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ISABELA PEIXOTO MARTINS

PROGRAMAÇÃO METABÓLICA E COMPORTAMENTAL EM DIFERENTES JANELAS CRÍTICAS DO DESENVOLVIMENTO: INSULTOS FARMACOLÓGICOS E NUTRICIONAIS

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

Orientador: Prof. Dr. Paulo Cezar de Freitas Mathias

Coorientador: Profa. Dra. Ananda Malta

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Aprovado em:

BANCA EXAMINADORA

_____

Prof. Dr. Paulo Cezar de Freitas Mathias
Universidade Estadual de Maringá

_____

Prof. Dr. Júlio Cezar de Oliveira
Universidade Federal do Mato Grosso - Sinop

_____

Prof. Dr. Jurandir Comar
Universidade Estadual de Maringá

_____

Profa. Dra. Adriana Torsoni
Universidade Estadual de Campinas

Profa. Dra Cristiane Matté
Universidade Federal do Rio Grande do Sul

Profa. Dra. Patrícia Lisboa
Universidade Estadual do Rio de Janeiro

BIOGRAFIA

Isabela Peixoto Martins nasceu em Maringá/PR em 21/06/1994. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá (2015). É mestre pelo Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá (06/2017). Atualmente é doutoranda do Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá e Professora Assistente no Departamento de Ciências Morfológicas da Universidade Estadual de Maringá. Tem experiência na área de Biologia Celular e Fisiologia, atuando principalmente nos seguintes temas: obesidade, desnutrição proteica e secreção de insulina.

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APRESENTAÇÃO

Esta tese é composta por dois artigos científicos, intitulados: 1- "**Methylphenidate treatment at adolescence malprograms metabolism and behavior at adulthood in male rats"** e 2- **"Low-protein diet induced HPA axis hyperactivation and altered milk composition imprints the metabolism of weaned male rat offspring".** Os trabalhos demonstram a importância de duas janelas críticas do desenvolvimento, lactação e adolescência, e como diferentes insultos podem impactar o metabolismo e o comportamento em roedores. Em consonância às regras do Programa de Pós-Graduação em Ciências Biológicas, os artigos 1 e 2 foram redigidos de acordo com as normas das revistas *Journal of Physiology*, com atual fator de impacto 4.54 (Qualis CB1: A2) e *European Journal of Nutrition*, com atual fator de impacto 4.66 (Qualis CB1: A2), respectivamente.

RESUMO GERAL

INTRODUÇÃO – O desenvolvimento de doenças metabólicas e distúrbios comportamentais na vida adulta têm sido associados à insultos no início da vida. O conceito DOHaD (Developmental Origins of Health ans Diseases) descreve, por meio de estudos experimentais e epidemiológicas, como eventos em fases sensíveis do desenvolvimento provocam alterações fisiológicas no organismo, programando-o para consequências a longo prazo. Gestação, lactação e adolescência têm sido consideradas janelas para a programação metabólica, uma vez que há plasticidade do Sistema Nervoso Central nestes períodos, além da susceptibilidade dos órgãos periféricos. Insultos nutricionais e farmacológicos podem programar o organismo nessas fases. Com isso, ratos adolescentes tratados com o psicoestimulante Metilfenidato, utilizado para o tratamento do Transtorno de Déficit de Atenção com Hiperatividade (TDAH) podem apresentar um fenótipo extremamente vulnerável ao desenvolvimento de doenças na vida adulta. Em relação aos insultos nutricionais, a restrição proteica durante a lactação é considerada um modelo bem estabelecido para o estudo da programação metabólica. O estresse causado nas mães pela desnutrição pode alterar seu comportamento em relação a prole, modificar a composição do leite e o perfil metabólico da prole desde o início da vida.

OBJETIVOS – Avaliar o impacto de insultos farmacológicos e nutricionais em fases críticas do desenvolvimento sobre o metabolismo e comportamento de ratos Wistar machos a curto e longo prazo.

MÉTODOS – Para a realização do primeiro artigo (insulto farmacológico), ratos Wistar machos foram tratados com Metilfenidato (grupo MPH, 1.0 mg/kg/dia, via oral) ou Salina (grupo SAL, 0.9%) dos 21 aos 51 dias de vida. Aos 52 dias, um lote de animais foi submetido aos procedimentos experimentais de comportamento e metabolismo. Outro lote de animais, ao término do tratamento, ficou 60 dias sem receber nenhuma droga, e então foi avaliado aos 110 dias de vida. Para a realização do segundo artigo (insulto nutricional), ratas prenhas foram alocadas em caixas individuais. Na ocasião do nascimento dos filhotes as ninhadas foram padronizadas para oito filhotes por mãe e foram divididas em dois grupos experimentais: mães que receberam dieta *low protein* nas primeiras duas semanas de lactação (grupo LP, 4% de proteínas) e mães que

receberam dieta comercial para ratos (grupo NP, 23% de proteínas). Mães e filhotes foram avaliados aos 7, 14 e 21 dias. Além disso, o leite das mães foi retirado nestes mesmos dias e analisados em relação a sua composição de macronutrientes. Durante todo o período experimental os animais foram mantidos sob temperatura (23 ± 2 5 ºC) e fotoperíodo (7:00 a.m. to 7:00 p.m., ciclo claro) controlados. Em ambos os trabalhos os dados obtidos foram expressos como média ± erro padrão e analisados através de test t de Student ou ANOVA de duas vias com pós teste de Holm-Sidak, com intervalo de confiança de 95%. O programa utilizado foi GraphPad Prism, versão 7.01.

RESULTADOS E DISCUSSÃO – O tratamento com Metilfenidato na adolescência provocou diminuição do consumo alimentar e hipoinsulinemia aos 52 dias, corroborando com relatos de caso da literatura. Além disso, observou-se um comportamento ansiogênico e antipânico nos animais jovens. O mecanismo envolvido na gênese destes comportamentos não foi elucidado. Na vida adulta, animais do grupo MPH apresentaram sobrepeso, aumento dos estoques de gordura corporal, hiperinsulinemia e dislipidemia. Essas alterações foram associadas ao aumento da atividade do Sistema Nervoso Parassimpático. Interessantemente, as alterações comportamentais se mantiveram na vida adulta. Em relação ao segundo artigo, demonstrou-se que o insulto nutricional na lactação é capaz de hiper ativar o eixo hipotálamo-pituitária-adrenal (HPA) da mãe. Com isso, as mães LP apresentaram modificações do comportamento materno em relação à prole e alterações na composição do leite, dentre elas a elevação dos níveis de corticosterona e de lipídeos totais. Associado a isso, os filhotes apresentaram um fenótipo magro durante a lactação, um aumento da concentração de corticosterona ao 7º dia e alterações morfológicas no tecido adiposo branco e marrom. Provavelmente, a elevação da atividade do eixo HPA e a alteração da composição do leite modificaram o metabolismo destes animais, de modo a aumentar seu gasto energético.

CONCLUSÃO – Insultos farmacológicos e nutricionais aplicados em fases sensíveis do desenvolvimento programam o metabolismo e o comportamento a curto e a longo prazo.

PALAVRAS – CHAVE – Adolescência; Lactação; Metilfenidato; Restrição proteica.

GENERAL ABSTRACT

INTRODUCTION - The development of metabolic diseases and behavioral disorders in adulthood has been associated with insults early in life. The DOHaD (Developmental Origins of Health ans Diseases) concept describes, through experimental and epidemiological studies, how events in sensitive stages of development cause physiological changes in the body, programming it for long-term consequences. Pregnancy, lactation, and adolescence have been considered windows for metabolic programming, since there is plasticity of the Central Nervous System in these periods, in addition to the susceptibility of peripheral organs. Nutritional and pharmacological insults can program the body in these phases. Thus, adolescent rats treated with the psychostimulant Methylphenidate, used for the treatment of Attention Deficit Hyperactivity Disorder (ADHD) may present an extremely vulnerable phenotype to the development of diseases in adulthood. In relation to nutritional insults, protein restriction during lactation is considered a well-established model for the study of metabolic programming. The stress caused in mothers by malnutrition can change their behavior in relation to offspring, modify the composition of milk and the metabolic profile of the offspring from the beginning of life.

AIMS - Evaluate the impact of pharmacological and nutritional insults at critical stages of development on the metabolism and behavior of male Wistar rats at short and long-term.

METHODS - For the first article (pharmacological insult), male Wistar rats were treated with Methylphenidate (MPH group, 1.0 mg/kg/day, via gavage) or Saline (SAL group, 0.9%) from 21 to 51 days of life. At 52 days, a batch of animals was submitted to experimental behavior and metabolism procedures. Another batch of animals, at the end of the treatment, was 60 days without receiving any drugs, and then was evaluated at 110 days of life. For the second article (nutritional insult), pregnant rats were placed in individual boxes. At birth, litters were standardized to eight puppies per mother and were divided into two experimental groups: mothers who received a low protein diet in the first two weeks of lactation (LP group, 4% protein) and mothers who received a normal protein diet (NP group, 23% proteins) through lactation. Mothers and puppies were evaluated at 7, 14 and 21 days. In addition, the mothers' milk was removed at the

same days and analyzed for its macronutrient composition. Throughout the experimental period, the animals were kept under temperature (23 ± 25 ºC) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle) controlled. In both studies, the data obtained were expressed as mean ± standard error and analyzed using Student's t test or two-way ANOVA with Holm-Sidak post-test, with a 95% confidence interval. The program used was GraphPad Prism, version 7.01.

RESULTS AND DISCUSSION - Treatment with methylphenidate in adolescence caused a decrease in food consumption and hypoinsulinemia at 52 days, corroborating with case reports in the literature. In addition, an anxiogenic and antipanic behavior was observed in young animals. The mechanism involved in the genesis of these behaviors has not been elucidated. At adulthood, MPH animals' group were overweight, increased body fat stores, hyperinsulinemia and dyslipidemia. These changes were associated with increased Parasympathetic Nervous System activity. Interestingly, behavioral changes have persisted into adulthood. In relation to the second article, it was shown that the nutritional insult during lactation is capable of hyper-activating the mother's hypothalamic-pituitary-adrenal (HPA) axis. As a result, LP mothers showed changes in their maternal behavior in relation to their offspring and changes in the composition of milk, including an increase in levels of corticosterone and total lipids. Associated with this, offspring showed a lean phenotype during lactation, an increase in the concentration of corticosterone on the 7th day and morphological changes in the white and brown adipose tissue. Probably, the increase in the activity of the HPA axis and the change in the composition of milk modified the metabolism of these animals, in order to increase their energy expenditure.

CONCLUSION - Pharmacological and nutritional insults applied at sensitive stages of development program the metabolism and behavior in the short and long term.

KEYWORDS - Adolescence; Lactation; Methylphenidate; Protein restriction.

Methylphenidate treatment at adolescence malprograms metabolism and behavior at adulthood in male rats

Isabela Peixoto Martins[1,2]; Jhonatan Christian Maraschin[3]; Camila Cristina Ianoni Matiusso[1]; Audrei Pavanello[1,4]; Laize Peron Tófolo[1]; Késia Palma-Rigo[1]; Caio Sestile[1]; Ana Maria Praxedes de Moraes[1]; Rodrigo Vargas[1,4]; Tatiane Aparecida Ribeiro[5]; Elisabeth Aparecida Audi[3]; Paulo Cezar de Freitas Mathias[1]; Ananda Malta[1]

[1]Department of Biotechnology, Genetics, and Cellular Biology, State University of Maringa, Maringa, PR, Brazil.

[2]Departament of Morphological Sciences, State University of Maringa, Maringa, PR, Brazil.

[3] Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, SP, Brazil.

[4]Health Sciences Center, Unicesumar, Maringa, PR, Brazil.

[5]Department of Biochemistry and Biomedical Science, McMaster University - Hamilton ON – Canada.

Correspondence to: Isabela Peixoto Martins; Department of Biotechnology, Genetic and Cell Biology; Laboratory of Secretion Cell Biology – Block H67, room 19, State University of Maringa/UEM - Phone/Fax + 55 (44) 3011 4892. Colombo Avenue 5790, 87020-900, Maringá/PR – Brazil. E-mail address: isa.peixotomartins@gmail.com.

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Abstract

Currently, attention deficit hyperactivity disorder (ADHD) affects many children and adolescents worldwide. Methylphenidate (MPH) is the mainly drug used to treat ADHD, once it inhibits the dopamine reuptake in the synaptic cleft. Recently, concern has been raised about the consequences of MPH use and abuse during adolescence, an important critical stage of development. We investigated the short- and long-term effects of MPH treatment during rat adolescence on body composition, metabolism, and anxiety-like behavior. To test this hypothesis, male Wistar rats were treated with MPH (1.0 mg /kg /day) or saline (0.9% NaCl) from postnatal day (P) 21 to P51. A batch of animals were used in the experiments at P52. Another batch of rats were untreated between P52 and P110 and in this age the experiments were conducted. MPH treatment provoked, at P52, reduced food intake, hypoinsulinemia, decreased total cholesterol and increased HDL-C. In addition, adolescent rats showed anxiogenic-like effect and antipanic response to behavioral tests. After a long time of drug discontinuation, MPH group had increased body weight, food intake, fat pad stores, dyslipidemia, and hyperinsulinemia. This phenotype was associated to elevated parasympathetic activity. Moreover, behavior tests showed no differences compared to the analysis at P52. We concluded that MPH treatment at adolescence programs male rats to obesity, metabolic dysfunction, and behavioral alterations at adulthood.

Keywords: methylphenidate; adolescence; behavior; obesity.

Introduction

MPH is a psychostimulant drug widely used for the treatment of ADHD (Lepelletier *et al.*, 2014; Montagnini *et al.*, 2016). This neurodevelopment disorder is characterized by hallmark symptoms including inattention, hyperactivity, and impulsivity (Jaboinski *et al.*, 2015; Somkuwar *et al.*, 2015). Although ADHD has multiple etiology, central catecholaminergic dysfunction, including dopaminergic and noradrenergic neurotransmission imbalance, are involved in the emerging of this condition (Wilens, 2008; Yang *et al.*, 2016). MPH acts by blocking the dopamine (DA) and norepinephrine (NE) transporters in the striatum and the prefrontal cortex, resulting in increases in synaptic DA and NE (Faraone, 2018).

