**ISABELA PEIXOTO MARTINS**

# DESNUTRIÇÃO PROTEICA PERINATAL: RESISTÊNCIA AO DESENVOLVIMENTO DE OBESIDADE

Dissertação 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 de Mestre em Ciências Biológicas.

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**Orientador**

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Dissertação apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de Mestre.

Aprovado em:

**BANCA EXAMINADORA**

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## BIOGRAFIA

**Isabela Peixoto Martins** nasceu em Maringá/PR em 21/06/1994. Possui graduação em Ciências Biológics pela Universidade Estadual de Maringá (2015). Atualmente é mestranda do Programa de Pós-Graduação em Ciências Biológicas 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 dissertação é composta de um artigo científico, intitulado **"Protein-restriction diet in suckling phase programs rat metabolism against obesity and insulin resistance exacerbation induced by high-fat diet in adulthood"**. O trabalho demonstra o impacto de uma dieta rica em gordura na vida adulta de animais previamente programados por desnutrição proteica no início da vida. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, o artigo foi redigido de acordo com as normas da revista *Journal of Nutritional Biochemistry*, com atual fator de impacto 4,66 (Qualis CB1: A2).

## RESUMO GERAL

**INTRODUÇÃO** – Insultos nutricionais no início da vida têm sido associados ao desenvolvimento de doenças metabólicas na vida adulta, como a obesidade e diabetes tipo 2. O conceito DOHaD (Developmental Origins of Health ans Diseases) descreve, por meio de pesquisas experimentais e epidemiológicas, como eventos em fases sensíveis do desenvolvimento provocam alterações fisiológicas no organismo, programando-o para consequências a longo prazo. Em roedores, o pico de desenvolvimento dos órgãos e sistemas, como Sistema Nervoso Central, ocorre nas primeiras semanas de lactação. A restrição proteica é considerada um modelo bem estabelecido para o estudo da programação metabólica, e sabe-se que a prole de mães que passaram por restrição de proteínas durante os 2/3 iniciais da lactação apresenta um fenótipo magro na vida adulta associado a intolerância à glicose, hipoinsulinemia, redução da secreção de insulina pelas ilhotas pancreáticas e alterações na atividade do Sistema Nervoso Autonômico. Este perfil pode ser extremamente vulnerável a uma mudança nutricional na vida adulta, principalmente à exposição a uma dieta hiperlipídica.

**OBJETIVO** – Avaliar o impacto de uma dieta rica em gordura no metabolismo de animais programados no início da vida com uma dieta hipoproteica.

**MÉTODOS** – Ratos Wistar machos e fêmeas, de 80 e 70 dias de vida, foram obtidos do Biotério Central da Universidade Estadual de Maringá e colocados para cruzar na proporção de um macho para três fêmeas no Biotério Serotial do Laboratório de Biologia Celular da Secreção, após cinco dias de adaptação. Detectada a prenhez, as ratas foram colocadas em caixas individuais e, ao nascimento, as ninhadas foram padronizadas para oito filhotes por mãe e foram divididas em dois grupos experimentais. Mães controles (n=12) receberam dieta comercial, normoproteica (NP; 23% de proteína; Nuvital®; Curitiba/PR, Brazil) por toda a lactação e mães desnutridas (n= 12) receberam dieta hipoproteica (LP; 4% de proteína) nos primeiros 14 dias de lactação. Aos 21 dias os animais foram desmamados e mantidos com dieta controle até os 60 dias de vida. Nessa idade, a prole de mães NP e LP recebeu dieta normolipídica (NF; 4.5% de gordura; Nuvital®; Curitiba/PR, Brazil) ou dieta hiperlipídica (HF; 35% de gordura) até os 90 dias. Durante todo o período experimental os animais foram mantidos sob temperatura (23 ± 2

ºC) e fotoperíodo (7:00 a.m. to 7:00 p.m., ciclo claro) controlados. Com 90 dias, os animais passaram pelo teste de tolerância à glicose intravenoso e teste de tolerância à insulina intraperitoneal, ambos realizados por meio de uma cânula implantada por procedimento cirúrgico na veia jugular direita do rato. Esses animais foram posteriormente anestesiados e eutanasiados para o isolamento das ilhotas pancreáticas com o objetivo de estudar a secreção de insulina frente a diferentes concentrações de glicose, antagonistas e agonistas muscarínicos e adrenérgicos. Outro grupo de animais, aos 90 dias de vida, foi submetido ao registro elétrico dos nervos vago e esplâncnico e posterior retirada dos principais estoques de gorduras. Os dados foram expressos como média ± erro padrão e analisados através de test t de Student ou ANOVA de duas vias com pós teste de Tukey, com intervalo de confiança de 95%. O programa utilizado foi GraphPad Prism, versão 6.01.

