Adjunta da CEUA/UEM
Universidade Estadual de Maringá

Av. Colombo, 5790, UEM-PPG, sala 4. CEP: 87020-900, Maringá-PR, whatsapp 3011-4597. - tel: 55 (44) 3011-4444
Horário de atendimento: 2ª a 6ª, das 8h às 11h40 e 14h às 17h30 : e-mail: ceea@uem.br
CEUA N 6328301019