ADHD is a neuropsychiatric pathology mainly affecting children and adolescents. Its worldwide epidemiological prevalence is of 12-15% in children and persisting into adulthood in 4-5% of individuals (Froehlich *et al.*, 2007; Somkuwar *et al.*, 2015). The rate of diagnosis of ADHD has increased by 41% over the last decade, especially in boys aged 14 to 17 (Jordan *et al.*, 2014; Jaboinski *et al.*, 2015). Approximately two thirds of diagnosed children are medicated with psychostimulants. MPH is also used illegally by high school and college students to improve the performance in studies (Thanos *et al.*, 2015). Chronic exposure to psychostimulants induces behavioral, biochemical, molecular, and morphological changes that are linked to central nervous system plasticity (Ponchio *et al.*, 2015).

The increasing use and abuse of MPH, especially during critical windows of development, raises concerns about the consequences on neural and behavioral development and in adult health (Thanos *et al.*, 2015). Epidemiological (Ravelli *et al.*, 1976; Barker, 1995) and experimental (Fagundes *et al.*, 2007; de Oliveira *et al.*, 2011) studies demonstrated that insults during childhood and adolescence can contribute to the

emergency of diseases later in life (Holder & Blaustein, 2014; Mantovani & Fucic, 2014; Ismail *et al.*, 2017). Notably, during adolescence, changes in synaptic pruning and myelination rates have been reported; in addition, extensive shaping of connectivity is occurring between brain regions in this phase (Tzanoulinou & Sandi, 2017; Di Miceli *et al.*, 2019). These remarks corroborate with Development Origins of Health and Disease (DOHaD) concept, which describes thought scientific studies how early environmental factors can program long-term consequences.

Although many studies shown that nutritional insults in early life are crucial to program diseases at adulthood, recently, other factors are also showed to determine long-term dysfunctions, likewise substance abuse such as psychostimulants medicines (Vaiserman, 2015; Korchynska *et al.*, 2020). The use of amphetamines during pregnancy was associated with low birth weight, prematurity, and increased maternal and child mortality (Costa Gde *et al.*, 2016). MPH during pregnancy permanently impairs the ability of insulin production by pancreatic β cells, leading to glucose intolerance in adult offspring female rats (Korchynska *et al.*, 2020) and administration of MPH or cocaine during early lactation impairs maternal behavior and program to increase anxiety-like behavior in adult offspring (Zimmerberg & Gray, 1992; Ponchio *et al.*, 2015).

Recent studies performed in rodents demonstrated that adolescent exposure to clinical oral doses of MPH may induce acute and long-lasting effects on monoamine neurotransmission (Amodeo *et al.*, 2017; Di Miceli *et al.*, 2019) reward-dependent learning and decisions stimuli (Bolanos *et al.*, 2003). Administration of MPH during adolescence also enhances anxiety, as well depressive-like symptoms later in life (Loureiro-Vieira *et al.*, 2017).

The literature is mainly focused on the cognitive, neurophysiological, neurological, psychosocial and behavior outcomes caused by exposure to amphetamine,

cocaine, and methylphenidate during critical windows of development. However, the acute and long-lasting effects on metabolism at adulthood have been poorly reported, although a link between prenatal psychostimulants exposure and an increased risk of developing obesity and type 2 diabetes in the offspring has been noted (Messiah *et al.*, 2011; Vaiserman, 2015; Korchynska *et al.*, 2020).

During MPH treatment at childhood and adolescence changes in body composition are observed, including fat loss and alterations in bone development (Poulton *et al.*, 2012). Anorexigenic effect of MPH had been demonstrated in preclinical and clinical studies, especially during the first three to six months of treatment in children (Bou Khalil *et al.*, 2017). According to a case report, an adolescent patient that discontinued MPH treatment presented an increase by five points in BMI (body mass index) and eating behavior disorders within one year of medicine cessation (Benard *et al.*, 2015). The consequences of acute and long-term adolescence MPH treatment on weight gain, metabolism and behavior have been poorly analyzed. Therefore, we investigated the short- and long-term effects of MPH treatment during rat adolescence on body composition, metabolism, and anxiety-like behavior.

Materials and Methods

Ethical approval

All experiments were conducted according to the ARRIVE guidelines (Kilkenny *et al.*, 2010) and with Brazilian Association for Animal Experimentation (COBEA) standards. Protocols were approved by the Ethics Committee in Animal Research of the State University of Maringa (protocol number 8597180117).

Animals and Methylphenidate (MPH) treatment

Wistar rats were provided by the central animal house of the State University of Maringa and were kept in the animal house of the laboratory of secretion cell biology. After one week of adaptation, female and male Wistar rats (70 and 80 days of age, respectively) were mated in a ratio of three females to each male. Pregnant females were transferred to individual cages. At birth, litters were standardized to nine pups per dam, preferentially male. At postnatal day 21 (P21), the male offspring were weaned, housed four per cage and assigned to either the control group that received a 0,9 % saline solution, (SAL group; n=12 litters) or the group that received Methylphenidate (Ritalin®, Novartis, Brazil) at a dose 1.0 mg/kg/day (MPH group; n=12 litters). MPH dose corresponds to a relatively low therapeutically recommended oral dose in humans (Seeman & Madras, 2002; Haleem *et al.*, 2015). Animals were weighted and treated via gavage daily for 30 days (P21-P51). Between P52 and P110 the animals of both groups did not receive treatment. Analyses were conducted at P52 and P110. Metabolism analyses were performed with the first lot of animals. Experimental procedures were effectuated at P52 (n=4 litters per group) and P110 (n=4 litters per group). In this lot, all the pups of the same litter received the same treatment. The second lot of animals (n=4 litters per group) were subjected to behavioural tests at P52 and P110; for this, in the same litter two rats received saline and two rats received MPH. During all the experimental period animals received water and food *ad*

157 *libitum* and were kept under controlled temperature (23 ± 2°C) and photoperiod (7:00
158 a.m. to 7:00 p.m., light cycle) conditions.

159

160 *Body weight, food intake and fat pad stores measurements*

161 Body weight (bw) was determined every day and food intake (fi) was measured three
162 times a week during treatment (P21-P51). After treatment (P52-P110) bw and fi were
163 determined three times a week. Food intake was calculated as the difference between the
164 amount of diet remaining (Df) and the amount presented previously (Di), divided by the
165 number of animals in the cage and by the number of days: [FI(g) = (Df –Di)/4/2]. The
166 area under the curve (AUC) was calculated for bw and fi. At P52 and P110, rats were
167 anaesthetized with thiopental (45 mg/kg of bw), decapitated and laparotomized to remove
168 their retroperitoneal, periepididymal and mesenteric fat pad stores (n= 4 litters per group).
169 The weight of fat pads was expressed in relation to the body weight of each animal (g/100
170 g of bw).

171

172 *Intraperitoneal insulin tolerance test (ipITT)*

173 At P52 and P110, a batch of animals (n= 4 litters per group) were submitted to 6-hour fast
174 to perform ipITT. They received an injection of insulin (1 U/kg of bw), and blood glucose
175 was measured using a glucometer, as previously reported (Lechner & Hess, 2019).
176 Glucose was determined at 0, 15, 30, 45 and 60 minutes. Subsequently, the rate of glucose
177 tissue uptake or the rate constant for plasma glucose disappearance (K_{itt}) was calculated
178 (Bonora *et al.*, 1989).

179

180 *Intravenous glucose tolerance test (ivGTT)*

Two days after the ipITT, animals (n= 4 litters per group) were subjected to a surgical procedure to perform the ivGTT, as previously described (de Oliveira *et al.*, 2011). After a 12-hour fast, blood samples were removed before the injection of glucose (1 g/kg of bw) (0 min) and 5, 15, 30 and 45 min afterward. Blood was collected, centrifugated and the plasma was stored at -20ºC for determination of glucose and insulin concentrations. The glucose and insulin responses during the test was calculated by AUC.

Blood glucose and insulin

Glucose concentration was measured by the glucose oxidase method using a commercial kit (GoldAnalisa®; Belo Horizonte, MG, Brazil) (Trinder, 1969). The insulin levels of plasma were measured by radioimmunoassay (RIA) (Scott *et al.*, 1981).

Lipid profile

Triglycerides, total cholesterol and HDL-C were measured in plasma samples by a colorimetric method using commercial kits (Gold Analisa®; Belo Horizonte, MG, Brazil). LDL-C and VLDL-C values were determined by the Friedewald formula (Simoes *et al.*, 2007). The dosages were performed at P52 and P110.

Autonomic nerve electrical activity

At P110, a batch of rats (n=4 litters per group, rats from behavioural tests) that has been fasted for 12 hours were anaesthetized with thiopental (45mg/kg of bw). A longitudinal surgical incision was made on the anterior cervical region of the animal. The left superior vagus nerve from the superior cervical ganglion was isolated. A sympathetic nerve bundle was dissected from the ventral surface of the right interscapular brown adipose tissue (BAT) pad and placed on a bipolar hook electrode, according to the method previously

described (Madden *et al.*, 2017). The electrode was connected to an electronic device (Bio-Amplificator; Insight Equipamentos, Ribeirão Preto, Brazil) that amplified the electrical signal prior to filtering out the frequencies lower than 1 kHz and higher than 80 kHz. The signal output was acquired using Insight software and stored on a computer. The animals were placed in a Faraday cage to avoid any electromagnetic interference during the experimental period (Barella *et al.*, 2015).

Elevated T-maze (ETM) behaviour

On P52 and P110 animals (n= 4 litters per group) were assessed for elevated T-maze behaviour. The ETM was made of wood and had three arms with equal dimensions (50x12 cm). One of the arms was enclosed by 40 cm high walls and was oriented perpendicularly to two opposite open arms. The whole apparatus was elevated 50 cm above the floor. To analyze the inhibitory avoidance, each rat was placed at the end of the closed arm, facing the intersection of the arms. The time used to leave this arm was assessed and registered at baseline (seconds). The same procedure was repeated twice with an interval of 30 seconds (inhibitory avoidance 1 and 2). Moreover, escape latency was evaluated putting animals in the open arms and assessing the latency to escape from this arm with four paws (escape 1-3). The maximum time considered for both tasks was 300 seconds. Twenty-four hours before the test, the animals were pre-exposed to one of the open arms of the ETM for 30 minutes, making the test more sensitive because it decreases the exploration of the animal during the test (Teixeira *et al.*, 2000).

Open field test

Locomotor activity of P52 and P110 rats (n= 4 litters per group) was measured by the ambulation of each animal in a circular arena (diameter 70 cm with 40 cm high walls).

Rats were placed inside the circular arena, facing the wall, for 5 min. The total distance travelled in meters by each rat was recorded and analyzed by ANY-maze video monitoring program (Stoelting, USA) (Sestile *et al.*, 2016).

Statistical analysis

The results are presented as the mean ± standard error of the mean (S.E.M). Statistical analysis was performed using Student's *t*-test. A P value < 0.05 was considered statistically significant. Repeated-measures analysis of variance (RMANOVA) was used to analyze avoidance data from the ETM, with treatment as independent factor and trials (baseline, avoidance 1 and 2) as the repeated measure. When appropriate, post hoc comparisons were performed by the Holm-Sidak's test. For escape data, latencies were merged, and data from each rat were analyzed as the mean ± standard error of the mean (S.E.M.) of the three performed trials. Merged escape index and the total distance travelled in the circular arena were analyzed by student's t-test. Analyses were conducted in GraphPad Prism version 7.01 for Windows (GraphPad Software, Inc. San Diego, CA, USA).

Results

Body weight, food intake and body composition

As shown in Figure 1, during MPH treatment no difference was observed in bw AUC (inset of Fig. 1A) and in final bw at P52 (Table 1). However, MPH animals presented a decrease of 12% ($p<0.01$) in food intake (inset of Fig. 1B) during this period. In the period after treatment, MPH group showed an 23% ($p<0.01$) and 114% ($p<0.05$) increase in bw (inset of Fig. 1A) and food intake (inset of Fig. 1B), respectively. Final bw at P110 was 6% higher in MPH group ($p<0.05$, Table 1) compared to SAL animals.

Figure 2A shows that fat pad stores of MPH treated animals were not altered at P52. However, at P110 MPH group presented elevated retroperitoneal, periepididymal and mesenteric fat stores by approximately 20% ($p<0.05$, Fig. 2B).

Biochemical parameters and lipid profile

Table 1 shows that MPH animals were normoglycemic, even though they presented 50% ($p<0.05$) lower fasting insulin levels at P52. 60 days after of treatment MPH animals showed an increase by 107% ($p<0.01$) in fasting insulin, without alterations in fasting glycemia.

Regarding lipid profile (Table 1), at P52, MPH group displayed increases of 18% ($p<0.05$) and 23% ($p<0.05$) in total cholesterol and HDL-C, respectively. Additionally, triglycerides, LDL-C and VLDL-C presented no difference. At P110 MPH animals showed 13% ($p<0.05$) and 34% ($p<0.05$) increase in total cholesterol and LDL-C respectively.

Glucose homeostasis during the glucose and insulin tolerance tests

Immediately after the treatment, at P52, MPH rats were normoglycemic during the ivGTT (Fig. 3A). As demonstrated in Figure 3B, MPH group presented lower insulin levels during ivGTT at 0, 30 and 45 minutes ($p<0.05$). The K_{itt} (Fig. 3C) showed similar insulin sensitivity between groups at P52. At adulthood (P110), MPH group displayed higher glucose levels at 15 ($p<0.05$), 30 ($p<0.05$) and 45 ($p<0.001$) minutes of ivGTT associated to lower levels of insulin at 0 ($p<0.01$) and 15 minutes ($p<0.05$), as demonstrated in Figure 3D and E. Moreover, MPH rats showed a reduction of 30% ($p<0.05$) in glucose disappearance rate (K_{itt}), indicating insulin resistance (Fig. 3F).