**RESULTADOS E DISCUSSÃO** – A restrição proteico-calórica na lactação provocou baixo peso corporal, menores estoques de gordura, intolerância à glicose, hipoinsulinemia, elevada sensibilidade à insulina, redução da secreção de insulina e alteração do Sistema Nervoso Autonômico na prole aos 90 dias de vida. Animais NP que ingeriram dieta HF na vida adulta apresentaram-se com elevado ganho de peso corporal, aumento nos estoques de gordura, alterações no metabolismo da glicose e na secreção de insulina pelas ilhotas pancreáticas. Interessantemente, os animais LP alimentados com dieta HF na vida adulta também apresentaram elevado peso corporal, acúmulo de gorduras e alterações na homeostase glicêmica, porém, com menor magnitude em relação ao grupo NP/HF. Além disso, animais LP/HF têm elevada sensibilidade à insulina. Esses resultados sugerem que os animais desnutridos no início da vida apresentam um mecanismo de proteção ao desenvolvimento de obesidade e disfunções metabólicas exacerbadas na vida adulta, induzidas por uma dieta obesogênica. Provavelmente, o aumento na expressão dos receptores de glicose dependentes de insulina, GLUT-4, e o aumento na atividade do Sistema Nervoso Simpático dos animais LP são as chaves do mecanismo de resistência apresentado frente a dieta HF.

**CONCLUSÃO** – Restrição proteica na lactação promove resistência ao desenvolvimento de obesidade exacerbada induzida por dieta hiperlipídica na vida adulta.

## GENERAL ABSTRACT

**INTRODUCTION** – Early life nutritional insults have been associated with the development of metabolic diseases in adulthood, such as obesity and type 2 diabetes. The DOHaD concept (Origins of Health Development and Disease) describes, through experimental and epidemiological research, how events in sensitive stages of development cause physiological changes in the body, scheduling it for long-term consequences. In rodents, the peak development of organs and systems, such as the Central Nervous System, occurs in the first weeks of lactation. Protein restriction is considered to be a well-established model for the study of metabolic schedule, and it is known that the offspring of mothers who have undergone protein restriction during the initial 2/3 of lactation has a lean phenotype in adulthood associated with glucose intolerance, hypoinsulinemia, reduction of insulin secretion by pancreatic islets and alterations in the activity of the Autonomic Nervous System. This profile can be extremely vulnerable to nutritional change in adult life, especially exposure to a high-fat diet.

**AIM –** Evaluate the impact of a high-fat diet on the metabolism of animals programmed in early life with a low-protein diet.

**METHODS** – Male and female Wistar rats, 80 and 70 days old, were obtained from the Central Biotério of the State University of Maringá and placed to cross in the ratio of one male to three females in the Serotial Biotério of the Laboratory of Cellular Biology of Secretion, after five days of adaptation. When the pregnancy was detected, the dams were placed in individual cages and, at birth, the litters were standardized to eight pups per dam and were divided into two experimental groups. Controlled mothers (n = 12) received a commercial diet (NP, 23% protein, Nuvital®, Curitiba / PR, Brazil) throughout the lactation and malnourished mothers (n = 12) received a low-protein diet (LP, 4% Protein) during the first 14 days of lactation. At 21 days, the animals were weaned and kept on a control diet until 60 days of age. At this age, the offspring of NP and LP mothers received a normolipid diet (NF, 4.5% fat, Nuvital®, Curitiba / PR, Brazil) or a high-fat diet (HF, 35% fat) until 90 days of age. Throughout the experimental period the animals were kept under controlled temperature (23 ± 2 ºC) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle). At 90 days, the animals underwent the intravenous glucose tolerance test and intraperitoneal insulin tolerance test, both performed by means of a cannula implanted by

surgical procedure in the right jugular vein of the rat. These animals were subsequently anesthetized and euthanized for the isolation of pancreatic islets to study the insulin secretion with different glucose concentrations, antagonists and agonists of muscarinic and adrenergic receptors. Another batch of animals, at 90 days of age, was submitted to electrical recording of the vagus and splanchnic nerves and subsequent removal of the main fat stores. Data were expressed as mean ± standard error and analyzed by Student t test or two-way ANOVA with Tukey test powders, with a 95% confidence interval. The program used was GraphPad Prism, version 6.01.

**RESULTS AND DISCUSSION** – Protein-caloric restriction during lactation caused low body weight, lower fat stores, glucose intolerance, hypoinsulinemia, high insulin sensitivity, reduced insulin secretion and alteration of the Autonomic Nervous System in offspring at 90 days of life. NP animals fed with HF diet in adulthood presented high body weight gain, increased fat stores, changes in glucose metabolism and insulin secretion by pancreatic islets. Interestingly, LP animals fed with HF diet in adult life also presented high body weight, accumulation of fats and alterations in glycemic homeostasis, but with smaller magnitude compared to NP/HF group. In addition, LP/HF animals have high insulin sensitivity. These results suggest that malnourished animals in early life present a protective mechanism against the development of obesity and exacerbated metabolic dysfunctions in adulthood, induced by an obesogenic diet. Increased expression of insulin-dependent glucose receptors, GLUT-4, and the increase in the activity of the Sympathetic Nervous System of LP animals are probably the keys to the resistance mechanism presented against the HF diet.

**CONCLUSION** – Protein restriction during lactation promotes resistance to the development of exacerbated obesity induced by high-fat diet at adulthood.