Autonomic nervous system activity

As shown in Figure 4, parasympathetic activity at P110 was increased 34% ($p<0.05$) in MPH group. No difference was observed between MPH and SAL groups in sympathetic activity.

Elevated T-maze - avoidance, merged escape and locomotion

Figure 5A shows that MPH facilitated the inhibitory avoidance acquisition at P52, suggesting an anxiogenic-like effect. RMANOVA revealed significant effects of trial [$F(2, 32) = 7.95$; $p = 0.002$], treatment [$F(1, 16) = 4.98$; $p = 0.04$] and an interaction between these factors [$F(2, 32) = 3.21$; $p = 0.05$]. Moreover, Figure 5B shows that MPH significantly increased the escape latency in the ETM [$t16=2.21$; $p = 0.04$], indicating an antipanic-like effect. MPH treatment did not affect locomotion in the circular arena at P52 [$t16 =1.40$; $p = 0.18$], as demonstrated by Figure 5C, indicating that the effects observed were not due to locomotor impairment.

Administration of MPH did not affect inhibitory avoidance acquisition and escape performance in the ETM or locomotion in the circular arena at P110 (Figure 5D, E, F).

RMANOVA revealed that the animals acquired inhibitory avoidance, observed by the significant effect of trial [$F(2, 32) = 7.79$; $p = 0.002$], but not of treatment [$F(1, 16) = 0.44$; $p = 0.52$] or a treatment x trial interaction [$F(2, 32) = 0.39$; $p = 0.68$].

Discussion

The increasing use of MPH in sensitive stages of development, especially adolescence, raises concerns about its immediate and long-term health effects. In the present study, we found that adult offspring rats exposed to MPH at adolescence displayed elevated body weight, food intake, and fat pad tissue. Moreover, insulin resistance and glucose intolerance can be associated with high parasympathetic activity. Altogether, these results suggest that MPH treatment at adolescence can be a possible predisposing factor for diabetes mellitus and obesity risk in later life.

Now is completely accepted that the nutritional or hormonal environment during gestation and or lactation phases of development can permanently affect neuroendocrine pathways and predispose the adult organism to metabolic disorders (Dorner & Plagemann, 1994; Bouret, 2012). In addition to these environmental factors, recent studies provide evidence that the use of psychostimulants during pregnancy and lactation can also poorly program the offspring. Pregnant mice treated with MPH exhibited an increase in resorptions and offspring presented increased rates of external, skeletal, and visceral malformations (Costa Gde *et al.*, 2016). Moreover, studies showed metabolic dysfunction and behavior alterations in the offspring of mothers treated with MPH or cocaine during pregnancy and /or lactation (Vaiserman, 2015; Korchynska *et al.*, 2020).

In addition to prenatal and postnatal periods, adolescence was recently reported as another window to metabolic programming since the neuroplasticity of puberty contribute to the vulnerability for the development of diseases. The increase of sexual hormones is a cause of structural and functional changes in the brain. Accordingly, nutritional, hormonal and drug abuse at adolescence can permanently changes

physiological function (Holder & Blaustein, 2014; Ibanez *et al.*, 2017; Ismail *et al.*, 2017; de Oliveira *et al.*, 2018).

In this study, during adolescence, MPH treatment caused a reduction in food intake, as previously reported (Davis *et al.*, 2012; Thanos *et al.*, 2015), however, no changes in body weight and fat pad stores in MPH rats. In rats, the anorexigenic effect of MPH has been demonstrated in a dose-dependent manner; for example, daily doses of 5 mg/kg do not induce body weight loss during development (Montagnini *et al.*, 2014). Whereas in humans, weight loss and reduced appetite are the most common adverse effect associated to MPH (Thanos *et al.*, 2015). Although the mechanisms involved in reduced food intake and weight loss by MPH are not totally understood, it is known that amphetamine-like drugs are sympathomimetic agents with marked central and peripheral stimulant properties (Mariotti *et al.*, 2013; Bou Khalil *et al.*, 2017).

Interestingly, metabolism impairment is evident in adult rats treated with MPH at the adolescence period. MPH rats presented high food intake, associated to elevated body weight gain and fat pad stores. Studies have been reporting that cessation of stimulant drugs use may cause a significant increase in body weight and appetite, accompanied by metabolic readaptation, which is interpreted as a growth rebound (Pizzi *et al.*, 1986; Benard *et al.*, 2015). Interestingly, after 3 months cessation of MPH treatment during adolescence significantly increased neuropeptide Y (NPY) levels in striatum, suggesting a correlation to weight gain (Gray *et al.*, 2007). However, contrary to our findings, some previous studies have shown that unlike neonatal rats, periadolescent rats treated with MPH failed to show any growth impairment. They suggest differential effects of MPH on growth in different phases of development (Sprague & Sleator, 1977; Gray *et al.*, 2007).

The hormones insulin and leptin act directly regulating adiposity via central nervous system (CNS) (Niswender & Schwartz, 2003). Interestingly, in this study, low levels of fasting insulin were observed at P52, which is closely related to a lean phenotype and normal glucose and insulin homeostasis. Conversely, increased fasting insulin levels and increased vagal parasympathetic activity were found at P110. An unbalanced autonomic nervous system (ANS), with high parasympathetic and low sympathetic activity, is associated to metabolic dysfunction and is particularly related to β-cell impairment in obese animals and humans (Balbo *et al.*, 2007). In this way, MPH treatment was able to alter parasympathetic activity, which is closely related to obesity showed in these animals at adulthood.

Although the overweight and metabolic dysfunction in adult rats were clearly observed in MPH group, little is known about the mechanisms that underlie these alterations. Prolactin is a hormone synthesized in the anterior pituitary gland which acts in β-cells activating its proliferation and increasing insulin secretion. Moreover, hyperprolactinemia can be associated to insulin resistance in humans and animals (Foa *et al.*, 1955; Bahceci *et al.*, 2003). Interestingly, the literature demonstrate that dopamine is involved in the regulation of prolactin secretion; the elevation of dopamine and/or stimulation of dopamine receptor D2 suppresses prolactin synthesis (Reis *et al.*, 1997; Park *et al.*, 2012). In this way, with the cessation of MPH treatment, and consequent decrease of dopamine in the synaptic cleft, probably caused an increase in prolactin release, collaborating to higher insulin secretion, insulin resistance and higher adiposity observed in MPH animals at P110. However, as a limitation of our study, we did not assess the dopamine and prolactin levels from these animals, which might exhibit changes caused by MPH treatment.

In the present study, at the end of MPH treatment, animals had an increased total cholesterol that can be attributed to the increase in HDL-C. Previously, it was demonstrated that MPH has a positive impact on the lipid and lipoprotein profile, it significantly decreases total cholesterol, triglycerides and LDL-C in patients diagnosed with ADHD treated for 3 months (Charach *et al.*, 2009). After discontinuation of MPH treatment, at P110, animals presented increased total cholesterol associated to an elevation in LDL-C levels. Psychostimulants long-term effects in lipid profile are not well understood, however, previously studies showed that MPH produces hepatic necrosis in mice indicating hepatotoxicity in a long-term use (Alam & Ikram, 2018). The precise long-term impact of MPH in liver and lipid profile requires more future investigation.

MPH treatment at a sensitive stage of development, as adolescence, may also affect anxiety and panic like behavior. In the present study, we showed the short and long-term effect of treatment in adolescent male rats on inhibitory avoidance, escape latencies and locomotor activity. Comparing inhibitory avoidance and escape latency from MPH-treated group at P52 and P110, we observed there was no difference, suggesting the first effects remained. Saline-treated group at P110 showed an increase in both latencies that could mistakenly suggest a possible anxiogenic- and an antipanic-like effect compared to the first time the same animals were submitted to the ETM. In addition, locomotor activity was reduced in both groups at P110 compared to P52, indicating a reduction in exploratory behavior. However, it is more likely these effects observed at P110 can be attributed to a long-term memory. The literature shows a wealth of evidence indicating ETM, specifically the inhibitory avoidance task, as a learning and a memory model (Bertoglio & Carobrez, 2000; Asth *et al.*, 2012). A previous investigation observed that rats locomotion in the open field was reduced after successive exposures in a similar way seen in our study (Djiogue *et al.*, 2018). This is a limitation of the model used; therefore,

the test performed at P52 is more accurate and precisely than the test at P110. Thus, the second test in the P110 could have retrieved the memory consolidated after the first test, reducing exploratory behavior in all tasks, as observed by increased avoidance and escape latencies and reduced locomotion.

Persistent alteration of monoaminergic transmission triggered by MPH chronic treatment at adolescence can potentially have developmental consequences in brain architecture and biochemical compounds. Indeed, the normal brain development requires a coordinated maturation of many processes and monoamines are important regulators (Gray *et al.*, 2007). We observed an anxiogenic-like effect in male rats on the last day of treatment, which persists until adulthood. Along the same line, previously studies observed anxiety-like behavior until adulthood in rats treated with 2.0 mg/kg of MPH during periadolescence and adolescence (Bolanos *et al.*, 2003; Britton *et al.*, 2007; Vendruscolo *et al.*, 2008). However, there are disagreements whether behavioral changes persist or not (Konrad-Bindl *et al.*, 2016). Some studies have showed that animals treated at adolescence with MPH presented anxiolytic-like behavior at adult life (Gray *et al.*, 2007; Boyette-Davis *et al.*, 2018).

Experimental studies in animal models showed that many brain regions are involved in anxiety symptoms, such as hippocampus, amygdala, prefrontal cortex, and nucleus accumbens. Various mechanisms and neurotransmitters are involved in the regulation of anxious states; it has been suggested that dopaminergic system may play a central role in regulating anxiety-like behaviors (Zarrindast & Khakpai, 2015). Several evidence suggests that the mesolimbic/cortical dopamine systems seem to be involved in drugs affecting anxiety. Thus, increased dopamine in the synaptic cleft, for example when there is inhibition of reuptake by MPH, can induce an anxiogenic effect (Nasehi *et al.*,

2011; Zarrindast & Khakpai, 2015). There is no specific study showing the action of dopamine transporter inhibition on the anxiogenic effect and their mechanisms.

At P52, we showed that MPH animals presented antipanic-like effect. Although MPH is commonly believed to affect primarily the dopamine system through blockage of dopamine transporter, evidence from neurochemical, histochemical and behavioral studies suggests that MPH can also affect noradrenergic and serotonergic systems (Gainetdinov *et al.*, 1999). Previously studies demonstrated short and long-term effects of MPH on frontal serotoninergic system (Daniali *et al.*, 2013) and MPH acting as an agonist of serotonin receptor (5-HT1A), an important pathway in antipanic-like response (Faraone, 2018). However, further studies are needed to clarify the effects of early methylphenidate treatment on panic behavior.

In conclusion, chronic treatment with a low dose clinically relevant of MPH at adolescence programs male rats to overweight, metabolic dysfunction and behavioral alterations at adulthood. The effect of discontinued treatment of MPH requires further examination in view to verify the mechanisms involved in the metabolism and behavior programming.

Author Contributions

I.P.M., P.C.d.F.M and A.M. and were responsible for the conception and design of the experiments. J.C.M.; C.C.I.M.; A.P.; L.P.T.; K.P.R.; C.S.; A.M.P.M.; R.V.; T.A.R.; E.A.A. were responsible for the collection, analysis and interpretation of the data. All authors were involved in drafting the article and critically revising it for intellectual content. All authors approved the final version of the manuscript submitted for publication.

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Figures

Figure 1

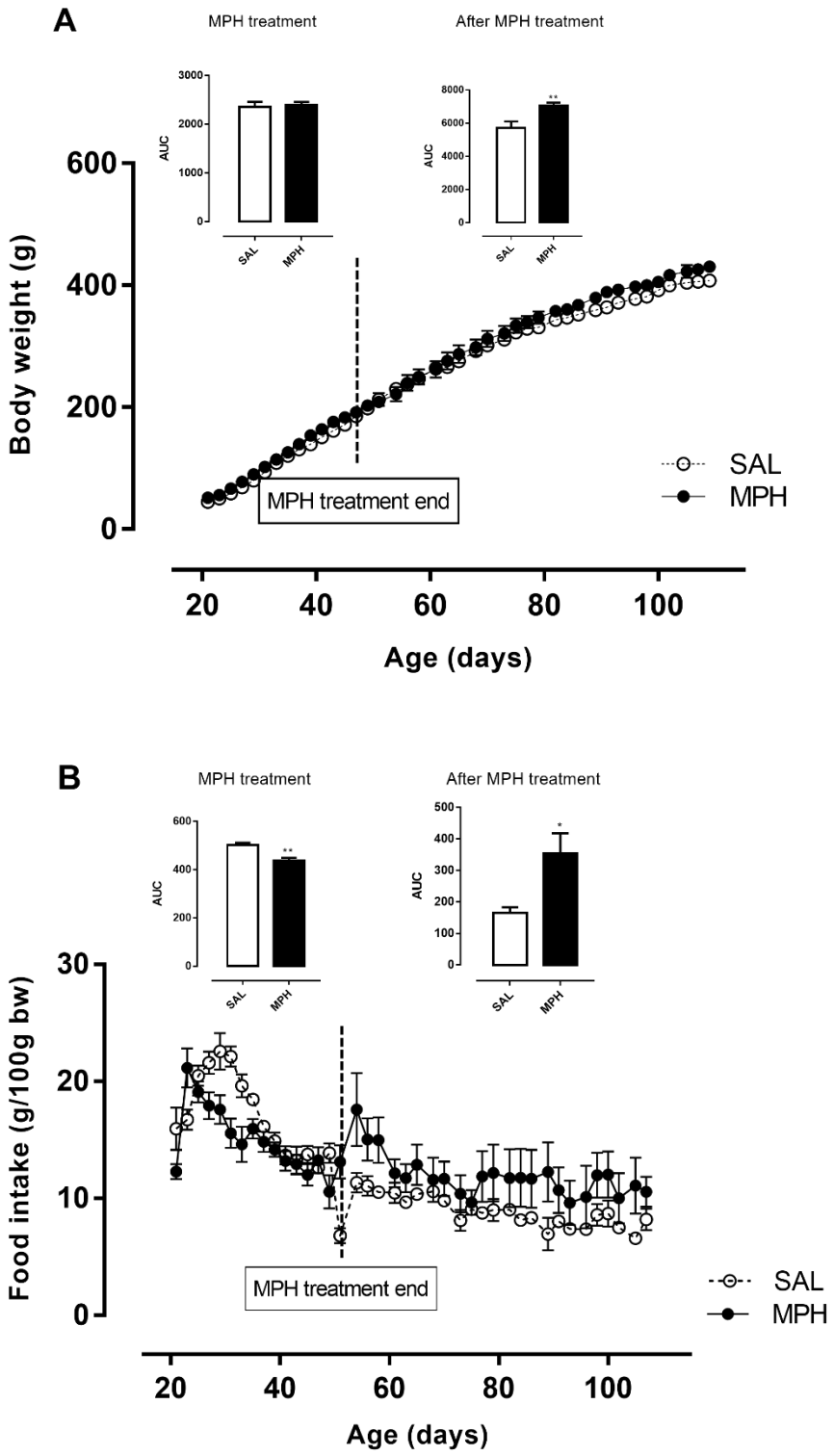

Figure 2

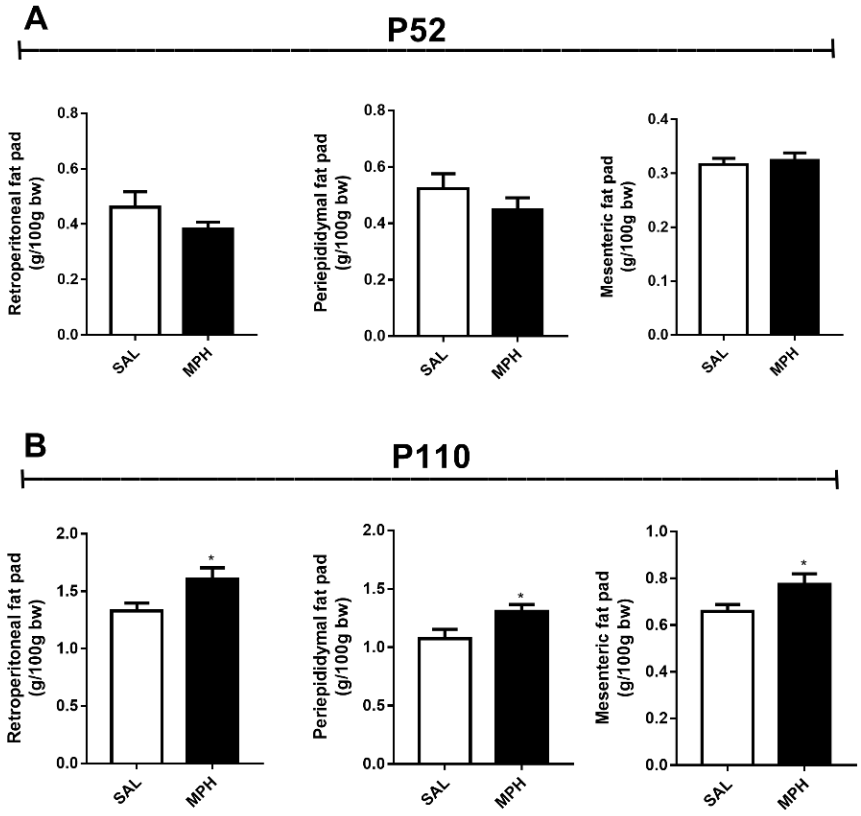

Figure 3

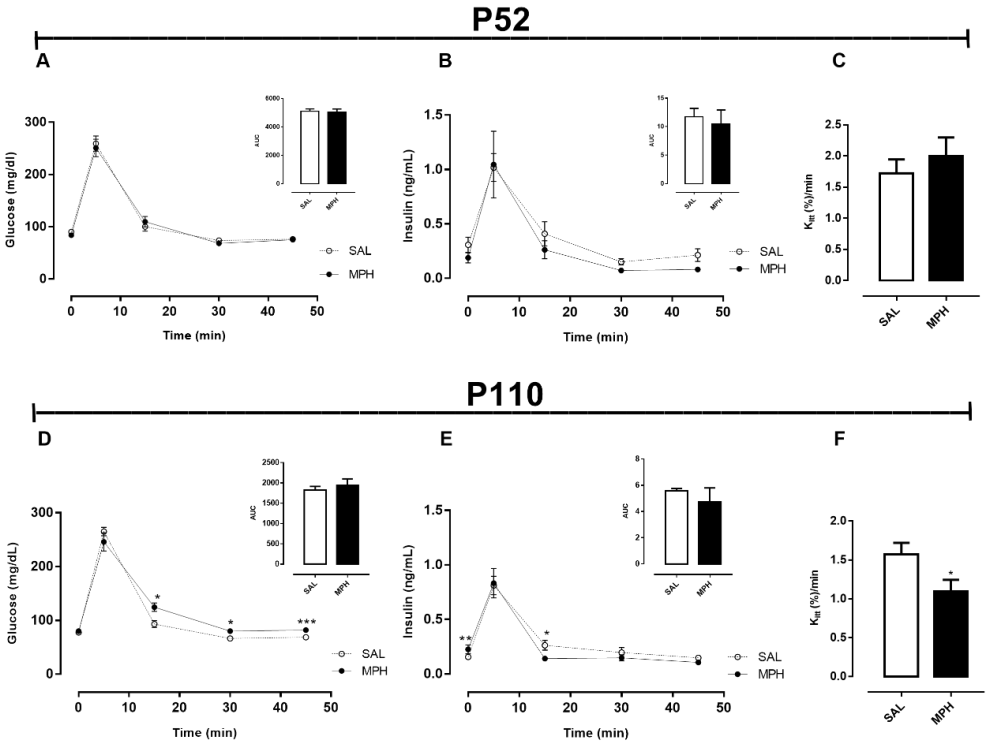

Figure 4

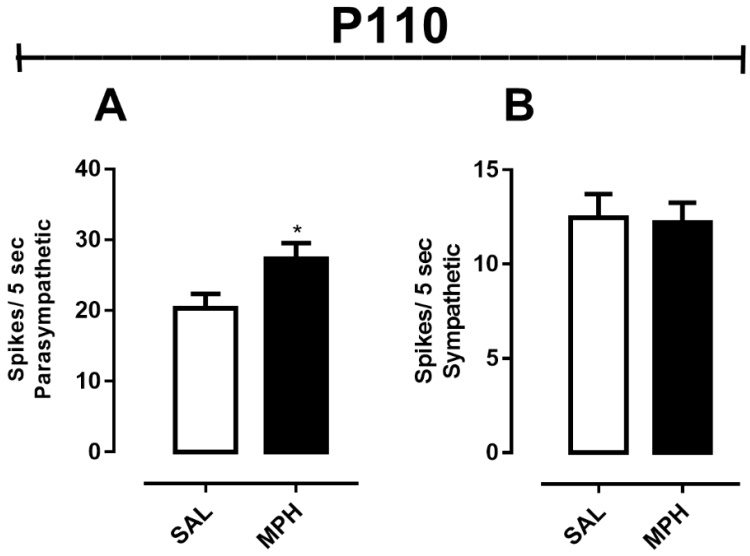

Figure 5

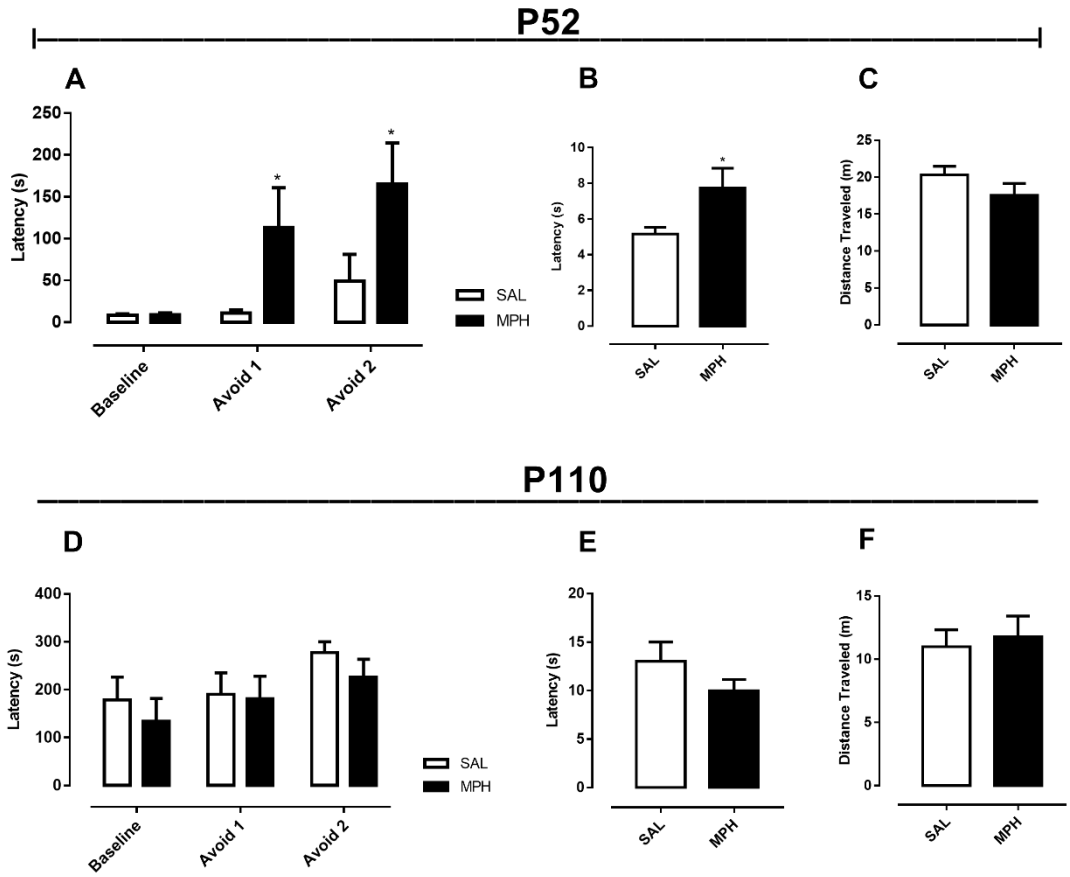

Table 1 – Biometric and biochemical parameters in the rats just after the MPH treatment (P52) and at adulthood (P110).

Parameters	P52		P110	
	SAL	MPH	SAL	MPH
Final body weight (g)	204.9±6	217.2±3.7	404.8±7	428.7±7.5*
Fasting glycemia (mg/dl)	90.2±4.9	83.7±2.9	78±3.4	83.1±2
Fasting insulinemia (ng/ml)	0.24±0.03	0.12±0.04*	0.14±0.01	0.29±0.03**
Total cholesterol (mg/dl)	66.2±3.2	78.6±3*	72.8±2	82.5±3.7*
Triglycerides (mg/dl)	36.2±4	36.6±3.7	63.8±2	59.1±2.7
HDL-C (mg/dl)	27.1±1.9	33.5±2.1*	38.1±2.2	39.2±1.7
LDL-C(mg/dl)	35.3±2	41.7±2.5	27.5±1.9	36.9±3.2*
VLDL-C (mg/dl)	7.2±0.8	6.7±0.4	12.7±0.4	11.8±0.5

Data are presented as the mean ± SEM obtained from 9-12 rats from 4 litters in each experimental group. Significant differences between SAL and MPH group are represented by *$p<0.05$ and ** $p<0.01$ by Student's *t* test. SAL, saline; MPH, methylphenidate; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol and VLDL-C, VLDL-cholesterol.

Figure Legends

Figure 1. Body weight (A) and relative food intake (B). Data are presented as the mean ± SEM of 12 rats from 4 different litters. The upper panels, as an inset to each figure depict the area under the curve (AUC) for both periods, during MPH treatment (21 to 51 days old) and after MPH treatment (52 to 110 days old). Significant differences between SAL and MPH group are represented by *p<0.05 and **p<0.01 by Student's *t* test. SAL, saline; MPH, methylphenidate.

Figure 2. Retroperitoneal, periepididymal and mesenteric fat pad stores at P52 (A) and P110 (B). Data are presented as the mean ± SEM of 9-12 rats from 4 different litters. Significant differences between SAL and MPH group are represented by *p<0.05 by Student's *t* test. SAL, saline; MPH, methylphenidate.

Figure 3. Blood glucose and insulin during ivGTT and K_{itt}. Glucose (A), insulin (B) and K_{itt} (C) of P52. Glucose (D), insulin (E) and K_{itt} (F) of P110. Data are presented as the mean ± SEM of 9-12 rats from 4 different litters. Significant differences between SAL and MPH group are represented by *p<0.05, **p<0.01 and ***p<0.001 by Student's *t* test. SAL, saline; MPH, methylphenidate.

Figure 4. Parasympathetic (A) and sympathetic (B) electrical nerve activity at P110. Data are presented as the mean ± SEM of 9-12 rats from 4 different litters. Significant differences between SAL and MPH group are represented by *p<0.05 by Student's *t* test. SAL, saline; MPH, methylphenidate.

Figure 5. Elevated T-maze - avoidance, merged escape and locomotion. ETM avoidance (A), merged escape (B) and locomotion (C) at P52. ETM avoidance (D), merged escape (E) and locomotion (F) at P110. Data are presented as the mean ± SEM of 8 rats from 4 different litters. Significant differences between SAL and MPH group are represented by *p<0.05 by Student's *t* test or Holm-Sidak's post hoc test. SAL, saline; MPH, methylphenidate.