# Protein-restriction diet during the suckling phase programs rat metabolism against obesity and insulin resistance exacerbation induced by a high-fat diet in adulthood

**Running title:** Early low protein diet prevents adult obesity

**Keywords:** protein restriction, lactation, obesity, metabolic programming.

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**Abstract**

Protein restriction during the suckling phase can malprogram rat offspring to a lean phenotype associated with metabolic dysfunctions later in life. We tested whether protein-caloric restriction during lactation can exacerbate the effect of a high-fat (HF) diet at adulthood. To test this hypothesis, we fed lactating Wistar dams with a low-protein (LP; 4% protein) diet during the first two weeks of lactation or a normal-protein (NP; 23% protein) diet throughout lactation. Rat offspring from NP and LP mothers received a normal-protein diet until 60 days old. At this time, a batch of animals from both groups were fed an HF (35% fat) diet while another received a normal-fat (NF; 7% fat) diet. Maternal protein-caloric restriction provoked lower body weight and fat pad stores, hypoinsulinaemia, glucose intolerance, higher insulin sensitivity, reduced insulin secretion and altered autonomic nervous system (ANS) function in adult rat offspring. At 90 days old, NP rats fed an HF diet in adulthood displayed obesity, impaired glucose homeostasis and altered insulin secretion and ANS activity. Interestingly, the LP/HF group also presented fat pad and body weight gain, altered glucose homeostasis, hyperleptinemia and impaired insulin secretion, but at a smaller magnitude than the NP/HF group. In addition, LP/HF rats displayed elevated insulin sensitivity. We concluded that protein-caloric restriction during the first 14 days of life programs the metabolism of male rats to be resistant to the exacerbation of obesity and insulin resistance induced by an obesogenic HF diet.

**Keywords**: protein restriction; lactation; obesity; metabolic programming.

## 1. Introduction

An environment of nutritional imbalance in early life is associated with an increased risk of developing metabolic diseases in adulthood. This process, termed metabolic programming [1], can be explained by the developmental origins of health and disease (DOHaD) concept, which describes through various scientific studies how early environmental factors can induce physiological changes in foetal, neonatal and infant individuals and program long-term post-natal consequences [2-4]. Among these environmental factors, nutrition is one factor that plays a major role in development. Poor nutrition during foetal or early postnatal development provokes metabolic adaptations based on these early environmental circumstances; this response, postulated by Barker (1992), is known as the predictive adaptive response or Thrifty Phenotype Hypothesis [5]. When this predictive adaptive response is challenged with a different nutritional environment later in life, the mismatch between early and later nutrition can lead to later health problems[4, 6].

Pregnancy and the suckling period make up a window of susceptibility to metabolic programming. In rodents, the development and maturation of the major organs and tissues, such as the endocrine system and central nervous system (CNS), occurs mainly during the first weeks after birth [7]. During this period, postnatal undernutrition exposure can affect the development of neural circuitry involved in metabolism, energy expenditure, and pancreatic beta cell function [8, 9], which manifests as impaired insulin secretion and metabolic dysfunction.

A protein-restriction diet in rats is a well-established model used to investigate the relationship between early nutritional insults and adult diseases [10]. However, the timing and duration of dietary manipulation is crucial for understanding the programming effects of early malnutrition. Nutritional restriction to the foetus, as in the case of a maternal low-protein diet during pregnancy, causes low birth weight in rat offspring and subsequent rapid “catch-up” growth,

which represents an important predictor for the development of adult metabolic diseases. In this context, a low-protein diet throughout gestation has been associated with hyperinsulinaemia, insulin resistance and glucose intolerance [11-13]. On the other hand, rat offspring undernourished by a low-protein diet during the suckling period do not develop adult obesity and show a lean phenotype throughout life. Moreover, significantly reduced pancreatic beta cell function is observed in rat offspring that received low-protein diet [14, 15]. These studies indicate that a maternal low-protein diet during different periods can cause differential programming of metabolism.

Previously, we have demonstrated that protein restriction during first two weeks of the lactation period caused low body weight, hypoinsulinaemia, a lower capacity for insulin secretion, high insulin sensitivity, impaired glucose homeostasis and reduced vagal activity [16]. Animals with this profile can be extremely vulnerable to postnatal dietary challenge, principally exposure to high-calorie diets, and therefore potentially display obesity and related diseases in adulthood [6]. Although many studies have shown the effects of early undernutrition on the offspring's metabolic parameters [11, 14, 15, 17], few studies precisely delineate the impact of undernutrition during the lactation period and exposure to caloric abundance in adulthood, a food environment still commonly faced today in developing countries. Thus, in this study we aimed to evaluate the long-term effects of a low-protein diet offered to dams during the first two weeks of lactation on the body composition, pancreatic function, lipid profile, glucose homeostasis and ANS of rat offspring submitted to an HF diet in adulthood.

## 2. Materials and Methods

### *2.1 Experimental design and diets*

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, and the pregnant females were transferred to individual cages and fed a standard diet. At birth, the litters were standardized to eight pups per dam, preferentially male, and divided into two experimental groups. The control dams (n = 12) received a normal-protein diet (20.5% protein; Nuvital®; Curitiba/PR, Brazil; NP group) throughout lactation, while the other group of mothers was fed a low-protein diet (n = 12, 4% protein; LP group) for the first 14 days of lactation. At postnatal day 21, the male offspring were weaned, housed four per cage and fed a standard diet. At sixty days of age, offspring from NP and LP dams were fed a normal-fat (NF) diet (4.5% fat; Nuvital®; Curitiba/PR, Brazil) or a high-fat (HF) diet (35% fat) until ninety days of age. Thus, the four experimental groups used were as follows: NP/NF, control offspring fed a normal-fat diet; LP/NF, low-protein offspring fed a normal-fat diet; NP/HF, control offspring fed a high-fat diet; and LP/HF, low-protein offspring fed a high-fat diet (n = 6 litters per group). The experimental procedures were conducted at ninety days of 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*. The compositions of the low-protein and high-fat diet have been described previously [18, 19].