Low-protein diet induced HPA axis hyperactivation and altered milk composition imprints the metabolism of weaned male rat offspring

Isabela Peixoto Martins[1,2]; Rodrigo Vargas[1,3]; Lucas Paulo Jacinto Saavedra[1]; Sarah Rickli[1], Camila Cristina Ianoni Matiusso[1]; Audrei Pavanello[1,3]; Lucas Casagrande[2]; Maria José Pastre[2]; Júlia Berno Oliveira[3]; Ariadny Martins de Almeida[3]; Júlio Cezar de Oliveira[4]; Ananda Malta[1]; Paulo Cezar de Freitas Mathias[1]

[1]Department of Biotechnology, Genetics, and Cellular Biology, State University of Maringa, Maringa, PR, Brazil.

[2]Departament of Morphological Sciences, State University of Maringa, Maringa, PR, Brazil.

[3]Health Sciences Center, Unicesumar, Maringa, PR, Brazil.

[4]Health Education and Research Center (NUPADS), Institute of Health Sciences, Federal University of Mato Grosso, Sinop, MT, Brazil.

Correspondence to: Isabela Peixoto Martins; Department of Biotechnology, Genetic and Cell Biology; Laboratory of Secretion Cell Biology – Block H67, room 19, State University of Maringa/UEM - Phone/Fax + 55 (44) 3011 4892. Colombo Avenue 5790, 87020-900, Maringá/PR – Brazil. E-mail address: isa.peixotomartins@gmail.com.

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Abstract

Maternal protein-caloric restriction during lactation can malprogram the offspring to a lean phenotype associated to metabolic dysfunction in early life and at adulthood. This programming is mediated by the milk offered to the pups and little is known about the modifications in milk composition derived from a nutritional insult. We investigated the relationship between nutritional stress, mother's behavior and metabolism, milk composition and offspring parameters. Moreover, we focused on the role of HPA axis hyperactivation through lactation. To this, dams were fed with low-protein diet (LP, 4% protein) during the first two weeks of lactation or a normal protein diet (NP, 20% protein) all lactation period. Dams, milk, and offspring analysis were conducted at postnatal day (P) 7, P14 and P21. We observed body weight and food intake decrease in dams, associated to reduced fat pad stores and increased corticosterone levels at P14. The stressed LP dams demonstrated alterations in behavior and offspring care. Despite nutritional deprivation, dams adapted the metabolism to provide adequate energy supply to milk, however, we demonstrated elevated corticosterone and total fat levels at P14. Male offspring also showed increased corticosterone at P7, associated to a lean phenotype and alterations in white and brown adipose tissue morphology development. In conclusion, protein restriction diet exposure of dams during lactation promotes an increase in glucocorticoids levels in dams, milk, and offspring, associated to maternal behavior and milk composition alterations. Altogether, glucocorticoids and milk composition could play an important role in the metabolic programming induced by maternal undernutrition.

Keywords: lactation; protein; milk; corticosterone.

Introduction

The increasing pandemic of cardiometabolic syndrome worldwide is evident and several of the diseases that appear in adulthood can have origins in early life [1,2]. The developmental origins of health and disease (DOHaD) concept describes through scientifical data the impact of maternal malnutrition [3], among other factors, in the physiological developmental and neuronal circuitry maturation of offspring.

A protein restriction diet in rats is a well-established model used to investigate the link between early malnutrition and adult metabolic disorders [4] once maternal food restriction is an important insult during perinatal life. In particular, the suckling period constitutes an important window of susceptibility in rodents once the maturation of central nervous system (CNS) and endocrine organs occurs at the first weeks after birth [5]. Our previous studies showed that maternal protein-caloric restriction during lactation programs adult offspring to a lean phenotype, hypermetabolic status, and resistance to obesity [6,7].

The main factor involved in the neonatal growth and development is the milk offered to the offspring once maternal milk is widely known as the gold standard and best feeding source for newborns [8]. According to the World Health Organization (WHO), breastfeeding shows neurodevelopment benefits in a short and long-term way [9,10]. Epidemiologic evidence strongly suggest that breastfeeding protects against infections in the first years of life. Moreover, recent studies pointed that maternal milk could shield the offspring against metabolic disorders and obesity in childhood and adulthood [11]. Despite all the benefits, little attention has been given to the quality of the milk available during lactation. The amount of macronutrients, micronutrients, and hormones levels may be involved in the offspring metabolic programing [12]. Nonetheless, the implications of

maternal undernutrition on milk composition and their consequences to neonatal development have been poorly studied.

The content of carbohydrates, lipids and proteins present in breast milk is regulated to guarantee the normal development of the offspring; therefore, a healthy nutritional environment in perinatal life is important to the quality of the milk. Physiologically, the concentration of macronutrients in the milk of healthy mothers changes through the stages of lactation. [13]. However, perinatal intake of maternal protein, for example, has an impact on the composition of milk protein in the middle of lactation [11]. In addition, the concentrations of lipids and carbohydrates can be also altered and, consequently, affect the total energy density in the milk, programming the offspring to metabolic disorders at adulthood [14].

Among the hormones found in milk, glucocorticoids, especially corticosterone, play a critical role in early development [15,16]. They have been highlighted as an important hormone involved in the link between stressful conditions at perinatal life, such as malnutrition, and cardiometabolic diseases at adulthood [17]. Glucocorticoids are essential to the development/maturation of tissues/organs in the intrauterine and perinatal life. In addition, they are involved in glucose metabolism, lipid biosynthesis and distribution, food intake and thermogenesis [18].

Hypothalamus-pituitary-adrenal (HPA) axis regulates as the negative feedback the production and secretion of glucocorticoids [19,20]. Some manipulations at postnatal life alter the functioning of the HPA axis at adulthood, including neonatal handling, maternal deprivation, exposure to synthetic glucocorticoids, modifications of maternal behavior and nutrient restriction [21,22]. The literature shows evidence about the strongly effect of dietary restriction on maternal behavior and HPA axis dysfunction [23]. It is known that modifications in maternal care is a critical influence in the development,

therefore, variations in maternal behavior regulate the neuroendocrine, behavioral, emotional, and cognitive development of pups [24]. However, the exactly impact of malnutrition during lactation over hyperactivation of HPA axis, changes in maternal behavior and hormonal content in milk has not been precisely studied.

Although previously studies have shown the impact of protein-caloric restriction during lactation on the offspring metabolism at weaning and adulthood [25,26], few studies observed the relationship between maternal behavior, milk composition and pups metabolism through lactation as a potential mechanisms behind this programming. Thus, in this study, we aimed to evaluate whether undernutrition, a stressful insult, can hyperactivate the HPA axis inducing changes in maternal behavior, milk composition and metabolic features in offspring at early life.

Materials and Methods

Ethical approval

All experiments were conducted according to the ARRIVE guidelines [27] and with Brazilian Association for Animal Experimentation (COBEA) standards. Protocols were approved by the Ethics Committee in Animal Research of the State University of Maringa (protocol number 5409020520).

Maternal dietary manipulation and animal groups

Lactating Wistar rat dams (n= 15 rat dams from each experimental group) were fed either normal-protein rodent chow containing 20.5% protein (NP group; Nuvilab®, Curitiba, PR, Brazil) throughout lactation or an isocaloric low-protein diet containing 4% protein (LP group) from delivery until the 14th day of lactation, returning to a normal diet for the remaining third part of the lactation period. The composition of low-protein diet has been previously described [28]. At birth, the litter size was adjusted to eight pups (four male and four female) per lactating dam. Only male offspring was analyzed in this experiment. Dams and male offspring of NP and LP groups were analyzed at postnatal day (P) 7, 14 and 21 (n= 5 litters per group at each age). Throughout the experimental period, the animals were kept under controlled temperature (23 ± 2°C) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle) conditions. The animals received water and food *ad libitum*.

Body weight, food intake and fat pad stores measurements

Rat dams and offspring were weighted every two days during the lactation period. Food intake of rat dams was determined every two days and calculated as the difference

between the amount of diet remaining (Df) and the amount presented previously (Di) divided by the number of days: [FI(g) = (Df –Di)/2]. The area under curve (AUC) was calculated to body weight (bw) and food intake (fi). At P7, 14 and 21 rat dams were anaesthetized (thiopental, 45 mg/kg of bw), decapitated and laparotomized to remove their retroperitoneal, uterine and ovarian fat pads stores. At P21, male offspring underwent the same procedure to removal their retroperitoneal, periepidydimal, mesenteric, brown fat pads and adrenal gland. The weight of fat pads and adrenal gland were expressed in relation to the bw of each animal (g/100 g of bw).

Glucose metabolism assessment of dams

At P7, 14 and 21 (n= 5 dams per group at each age) of lactation, rat dams were submitted to 6-hour fast to perform intraperitoneal insulin tolerance test (ipITT). They received an injection of insulin (1 U/kg of bw), and blood glucose was measured using a glucometer, as previously reported [29]. Glucose was determined at 0, 15, 30, 45 and 60 minutes. Subsequently, the rate of glucose tissue uptake or the rate constant for plasma glucose disappearance (K_{itt}) was calculated. Additionally, after two days, dams were subjected to the intraperitoneal glucose tolerance test (ipGTT), as previously described [30]. After a 12-hour fast, blood samples were removed by the tail before the injection of glucose (2 g/kg of bw) (0 min) and 15, 30, 60 and 120 min afterward. Blood glucose was measured using a glucometer. The glucose response during the test was calculated by AUC.

Maternal behavior analysis

The maternal behavior of lactating dams was scored in alternate days during four periods of 72-min observation sessions during 21 days of lactation (starting from P2, until P20).

178 Observations occurred at regular times with three periods during the light phase (8:00
179 AM, 12:00 AM and 16:00 AM) and one period during the dark phase (20:00 PM) of the
180 light-dark cicle. Within each session, the behavior of each mother was scored every 3
181 minutes (25 observations per 4 period per day for a total of 100 observations per mother
182 per day) we identified five parameters considered maternal and four non-maternal
183 parameters, as following: (1) licking pups (either its body surface or its anogenital region),
184 (2) nursing pups in an arched-back posture, (3) "blanket" posture in which the mother
185 lays over the pups, (4) passive posture in which the mother is lying either on her back or
186 side while the pups nurse, (5) nest building, (6) feeding, (7) exploring the cage housing,
187 (8) movement away from the pups and (9) self-grooming [24,31]. Data are reported as
188 the percentage of observations in which pups received the target behavior (number of
189 observations in which the target behavior was recorded divided by the total number of
190 observations × 100).

191

192 *Milk sample collection and nutritional analysis*

193 For milk sample collection, dams at P7, 14 and 21 (n= 5 dams per group at each age) of
194 lactation were separated from their pups for 2 hours before the procedure. The fed dams
195 were anesthetized (thiopental, 45 mg/kg of bw, i.p.) and received an injection (2.5 UI/kg
196 of bw, i.p.) of synthetic oxytocin (Oxytocin®, Chemical Union, Embu, São Paulo, Brazil)
197 to induce milk secretion. Breast milk samples were collected by manually massaging the
198 nipple (0.5ml/dam) and stored at −20 °C for subsequent analysis. Milk samples were
199 diluted (1:20 v/v) in saline solution (0.9% NaCl) for measurements [32].

200 Total protein content was evaluated by enzymatic colorimetric method by a commercial
201 kit (Gold Analisa® Belo Horizonte, Minas Gerais, Brazil), according to the

manufacturer's instructions [33]. Total carbohydrate content in milk was analyzed using the phenol-sulfuric acid method in microplate format, as previously described [34]. Total fat content in milk samples was measured by the Folch method [35,36].

Biochemical detections in plasma and milk

Dams and pups serum glucose concentration were measured by the glucose oxidase method using a commercial kit (GoldAnalisa®; Belo Horizonte, MG, Brazil) [37]. Triglycerides and protein were measured in plasma samples by colorimetric method using commercial kits (Gold Analisa®; Belo Horizonte, MG, Brazil) [33,38].

Corticosterone levels in plasma and milk

The plasma levels of corticosterone (catalogue number ADI-900-097, Enzo® Life Sciences, Plymouth Meeting, PA, USA) was quantified by commercial ELISA kit following the manufacturer's recommendations. The intra- and interassay coefficients of variation were 7.7% and 9.7% [26,39].

Histology of white adipose tissue (WAT) and brown adipose tissue (BAT)

At euthanasia, P21 pups had the retroperitoneal white adipose tissue (rWAT) and interscapular brown adipose tissue (iBAT) samples removed, placed in 4% paraformaldehyde, fixed for 24 hours, and then embedded in paraffin, as previously described [40]. Five µm sections for every 30 µm interval were made using a microtome and placed on glass slides. The slices were stained with hematoxylin and eosin, and the sections were examined using light microscopy (5 optic zones of 40x per sections).

Statistical analysis

The results are given as the mean ± the SEM and were subjected to Student's t-test, where $P<0.05$ was considered statistically significant. In maternal behavior parameters differences between groups was analyzed by repeated measures two-way ANOVA with lactation days and LP diet as factors. Post hoc comparisons were performed by the Holm-Sidak's test. Tests were performed using GraphPad Prism version 7.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

Maternal body composition, food intake and biochemical parameters.

As shown in AUC of Figure 1A, during lactation, LP dams had 17.6% ($P<0.001$) of reduction in body weight compared to NP mother. Associated with that, we observed a 48.4% lower food intake in LP dams compared to the control group ($P<0.0001$, Fig. 1B). Table 1 shows that LP dams displayed smaller retroperitoneal and uterine fat pads at P21 (32% and 11%, respectively, $P<0.05$) than NP dams.

In comparison with NP dams, in fasting conditions, LP mothers showed a reduction in protein levels at P14 (38%, $P<0.01$, Table 1). At the same stage of lactation (P14), LP dams presented 97% ($P<0.01$) higher triglycerides levels compared to NP group. In addition, LP dams displayed hyperglycemia at P21 (10.7%, $P<0.05$, Table 1) and presented less glucose levels during ipGTT at P7 (16.1%, $P<0.01$, Table 1) and 14 (14.1%, $P<0.05$, Table 1). Insulin sensitivity was not altered in LP dams through lactation, as demonstrated by K_{itt} in Table 1. As shown in Figure 1C, LP dams have higher levels of corticosterone at P14 (412%, $P<0.01$) than NP mothers.

Maternal behavior through lactation

According to two-way ANOVA, there was no significant effect of LP diet, lactational day (LD) or interaction between factors on observation percentage of blanket nursing (LP diet factor: $P=0.67$, LD factor: $P=0.06$, interaction: $P=0.44$, Fig. 2D). There is a significant effect of LD, but no significant effect of LP diet and interaction between factors on observation percentage of nest building (LP diet factor: $P=0.24$, LD factor: $P<0.001$, interaction: $P=0.19$, Fig. 2A), licking pups (LP diet factor: $P=0.06$, LD factor: $P<0.0001$, interaction: $P=0.09$, Fig. 2B) and total maternal behavior (LP diet factor: $P=0.53$, LD

factor: P<0.0001, interaction: P=0.22, Fig. 2F). There is an effect of LP diet and LD, without difference in interaction on observation of passive nursing (LP diet factor: P<0.001, LD factor: P<0.01, interaction: P= 0.08, Fig. 2E). We observed an effect of LP diet, LD, and interaction between factors on observation percentage of arched nursing (LP diet factor: P<0.05, LD factor: P<0.0001, interaction: P<0.01, Fig. 2C).

Regarding non-maternal behavior, we showed no significant effect of LP diet, LD, and interaction between factors in the percentage of self-grooming (LP diet factor: P=0.35, LD factor: P=0.07, interaction: P=0.3, Fig. 3D). There is an effect of LD and an interaction on observation percentage of feeding (LP diet factor: P=0.39, LD factor: P<0.0001, interaction: P<0.001, Fig. 3A). It was observed an effect of LD and interaction on observation percentage of total non-maternal behavior (LP diet factor: P=0.71, LD factor: P<0.0001, interaction: P<0.05, Fig. 3E). Finally, there were a significant effect in LP diet, LD and interaction between factors on exploring behavior (LP diet factor: P<0.05, LD factor: P<0.0001, interaction: P<0.0001, Fig. 3B) and non-exploring (LP diet factor: P<0.05, LD factor: P<0.0001, interaction: P<0.0001, Fig. 3C).

Milk composition assessment

Figure 4 shows the nutritional and hormonal parameters of LP dams milk. Protein concentration was increased at P7 (135%, P<0.01, Fig. 4A) and decreased at P21 (34%, P<0.05, Fig. 4A) in LP dams milk, without change at P14. Total fat was increased in LP milk at P7 and 14 by 83.3% (P<0.01, Fig. 4B) and 111% (P<0.0001, Fig. 4B) respectively. At P21, total fat content at LP milk was 43.4% (P<0.05, Fig. 4B) reduced when compared to NP samples. Furthermore, as showed in Figure 4C, total carbohydrates at LP milk were increased at P7 (53.5%, P<0.01) compared to NP milk. At P21, this parameter was

294 decreased by 68% (P<0.0001) in LP dams milk. At P14 not significantly change was
295 observed in total carbohydrates concentrations. In Figure 4D, we showed elevated
296 corticosterone levels in milk samples of LP group at P14 (133.3%, P<0.05).

297

298 *Pups body composition*

299 As expected, the body weight of LP pups through lactation was decreased by 40.5%,
300 indicated in AUC (P<0.001, Fig. 5A) and by 35% at weaning (P<0.001, Table 2). At P21,
301 LP rats presented smaller retroperitoneal (41.19%, P<0.001), periepidydimal (33.98%,
302 P<0.001), mesenteric (17.89%, P<0.05) and brown (15.94%, P<0.01) fat pads than NP
303 rats (Table 2). Adrenal gland weight was similar between groups, as demonstrated in
304 Table 2.

305

306 *Pups' biochemical parameters*

307 Table 3 shows that LP pups reduced protein plasmatic levels at P7 and 14 (12.25%,
308 P<0.05 and 26.38%, P<0.0001, respectively). At P21 there is no difference between
309 groups in protein concentration. Serum triglycerides was higher in LP pups at P21
310 (35.74%, P<0.01. Table 3) than control group. Moreover, LP pups are hyperglycemic at
311 P7 (54.26%, P<0.01, Table 3). According to the Figure 5B, LP pups showed elevated
312 levels of corticosterone at P7 (92.22%, P<0.05), without significantly alteration at P14.

313

314 *Pups' morphometric analysis of rWAT and iBAT*

315 According to Figure 6, LP rats had white adipocytes area 45.28% lower than NP rats
316 (P<0.0001, Fig. 6A) and the number of cells was 64.9% higher in LP group (P<0.0001,

Fig. 6B). About iBAT, Figure 6C and 6D demonstrated an increase by 30.75% and 37.7% in adipocytes area and number of cells in LP rats compared to NP ones, respectively ($P<0.0001$).

Discussion

There is a gap in the understanding of the relationship between maternal nutrition during lactation, milk composition and metabolic programming features in offspring. Thus, in the present study, we analyzed the impact of a low-protein diet at the first two weeks of lactation on maternal metabolism and behavior and the correlation with milk composition and offspring metabolism through lactation period. The major finding was that stress nutritional provoked a hyperactivation of HPA axis in dams and in offspring, through elevated corticosterone in milk. In addition, macronutrients balance in milk was modified by maternal protein-caloric restriction and this may be related to changes in metabolism and tissue development in the offspring.

A low-protein diet during lactation provoked low body weight and food intake in dams and in the offspring through this period, as previously observed [12,41]. The maternal reduced weight can be attributed to the lower amount of fat stores. In addition, numerous metabolic adaptations occur during lactation in dams to support adequate milk synthesis with the necessary balance of compounds to offspring development [42]. In this study, malnourished mothers probably undergo metabolic adaptations, including increased muscle proteolysis and lipolysis. Likewise, the high concentration of glucose and triglycerides in maternal plasma, observed also in 10% protein restriction at perinatal life [35] consists of an adaptation to guarantee the energy supply to offspring through milk.

After delivery, dams show the ability to respond immediately to their offspring through maternal care. This maternal behavior is regulated by circulating hormones such as estrogen, progesterone, oxytocin, prolactin, and corticosterone [43]. Interestingly, in this study, in the middle of lactation LP dams presented elevated corticosterone levels. It is already known that caloric restriction diet increases total daily glucocorticoids release

[44], indicating an activation of HPA axis. Experiments showed that the removal of adrenal gland, the source of corticosterone, reduced maternal behaviors such as licking pups and arched nursing. Furthermore, there is evidence that corticosterone improves the mothers memory about pups in the postpartum period [45]. In our study, LP dams showed increase in some maternal behaviors during lactation, however, they spent more time exploring the environment compared to NP mothers. The maternal response to stress during perinatal period requires further investigation, since some studies indicate a state of anxiety that leads to greater care to offspring [46] and other studies show maternal neglect and increased non-maternal behaviors [47].

There is evidence about retardation in the decline of maternal behavior in low protein fed dams [23]. One of the probable reasons is that milk production was lower [12], which can contribute to low offspring milk consumption and, to compensate this, pups present increase in their feeding behavior. Associated to maternal metabolic and behavioral changes during lactation, milk composition of malnourished mothers was altered. At the early stage of lactation, nutrients and adequate energy supply must be given to the pups, which cannot synthetize many important metabolites to their development [48]. In this way, we observed that malnourished mothers adapted their metabolism, despite inadequate food intake, to maintain adequate protein levels in milk for as long as possible through lactation. The utilization of tissue protein reserves, especially muscles, has been suggested to be important in allowing lactating rats to sustain lactational performance under bad nutritional conditions [49].

The main carbohydrate in mammalian milk is generally the disaccharide lactose, synthetized in the mammary glands [50] and maternal plasma glucose is the predominant source of carbon for lactose synthesis [51]. We observed an elevation of total carbohydrates in the beginning of lactation, however, at weaning low protein fed dams

milk had lower carbohydrate content. At the same time, plasma glucose of dams was increased, as previously reported [35]. Indeed, in the period of nutritional recovery, although there is a high concentration of plasma glucose, communication of this nutrient with mammary gland was impaired. The mechanisms behind this process are unknown, however, it is important to point that the time and duration of low protein diet offered to the dams may result in different alterations in milk composition.

Interestingly, until P14 milk from low protein fed dams showed an elevation in total fat content, as already observed by other studies [11,35,52]. It has been demonstrated an increase in fatty acid mobilization from the mammary glands in malnourished dams, even though the low protein diet impairs the glands differentiation, proliferation, and development during lactation [35,53]. Adipose tissue and liver may be involved in the release of fat to mammary glands once protein restriction was shown to cause a fatty liver phenotype in dams [54,55]. The exact mechanisms by which the mother and their mammary glands mobilize more fat for milk are not known, this subject requires further investigation.

After birth, the pups undergo metabolic changes that are key in their healthy development. Fetal life is characterized by the predominant use of glucose as a metabolic fuel, however, in lactation there is a shift to a lipid-based diet, as lipids are very abundant in milk [56]. For this, a coordinate regulation of key genes expression occurs at the beginning of lactation to allow the pups to deal with the large amount of fatty acids available from milk and PPARα has a major role in this process. The higher quantity of free fatty acids provided by LP dams milk activates offspring PPARα expression more than in control offspring [57]. Fibroblast growth factor 21 (FGF21) is a recent found metabolic regulator, which secretion by the liver are controlled by PPARα. FGF21 stimulates lipolysis in white adipose tissue and enhances thermogenesis in brown adipose

423 tissue. Physiologically, at the first week of lactation, FGF21 rises in pups and decrease in
424 the second week [56,58]. An elevated percentage of lipids in milk may prolong the
425 duration of the FGF21 peak in lactation, programming the offspring to an elevated status
426 of lipolysis and lean phenotype at weaning and adulthood [6]. However, we did not
427 measure the levels of FGF21 and PPARα in pups in the present study, therefore this
428 hypothesis needs more study.

429 The levels of glucocorticoids at perinatal life influence the growth and
430 differentiation of many tissues [59]. To adequate development, activity of adrenal gland
431 decreases after birth and consequently corticosterone concentration in rat's plasma stays
432 low in the first 12 days. Moreover, mammary gland present a mechanism to keep a low
433 and stable glucocorticoids concentration in milk [16]. In the present study, we found
434 elevated corticosterone levels in milk and in offspring of LP group in the very beginning
435 of lactation, indicating a perturbation of HPA axis and consistently to plasma glucose
436 increase in rats at P7 [60]. It is interesting that glucocorticoids and FGF21 are regulated
437 in a feed-forward way, indeed, in a food privation situation chronically elevated FGF21
438 levels increases corticosterone production, and, in the same line, glucocorticoids directly
439 regulate the expression of FGF21 gene and their release [61].

440 At adult life, low protein during lactation rats shows elevated sympathetic activity
441 and vagal hypoactivity [6,7]. Those physiological alterations were probably programmed
442 at early life as a consequence of stress exposure and adipose tissue differentiation and
443 function was affected in a short and long-term way [62]. At weaning, low protein rats
444 have lower adipocyte area in WAT, consistently with smaller fat pads. Also, LP rats
445 present reduced weight, increased number of cells and adipocytes area in BAT, which is
446 associated to higher thermogenesis and energy expenditure [63,64]. Recently, was
447 demonstrated that stress, through HPA axis and Sympathetic Nervous System (SNS)

activation, result in a lean phenotype and/or obesity resistance whether brown adipose tissue is recruited, and thermogenesis is increased [62].

In conclusion, protein restriction diet exposure of dams at the lactational phase promotes an increase in corticosterone plasma levels in dams, offspring and milk. In addition, maternal behavior was altered in response to a nutritional stress condition. Altogether, increased HPA axis activity in dams and offspring, associated to high fat content in milk at the first days of lactation could play an important role in the metabolic programming induced by maternal undernutrition, including the obesity resistant phenotype of these animals at adulthood. Thereby, further studies are needed to clarify the mechanisms involved in programming through nutritional stress during lactation.

Author Contributions

I.P.M., R.V.; A. M. and P.C.d.F.M. and were responsible for the conception and design of the experiments. S.R.; L.P.J.S.; C.C.I.M.; A.P.; L.C.; M.J.P.; J.B.O.; A.M.A. were responsible for the collection, analysis and interpretation of the data. All authors were involved in drafting the article and critically revising it for intellectual content. All authors approved the final version of the manuscript submitted for publication.