### *2.2 Body weight gain, caloric intake and fat pad store measurements*

Body weight (bw) and food intake were determined every two days from weaning until 90 days of age. Food intake was calculated as the difference between the amount of diet remaining ($D_f$)

and the amount presented previously ($D_i$), divided by the number of animals in the cage and by the number of days: [FI(g) = ($D_f$ –$D_i$)/2/3]. Considering that the energetic values of the diets are different, food intake was presented in calories (Kcal/g bw). The area under the curve (AUC) was calculated. At 90 days of age, the rats were anaesthetized with thiopental (45 mg/kg bw), decapitated and laparotomized to remove their retroperitoneal, periepididymal and mesenteric fat pad stores. The weight of fat pads was expressed in relation to the bw of each animal (g/100 g bw). Body length and final bw were assessed to evaluate the Lee index through the following calculation, which was previously described: Lee index = 1/3 [bw (g)/body length (cm)] [20].

*2.3 Intravenous glucose tolerance test (ivGTT)*

At 90 days of age, a batch of animals (n = 3 litters per group) were subjected to a surgical procedure to perform the ivGTT, as previously described [16]. After a 12-hour fast, blood samples were removed before the injection of glucose (1 g/kg bw) (0 min) and 5, 15, 30 and 45 min afterward. The glucose response during the test was calculated by AUC.

*2.4 Intraperitoneal insulin tolerance test (ipITT)*

Another batch of animals (n = 3 litters per group) were cannulated, and the ipITT was performed after a 6-hour fast. They received an injection of insulin (1 U/kg bw), and blood samples were collected, as previously reported [21]. Subsequently, the rate of glucose tissue uptake or the rate constant for plasma glucose disappearance ($K_{itt}$) was calculated [22].

*2.5 Pancreatic islets isolation*

Ninety-day-old rats (n = 3 litters per group) had their pancreatic islets isolated by the collagenase method, as previously described [23]. To adapt insulin secretion to a baseline glucose

concentration (5.6 mmol/l), we preincubated the islets (four islets per well) for 60 min at 37°C in Krebs solution containing 5.6 mmol/l of glucose at pH 7.4, bubbled with a mixture of $CO_2$ (5%) and $O_2$ (95%) [24].

*2.6 Insulin secretion stimulation*

To study the insulinotropic effects of different glucose and acetylcholine (ACh) concentrations, after 60 min of preincubation, we incubated the islets for an additional 60 min with 5.6, 8.3 or 16.7 mmol/l of glucose or 8.3 mmol/l of glucose plus 10 µmol/l of ACh in the presence of neostigmine (10 µmol) to block acetylcholinesterase activity. Another batch of islets was used to study muscarinic acetylcholine receptor (mAChR) function. To block mAChR function, we used one of the following cholinergic antagonists in a solution with 8.3 mmol/l of glucose plus 10 µmol/l of ACh: the non-selective mAChR antagonist atropine (Atr, 10 µmol/l), the selective mAChR subtype M3 antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 100 µmol/l) or the selective mAChR subtype M2 antagonist methoctramine (MTT, 1 µmol/l).

To study adrenoceptor function, after preincubation, we incubated another batch of islets with a high glucose concentration (16.7 mmol/l) in the presence of the agonist adrenaline (Adr, 1 µmol/l) or glucose (16.7 mmol/l), Adr (1 µmol/l) plus the $\alpha_2$-adrenoceptor antagonist yohimbine (Yoh, 10 µmol/l). The supernatants from the incubations were collected and stored at –20°C for further insulin measurements.

*2.7 Insulin level analyses*

The insulin levels of plasma and supernatants from the incubation of pancreatic islets were measured by radioimmunoassay (RIA) [25].

*2.8 Quantification of blood glucose and leptin levels*

Glucose concentration was measured by the glucose oxidase method using a commercial kit (GoldAnalisa®; Belo Horizonte, MG, Brazil) [26]. Leptin plasma levels were determined with an ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA, USA).

*2.9 Lipid profile*

Triglycerides, total cholesterol and HDL-C were measured in plasma samples by a colourimetric method using commercial kits (Gold Analisa®; Belo Horizonte, MG, Brazil). LDL and VLDL cholesterol values were determined by the Friedewald formula [27].

*2.10 Autonomic nerve electrical activity*

At 90 days of age, a batch of rats that had been fasted for 12 h (n = 3 litters per group) were anaesthetized with thiopental (45 mg/kg 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 laparotomy was performed to isolate the branch of the sympathetic nerve that is located in the splanchnic region [28]. A pair of silver recording electrodes (0.6 mm diameter) were placed under the nerve, which was covered with silicone oil to prevent dehydration. The electrode was connected to an electronic device (Bio-Amplificator; Insight Equipamentos, RibeirãoPreto, 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 [29].

*2.11 Statistical analysis*

The results are presented as the mean with the standard error (SEM). Statistical analysis was performed using Student's *t*-test or two-way ANOVA (analysis of variance) followed by Tukey's post hoc test. A P value $< 0.05$ was considered statistically significant for the effects of a low-protein diet (LP), a high-fat diet (HF) or the interaction (I) of low protein and high fat. Analyses were conducted in GraphPad Prism version 6.01 for Windows (GraphPad Software, Inc. San Diego, CA, USA).

## 3. Results

### *3.1 Body weight, food intake and body composition*

As shown in Table 1, protein restriction during lactation caused a decrease of 56% and 23% in the bw of the offspring at 21 and 90 days old, respectively ($P<0.0001$). Both groups that were fed a high-fat diet in adulthood had increased bw at 90 days old, with increases of 20% ($P<0.0001$) and 15% ($P<0.05$) for NP/HF and LP/HF, respectively. However, LP/HF rats showed a 26% lower bw than the NP/HF group ($P<0.0001$). No difference was observed in the Lee index of LP/NF rats. The NP/HF group showed a 6% increase in this parameter ($P<0.0001$), and the LP/HF group exhibited 4% lower Lee index than the NP/HF group ($P<0.001$).

The offspring of dams fed a low-protein diet showed a 25% lower bw gain than offspring of control dams until 60 days of age (Fig. 1A; $P<0.0001$), although they had a higher food intake compared with NP (Fig. 1B; +23%; $P<0.0001$). High-fat diet consumption at adulthood resulted in increased bw gain in both groups. NP/HF animals gained 46% more bw than NP/NF (Fig. 1C; $P<0.0001$), and the LP/HF group presented a 34% increase with respect to LP/NF (Fig. 1C; $P<0.0001$). However, LP/HF rats gained 28% less bw than NP/HF rats ($P<0.0001$). There was no difference between groups in caloric intake from 60 to 90 days old (Fig. 1D).

As shown in Fig. 2, the white adipose tissue mass was lower in the LP/NF rats than in the control group, with a reduction of 33% for periepididymal ($P<0.01$), 31% for retroperitoneal ($P<0.05$) and 41% for mesenteric fat ($P<0.05$). As expected, NP/HF animals had larger stocks of fat than NP/NF, as demonstrated by the periepididymal (Fig. 2A; +130%; $P<0.0001$), retroperitoneal (Fig. 2B; +150%; $P<0.0001$) and mesenteric (Fig. 2C; +136%; $P<0.0001$) fat pads. The LP/HF group also showed an increase in body fat composition (Fig. 2; +92%, 126% and 123%, respectively; $P<0.0001$) compared with LP/NF; however, LP/HF rats presented smaller fat pads than NP/HF by approximately 40% ($P<0.0001$).

247

248 *3.2 Biochemical parameters and lipid profile*

249 Table 1 shows that fasting glycaemia was increased by 24% in NP/HF and by 20% in LP/HF rats
250 compared with NP/NF rats ($P<0.001$). No effect was observed in malnourished rats fed a high-
251 fat diet compared with their counterparts. LP/NF animals exhibited a decrease of 57% in
252 insulinaemia compared with NP/NF ($P<0.05$). Both groups fed with HF diets showed an increase
253 of insulinaemia compared with their counterparts; however, the LP/HF group exhibited 38%
254 lower fasting insulinaemia than the NP/HF ($P<0.01$).

255 There was no significant difference in the plasma leptin of LP/NF rats compared with NP/NF;
256 however, as expected, animals that received a high-fat diet presented higher levels of leptin than
257 their controls (NP/HF, $P<0.0001$ and LP/HF, $P<0.05$). Still, the leptin levels were 44% lower in
258 the LP/HF group than in the NP/HF group (Table 1; $P<0.05$).

259 Regarding lipid profile (Table 1), LP/NF animals had lower levels of triglycerides and total
260 cholesterol than NP/NF ones ($P<0.05$). Control animals fed a hypercaloric diet exhibited
261 increases of 30% ($P<0.01$) and 36% ($P<0.0001$) in triglycerides and total cholesterol,
262 respectively. LP/HF rats presented elevated total cholesterol compared with LP/NF rats (+24%,
263 $P<0.0001$). Perinatal undernutrition caused a reduction of 42% in HDL-C ($P<0.01$), an increase
264 of 109% in LDL-C ($P<0.0001$) and a reduction of 23% in VLDL-C compared with the control
265 group ($P<0.01$). An HF diet in adulthood caused increased LDL-C (+47%, $P<0.05$) in control
266 animals; however, LP rats did not present differences in these parameters after HF consumption.

267 *3.3 Glucose homeostasis during the glucose and insulin tolerance tests*

268 During the ivGTT, as demonstrated by the AUC, the glycaemia of LP/NF animals was increased
269 by 19% (Fig. 3A; $P<0.05$), even though they displayed higher peripheral insulin sensitivity than
270 NP/NF, as indicated by $K_{itt}$ (Fig. 3B; $P<0.01$). Control animals fed an HF diet were glucose

intolerant (Fig. 3A; +41%; P<0.0001) and insulin resistant as indicated by $K_{itt}$ (Fig. 3B; -67%; P<0.01). Although hyperglycaemic during the test, LP/HF rats did not present any difference in glycaemia compared with LP/NF and showed insulin sensitivity as indicated by $K_{itt}$ (Fig. 3; +48%; P<0.0001).

*3.4 Glucose-induced insulin secretion and the muscarinic and adrenergic responses in pancreatic isolated islets*

Fig. 4A shows that the isolated islets of malnourished offspring secrete less insulin than those of the NP/NF group at a low glucose concentration (5.6 mmol/l) and under postprandial conditions (8.3 and 16.7 mmol/l). The islets of NP/HF animals presented increases of 54% ($P_{HF}$<0.01), 34% and 10% in insulin secretion at 5.6, 8.3 and 16.7 mmol/l glucose, respectively, compared with NP/NF. The islets of the LP/HF group exhibited increases of 85%, 29% and 32% in insulin secretion compared with LP/NF at the same glucose concentrations. However, LP/HF rats secreted approximately 27% less insulin at all glucose concentrations compared with NP/HF (Fig. 4A; P<0.01).