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Figures

Figure 1

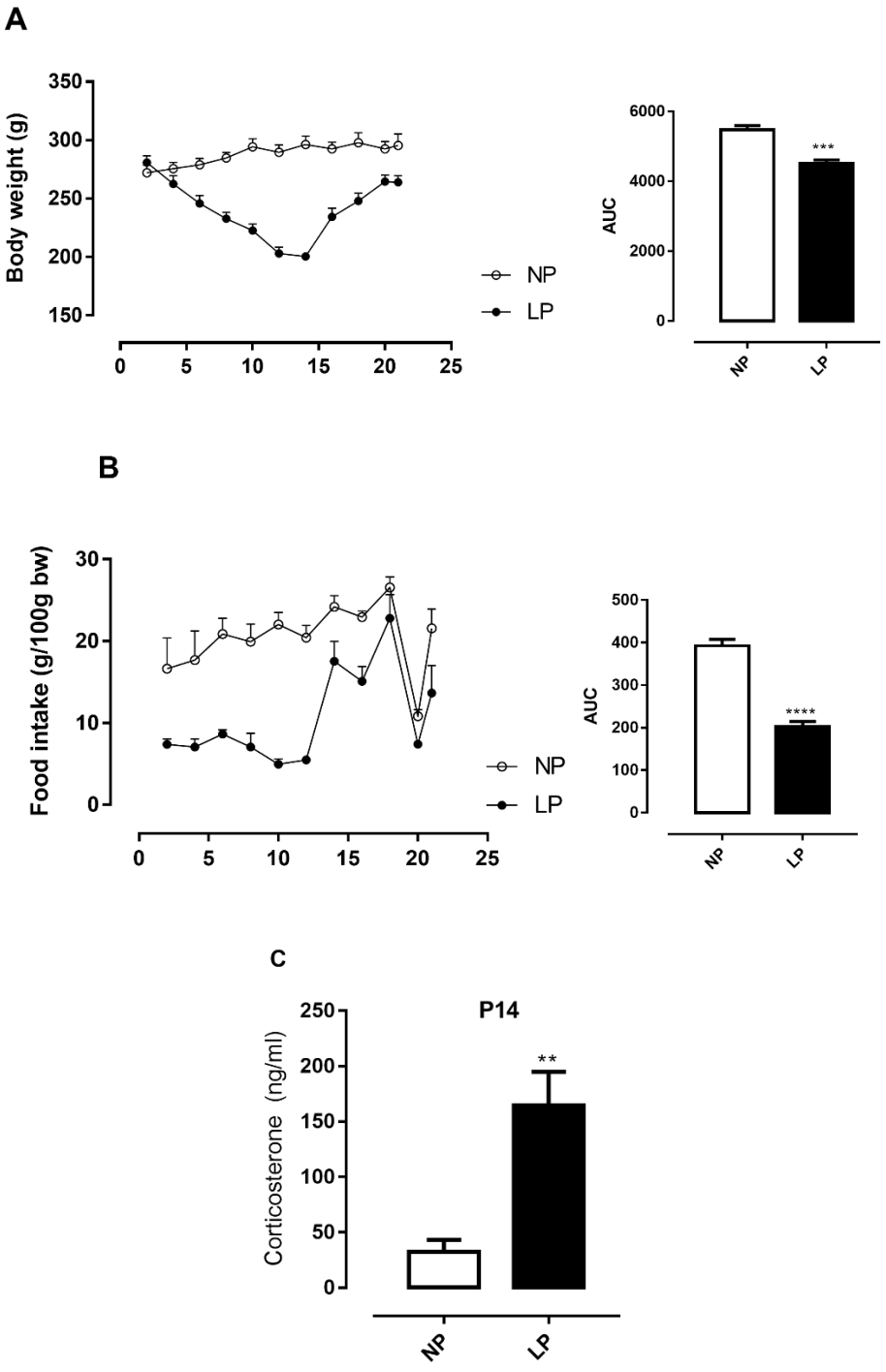

Figure 2

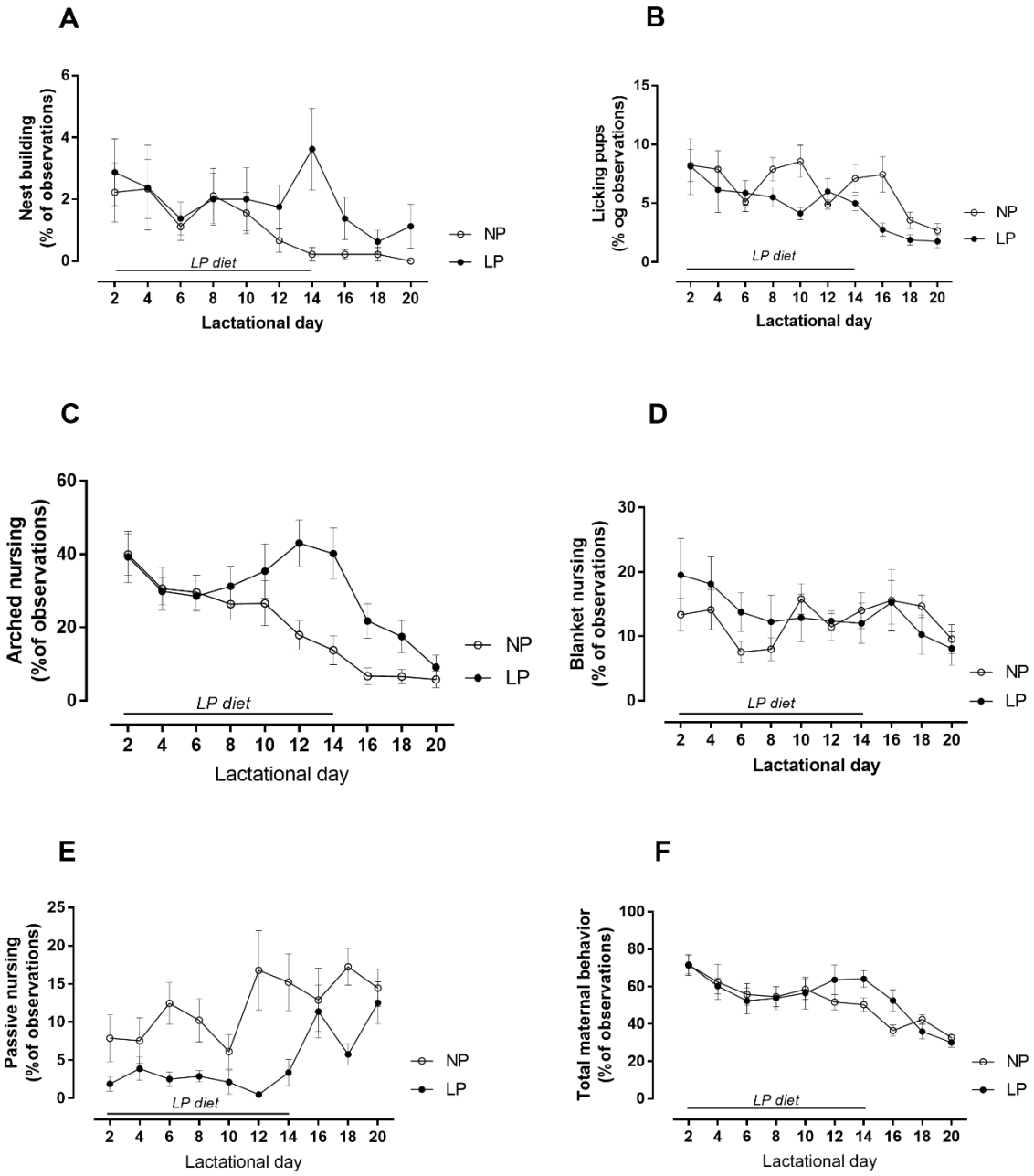
A
Nest building (% of observations)
LP diet
NP
LP
Lactational day
B
Licking pups (% og observations)
LP diet
NP
LP
Lactational day
C
Arched nursing (%of observations)
LP diet
NP
LP
Lactational day
D
Blanket nursing (% of observations)
LP diet
NP
LP
Lactational day
E
Passive nursing (%of observations)
LP diet
NP
LP
Lactational day
F
Total maternal behavior (%of observations)
LP diet
NP
LP
Lactational day

Figure 3

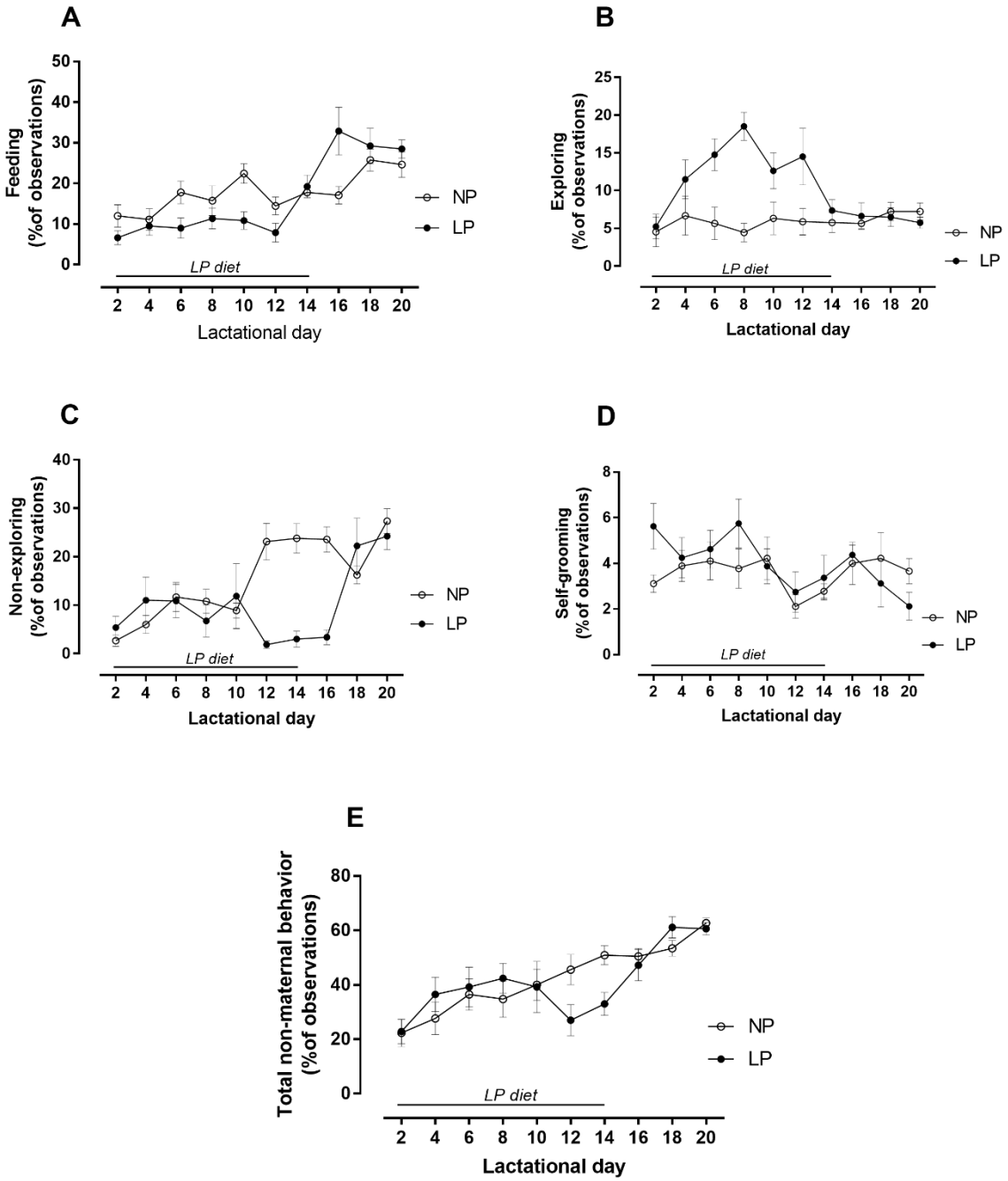
A
Feeding (%of observations)
50
40
30
20
10
0
NP
LP
LP diet
2 4 6 8 10 12 14 16 18 20
Lactational day
B
Exploring (%of observations)
25
20
15
10
5
0
NP
LP
LP diet
2 4 6 8 10 12 14 16 18 20
Lactational day
C
Non-exploring (%of observations)
40
30
20
10
0
NP
LP
LP diet
2 4 6 8 10 12 14 16 18 20
Lactational day
D
Self-grooming (%of observations)
8
6
4
2
0
NP
LP
LP diet
2 4 6 8 10 12 14 16 18 20
Lactational day
E
Total non-maternal behavior (%of observations)
80
60
40
20
0
NP
LP
LP diet
2 4 6 8 10 12 14 16 18 20
Lactational day

Figure 4

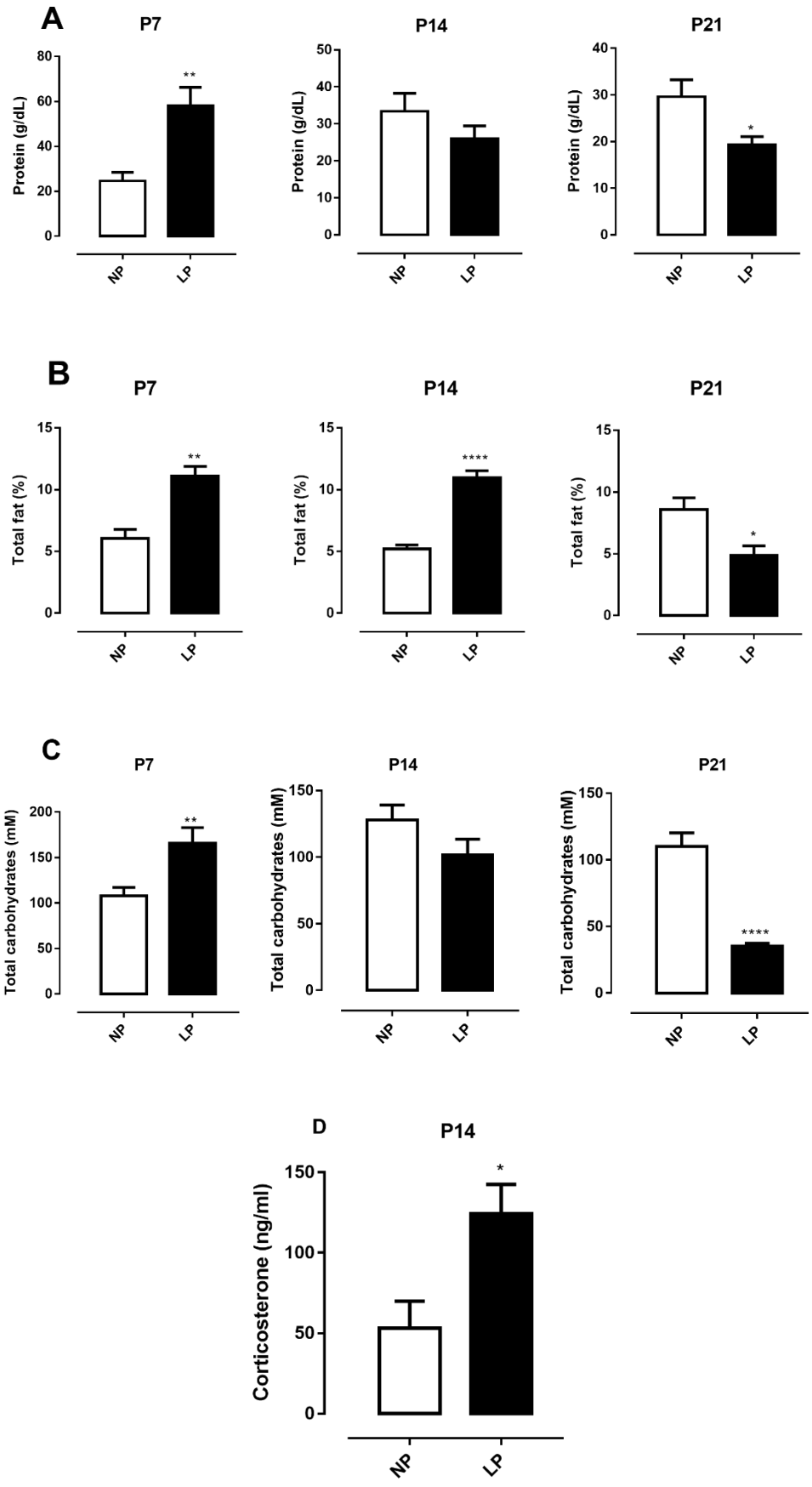
A
P7
P14
P21
Protein (g/dL)
NP
LP
B
Total fat (%)
C
Total carbohydrates (mM)
D
Corticosterone (ng/ml)

Figure 5

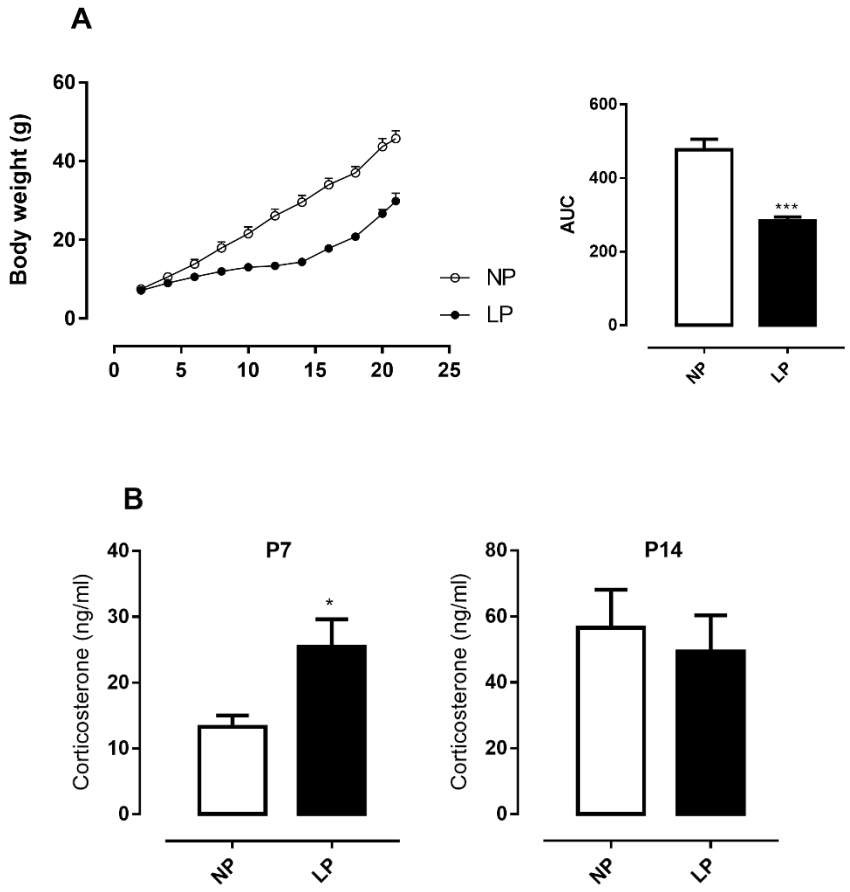
A
Body weight (g)
60
40
20
0
0
5
10
15
20
25
NP
LP
AUC
600
400
200
0
***
NP
LP
B
P7
Corticosterone (ng/ml)
40
30
20
10
0
*
NP
LP
P14
Corticosterone (ng/ml)
80
60
40
20
0
NP
LP

Figure 6

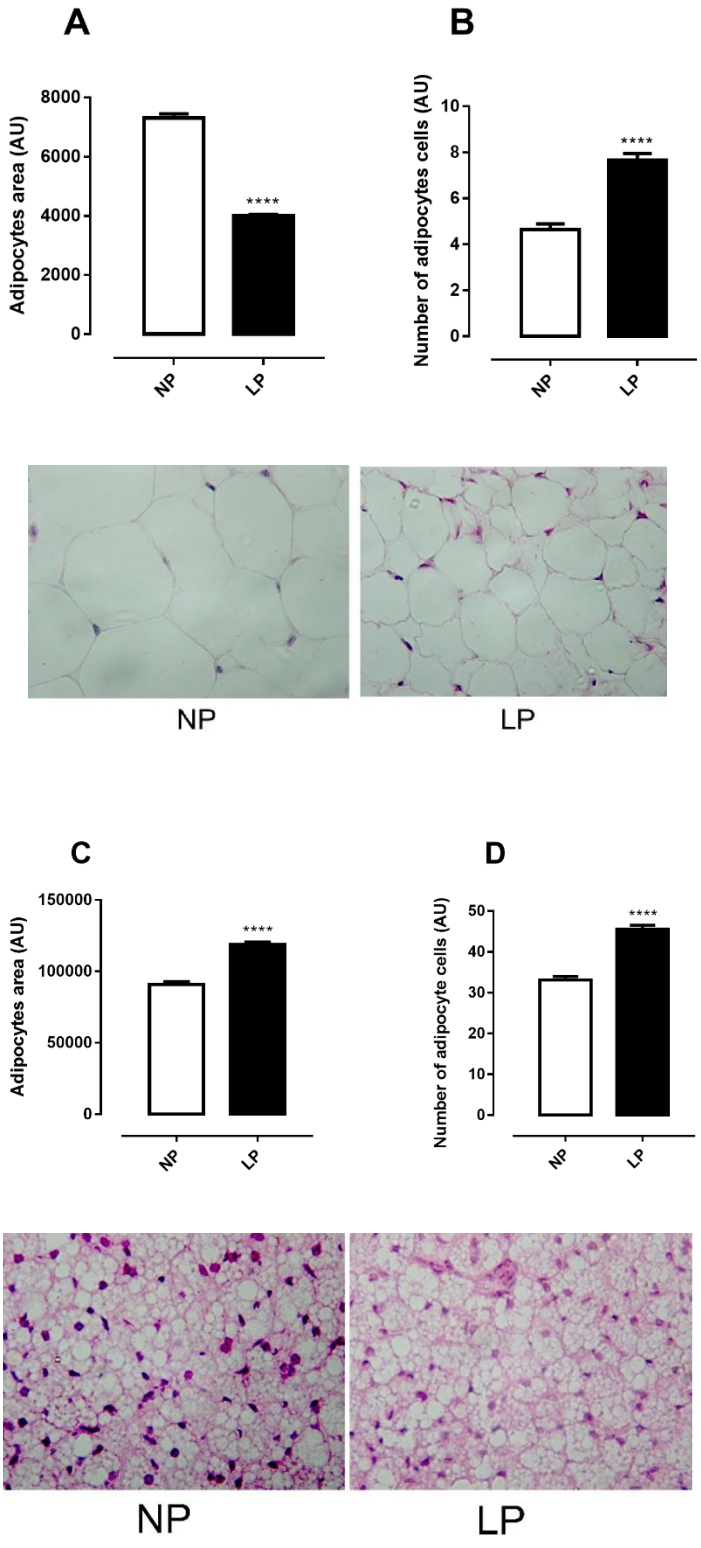
A
B
Adipocytes area (AU)
8000
6000
4000
2000
0
****
NP
LP
Number of adipocytes cells (AU)
10
8
6
4
2
0
****
NP
LP
NP
LP
C
D
Adipocytes area (AU)
150000
100000
50000
0
****
NP
LP
Number of adipocyte cells (AU)
50
40
30
20
10
0
****
NP
LP
NP
LP

Table 1 – Biometrical and biochemical parameters of dams during lactation.

Parameters	P7		P14		P21	
	NP	LP	NP	LP	NP	LP
Retroperitoneal fat pad (g/100g bw)	1.15±0.06	1.28±0.07	1.09±0.14	0.96±0.13	0.80±0.07	0.54±0.08*
Uterine fat pad (g/100g bw)	0.75±0.04	0.70±0.02	0.74±0.1	0.59±0.01	0.43±0.01	0.37±0.02*
Ovarian fat pad (g/100g bw)	0.75±0.11	1.07±0.11	0.68±0.07	0.62±0.07	0.50±0.04	0.40±0.04
Serum protein (g/dl)	5.68±0.32	5.32±0.23	7.06±0.36	4.31±0.48**	6.43±0.15	6.52±0.20
Serum triglycerides (mg/dl)	93.25±11.5	89.75±8.45	85.75±7.5	168.9±23.7**	99.38±13.31	108.5±11.69
Serum glucose (mg/dl)	103.2±3.83	99±3.01	99.67±5.27	97±1.26	79.2±1.71	87.71±1.92*
K_{itt} (%) min	1.93±0.16	2.05±0.16	1.12±0.14	1.16±0.09	1.58±0.08	1.73±0.22
AUC ipGTT	19605±462	16431±745**	18731±467	16080±649*	13424±465	15010±623

Data are presented as the mean ± SEM obtained from 5-8 dams in each experimental group. Significant differences between NP and LP group are represented by *p<0.05 and ** p<0.01 by Student's *t* test. NP, normal-protein; LP, low-protein; K_{itt} (%) min, glucose disappearance rate at insulin tolerance test; AUC ipGTT, AUC from glucose tolerance test.

Table 2 – Biometrical parameters of pups at P21.

Parameters	P21	
	NP	LP
Body weight (g)	45.2±1.8	29.2±2.0***
Retroperitoneal fat pad (g/100g bw)	0.15±0.01	0.09±0.01***
Periepidydimal fat pad (g/100g bw)	0.16±0.009	0.10±0.008***
Mesenteric fat pad (g/100g bw)	0.31±0.02	0.25±0.02*
Brown fat pad (g/100g bw)	0.28±0.007	0.23±0.01**
Adrenal weight (g/100g bw)	0.0176±0.001	0.0175±0.001

Data are presented as the mean ± SEM obtained from 14-17 rats from 4 different litters in each experimental group. Significant differences between NP and LP group are represented by *p<0.05, ** p<0.01 and *** p<0.001 by Student's *t* test. NP, normal-protein; LP, low-protein.

Table 3 - Biochemical parameters of pups through lactation.

Parameters	P7		P14		P21	
	NP	LP	NP	LP	NP	LP
Serum protein (g/dl)	3.1±0.13	2.72±0.11*	4.32±0.12	3.18±0.11****	4.53±0.09	4.53±0.09
Serum triglycerides (mg/dl)	90.88±14.96	70.38±8.77	123.4±6.71	132.8±14.53	132.6±6.5	180±15.13**
Serum glucose (mg/dl)	71.63±3.79	110.5±12.18**	110.8±4.72	117±11.53	115.7±4.16	114.1±4.29

Data are presented as the mean ± SEM obtained from 8 pups from 4 different litters in each experimental group. Significant differences between NP and LP group are represented by *$p<0.05$, ** $p<0.01$ and **** $p<0.0001$ by Student's *t* test. NP, normal-protein; LP, low-protein.

Figure Legends

Figure 1- Body weight (A), food intake (B) and corticosterone (C) of dams during lactation. Data are presented as the mean ± SEM of 4 – 6 dams per group. The lateral panels, as an inset to figures A and B depict the area under the curve (AUC). Figure C shows corticosterone levels at P14. Significant differences between NP and LP group are represented by **p<0.01, ***p<0.001 and ****p<0.0001 by Student's *t* test. NP, normal-protein; LP, low-protein.

Figure 2 - Composite maternal behavior of lactating rats. Nest building (A), licking pups (B), arched nursing (C), blanket nursing (D), passive nursing (E) and total maternal behavior (F). Data are presented as the mean ± SEM of percentage of episodes across 100 observations per day. NP, normal-protein; LP, low-protein.

Figure 3 – Composite non-maternal behavior of lactating rats. Feeding (A), exploring (B), non-exploring (C), self-grooming (D) and total non-maternal behavior (E). Data are presented as the mean ± SEM of percentage of episodes across 100 observations per day. NP, normal-protein; LP, low-protein.

Figure 4 – Protein (A), total fat (B), total carbohydrates (C) and corticosterone (D) of milk during lactation. Data are presented as the mean ± SEM of 5 – 10 milk samples per group. Nutritional parameters of milk were measured at P7, 14 and 21 (A, B, C). Figure D shows corticosterone levels at P14. Significant differences between NP and LP group are represented by *p<0.05, **p<0.01 and ****p<0.0001 by Student's *t* test. NP, normal-protein; LP, low-protein.

Figure 5 – Body weight (A) and corticosterone levels (B, C) of pups during lactation. Data are presented as the mean ± SEM of 8 – 10 pups from 4 different litters per group. Figures B and C shows corticosterone levels at P7 and P14. Significant differences between NP and LP group are represented by *p<0.05 and ***p<0.001 by Student's *t* test. NP, normal-protein; LP, low-protein.

Figure 6 - Histology of retroperitoneal white adipose tissue (rWAT) and interscapular brown adipose tissue (iBAT) at P21. White adipocytes area (A), number of cells (B). Brown adipocytes area (C) and number of cells (D). Data are presented as the mean ± SEM of 5-6 pups from 4 different litters per group. Significant differences

between NP and LP group are represented by ****p<0.0001 by Student's *t* test. NP, normal-protein; LP, low-protein.