The cholinergic response was reduced in malnourished rats (Fig. 4B; P<0.01) as well as in NP/HF and LP/HF. NP/HF rats showed a reduction of 43% (Fig. 4B; P<0.05) in ACh response compared with NP/NF, while LP/HF presented a small increase over LP/NF in ACh response on insulin secretion (Fig. 4B; P< 0.05).

When the nonselective mAChR antagonist Atp was added, insulin secretion was blocked by 57% in NP/NF. LP/NF showed only 8% inhibition (Fig. 4C; P<0.0001); NP/HF animals presented a 33% reduction; and the LP/HF group recovered this parameter, since insulin secretion was inhibited by 57% (Fig. 4D; P<0.0001). 4-DAMP, a selective antagonist of the muscarinic M3 receptor, decreased insulin secretion in islets from all groups; however, secretion was inhibited

by 58% in the NP/NF group, 29% in LP/NF, 59% in NP/HF and 61% in LP/HF (Fig. 4D; P<0.0001). Incubation with MTT increased insulin secretion by 31% in the NP/NF group, 117% in LP/NF, 30% in NP/HF and 25% in LP-HF (Fig. 4E; P<0.0001).

Incubation with Adr (Fig. 4F) blocked 53% of insulin secretion in NP/NF islets. The LP/NF group had a 33% decrease in insulin release (P<0.0001), and NP/HF had a 45% reduction (P<0.0001). However, the islets of LP/HF rats showed only a 13% of block in insulin secretion (P<0.0001). Yoh promoted a 48% increase in insulin secretion in NP/NF islets, and LP/NF islets showed a 27% increase (Fig. 4G; P<0.01). Both groups that were fed an HF diet were less responsive to Yoh, with increases of 22% and 13% in NP/HF and LP/HF, respectively (P<0.01).

*3.5 Autonomic nervous system activity*

As shown in Fig. 5, parasympathetic activity was decreased 26% in malnourished rats (P<0.05), while both groups that were fed an HF diet had increases in this parameter: 28% for NP/HF (P<0.05) compared with NP/NF and 64% for LP/HF compared with LP/NF (P<0.001). No difference was observed between the NP/HF and LP/HF groups.

Sympathetic activity was increased 34% in LP/NF animals compared with NP/NF (Fig. 5; P<0.05). There was no difference between the NP/NF and NP/HF groups. However, LP animals maintained their increased activity when fed an HF diet. We observed that the LP/HF group showed a 24% increase in sympathetic activity compared with NP/HF (P<0.05).

## 4. Discussion

In the present study, we evaluated the long-term impact of undernutrition during the first two weeks of lactation induced by a maternal low-protein diet and the subsequent vulnerability to caloric excess induced by an HF diet. Herein, the major finding was that rats whose mothers were fed a protein-caloric-restricted diet during the lactation period are resistant to developing the HF diet-induced metabolic dysfunction that rats given an unrestricted diet develop. This outcome contradicts the hypothesis that rats nursed by protein-caloric restricted during lactation period appeared to be more vulnerable to an adverse adult environment.

A low-protein diet during lactation provoked low early body weight gain and lower body fat in the offspring throughout the lactation period, as previously observed [30]. The low bw can be attributed to milk consumption. In mothers fed a low-protein diet during lactation, milk production was lower, which can contribute to low offspring milk consumption [31]. Altered milk content can also be associated with this lean phenotype [32].

After weaning, LP animals had a peak in food intake that corroborates with previous data [12, 16], and this increase was not maintained during adulthood. No changes in food intake were observed between groups when HF was administered. In addition, LP animals maintained lower bw gain and fat pad stores during growth compared with unrestricted rats. Studies have demonstrated that perinatal malnutrition leads to alterations in the central pathway governing food intake and energy expenditure [14, 33, 34]. Additionally, a decrease in the mRNA levels of neuropeptide Y, an orexigenic hypothalamic neuropeptide, has been associated with better response to an HF diet in rats nursed by moderately calorie-restriction dams [35].

Catch-up growth after poor foetal or neonatal growth is associated with increased risks for the development of obesity, type 2 diabetes and cardiometabolic diseases [36-38]. Pups from mothers fed a low-protein diet (4% protein) during the last week of pregnancy showed low birth

weight and fast catch-up growth. Indeed, these intrauterine restricted rats had exacerbated high fat diet-induced metabolic dysfunction (data not published). In contrast to the intrauterine malnutrition animals, the offspring of the protein-restricted rats in this study did not exhibit catch-up growth. This indicates that protein restriction in the suckling period of rodents blocks catch-up growth and reduces the risk of obesity and related disorders [39].

It is well established that HF diet consumption leads to obesity associated with hyperinsulinaemia, hyperleptinemia, dyslipidaemia, glucose intolerance, insulin resistance and impaired endocrine-pancreatic function in rats [28, 40, 41] as well as disruption in the control of energy balance [42]. Nevertheless, in the current work, LP/HF rat offspring showed fat pad gains, altered lipid profiles, hyperglycaemia, hyperleptinemia and hyperinsulinaemia associated with high dietary demand, but with a much smaller magnitude than NP/HF rats. Indeed, LP/NF rat offspring were not insulin resistant; instead, they presented high insulin sensitivity, which was maintained even when they were fed an HF diet. Thus, our data shows that maternal protein restriction during the first 14 days of lactation blocks the deleterious effects of an HF diet upon energy metabolism in rats, which seems to oppose the prediction of the thrifty phenotype hypothesis [43, 44]. Along the same lines, a previous study showed that mice from mothers fed a low-protein diet (10% protein) during pregnancy and lactation are protected against high-fat diet-induced obesity. These animals showed an increase in energy expenditure due to increased mitochondrial function in skeletal muscle and increased mitochondrial density in white adipose tissue [45]. In all cases, experimental findings point to the insight that the effects of undernutrition in early life depend not only on the severity of diet restriction but also on the timepoint and/or the exact stage of life development in which it occurs, both of which can drastically influence metabolic imprinting or not [46].

Active adipogenesis in rodents occurs in later pregnancy and continues at weaning [47]. The hormones leptin and insulin act directly by regulating adiposity via the CNS [48]. Alteration in the levels of these hormones due to early nutritional imbalance can immediately modulate energy regulatory circuitry and body adiposity [48]. In this study, low fasting insulin levels and reduced fat tissue mass were observed in LP rat offspring. Indeed, these animals presented high fasting glucose levels and glucose intolerance during ivGTT, which can be explained by low insulin release from isolated pancreatic islets, as also previously reported in early protein-restricted rats [6, 49]. Interestingly, protein-restricted rats displayed high insulin sensitivity, which has been suggested to contribute to further development of insulin resistance due to high caloric abundance [50]. However, as shown here, one important point to highlight is that even on a high-fat diet, these LP rat offspring maintained high insulin sensitivity as well as high glycaemia. Furthermore, leptin levels and insulin secretion from isolated islets were increased in response to high caloric demand, but to a lesser extent than NP/HF. Along these lines, in contrast to LP/HF rat offspring, NP/HF offspring exhibit high insulin resistance and glucose intolerance associated with pancreatic beta cell dysfunction after HF intake. Caloric restricted rats during lactation exhibited beneficial gene expression related to central and peripheral leptin and insulin sensitivity, which may be involved in a better response to an HF diet [35].

These results suggest that a protein restriction diet during the suckling phase improves tissue glucose uptake induced by circulating insulin [35]. A protein-free diet during the first 10 days of lactation leads to higher membrane GLUT-4 expression and an altered insulin signalling response in adipocytes and muscle cells of adult rats. The high insulin sensitivity of malnourished animals is probably related to the increase in glucose uptake in these tissues [51, 52]. Accordingly, we propose that GLUT-4 expression in LP rats was programmed to exhibit

higher levels, which were maintained in adulthood. This would explain their high insulin sensitivity and the protection of their metabolism from the deleterious effects of an HF diet.

Pancreatic beta cell structure and function in rat offspring are significantly affected by a maternal low-protein diet [17, 53, 54]. As observed, rats whose dams are fed a low-protein diet during the first 14 days of lactation are programmed to exhibit beta cell dysfunction associated with changes in mAChR (low mAChR subtype M3 and high subtype M2) in pancreatic islets and low vagal activity in adulthood [6, 16]. In contrast, we show in the present study that HF diet-induced obese rats presented glucose-induced insulin release, which can be explained by high m3AChR levels in beta cells associated with high parasympathetic activity, as also observed in other obese rat models [21, 55, 56]. Under HF diet conditions, LP rat offspring had increased glucose-stimulated insulin secretion. However, this increase was lower than in NP rats. Interestingly, insulin secretion under the action of ACh, Atr and 4-DAMP was increased in the islets from LP/HF compared with LP/NF, whereas the insulinotropic effect of MTT was reduced compared with LP/NF. Indeed, we also observed low adrenergic activity by reduced insulinostatic effects of Adr and insulinotropic effects of Yoh. Taken together, these findings suggest a modulation of insulin secretion, which may be caused by changes in the composition of mAChR and adrenergic receptors and/or high receptor sensitivity or cellular signalling, associated with an altered ANS in response to high caloric demand [57]. In fact, LP/HF rats showed an increase in parasympathetic nervous system (PNS) activity provoked by HF diet consumption, which contributed to improved insulin secretion under obesogenic conditions. At the same time, we can observe that these animals were programmed to exhibit elevated activity in the sympathetic nervous system (SNS), which is consistent with their observed resistance to whole-body fat accumulation. This resistance to high adiposity may represent a key factor responsible for the

observed improvements in glucose tolerance and insulin sensitivity. However, the precise mechanism underlying this increase in sympathetic tone remains unclear at present.

Although the animals appear to be resistant to the development of obesity, little is known about the mechanisms that underline these alterations and whether the prolonged intake of an HF diet and/or ageing could lead to beta cell depletion and the development of type 2 diabetes in protein-restricted rats.

In conclusion, a maternal protein restriction diet during the suckling period in rats protects the offspring from the development of excessive HF diet-induced obesity by modulating peripheral insulin sensitivity, pancreatic islet function, and ANS activity even under caloric abundance. However, it is questionable whether the prolonged consumption of an HF diet in combination with ageing could drastically affect the metabolism of LP rats. Finally, the nature and range of the effects of a protein restriction diet during lactation in rats requires further examination.

**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.d.O., A.P., C.C.I.M., C.P, L.P.T, T.A.R., C.C.S.F., R.A.M., K.V.P., V.S.A., F.A.F. and A.M.P.M., 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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## References


[1] Holness MJ, Langdown ML, Sugden MC. Early-life programming of susceptibility to dysregulation of glucose metabolism and the development of Type 2 diabetes mellitus. The Biochemical journal. 2000;349 Pt 3:657-65.

[2] Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? The Journal of physiology. 2004;561:355-77.

[3] Lucas A. Programming by early nutrition: an experimental approach. The Journal of nutrition. 1998;128:401S-6S.

[4] Howie GJ, Sloboda DM, Kamal T, Vickers MH. Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. The Journal of physiology. 2009;587:905-15.

[5] Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia. 1992;35:595-601.

[6] Oliveira JC, Miranda RA, Barella LF, Torrezan R, Agostinho AR, Ribeiro TA, et al. Impaired beta-cell function in the adult offspring of rats fed a protein-restricted diet during lactation is associated with changes in muscarinic acetylcholine receptor subtypes. The British journal of nutrition. 2014;111:227-35.

[7] Lesage J, Sebaai N, Leonhardt M, Dutriez-Casteloot I, Breton C, Deloof S, et al. Perinatal maternal undernutrition programs the offspring hypothalamo-pituitary-adrenal (HPA) axis. Stress. 2006;9:183-98.

[8] Morimoto S, Calzada L, Sosa TC, Reyes-Castro LA, Rodriguez-Gonzalez GL, Morales A, et al. Emergence of ageing-related changes in insulin secretion by pancreatic islets of male rat offspring of mothers fed a low-protein diet. The British journal of nutrition. 2012;107:1562-5.

[9] de Moura EG, Passos MC. Neonatal programming of body weight regulation and energetic metabolism. Bioscience reports. 2005;25:251-69.

[10] Zohdi V, Lim K, Pearson JT, Black MJ. Developmental programming of cardiovascular disease following intrauterine growth restriction: findings utilising a rat model of maternal protein restriction. Nutrients. 2014;7:119-52.

[11] de Oliveira JC, Gomes RM, Miranda RA, Barella LF, Malta A, Martins IP, et al. Protein Restriction During the Last Third of Pregnancy Malprograms the Neuroendocrine Axes to Induce Metabolic Syndrome in Adult Male Rat Offspring. Endocrinology. 2016;157:1799-812.

[12] Zambrano E, Bautista CJ, Deas M, Martinez-Samayoa PM, Gonzalez-Zamorano M, Ledesma H, et al. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. The Journal of physiology. 2006;571:221-30.

[13] Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, Ozanne SE. Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. American journal of physiology Regulatory, integrative and comparative physiology. 2005;288:R368-73.

[14] Fagundes AT, Moura EG, Passos MC, Oliveira E, Toste FP, Bonomo IT, et al. Maternal low-protein diet during lactation programmes body composition and glucose homeostasis in the adult rat offspring. The British journal of nutrition. 2007;98:922-8.

[15] de Souza Caldeira Filho J, Moura AS. Undernutrition during early lactation period induces metabolic imprinting leading to glucose homeostasis alteration in aged rats. Research communications in molecular pathology and pharmacology. 2000;108:213-26.

[16] de Oliveira JC, Scomparin DX, Andreazzi AE, Branco RC, Martins AG, Gravena C, et al. Metabolic imprinting by maternal protein malnourishment impairs vagal activity in adult rats. Journal of neuroendocrinology. 2011;23:148-57.
[17] Su Y, Jiang X, Li Y, Li F, Cheng Y, Peng Y, et al. Maternal Low Protein Isocaloric Diet Suppresses Pancreatic beta-Cell Proliferation in Mouse Offspring via miR-15b. Endocrinology. 2016;157:4782-93.
[18] Malta A, de Moura EG, Ribeiro TA, Tofolo LP, Abdennebi-Najar L, Vieau D, et al. Protein-energy malnutrition at mid-adulthood does not imprint long-term metabolic consequences in male rats. European journal of nutrition. 2015.
[19] Barella LF, de Oliveira JC, Branco RC, Camargo RL, Gomes RM, Mendes FC, et al. Early exposure to a high-fat diet has more drastic consequences on metabolism compared with exposure during adulthood in rats. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2012;44:458-64.
[20] Bernardis LL, Patterson BD. Correlation between 'Lee index' and carcass fat content in weanling and adult female rats with hypothalamic lesions. The Journal of endocrinology. 1968;40:527-8.
[21] Miranda RA, Agostinho AR, Trevenzoli IH, Barella LF, Franco CC, Trombini AB, et al. Insulin oversecretion in MSG-obese rats is related to alterations in cholinergic muscarinic receptor subtypes in pancreatic islets. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2014;33:1075-86.
[22] Bonora E, Moghetti P, Zancanaro C, Cigolini M, Querena M, Cacciatori V, et al. Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. The Journal of clinical endocrinology and metabolism. 1989;68:374-8.
[23] de Oliveira JC, Lisboa PC, de Moura EG, Barella LF, Miranda RA, Malta A, et al. Poor pubertal protein nutrition disturbs glucose-induced insulin secretion process in pancreatic islets and programs rats in adulthood to increase fat accumulation. The Journal of endocrinology. 2013;216:195-206.
[24] Gravena C, Mathias PC, Ashcroft SJ. Acute effects of fatty acids on insulin secretion from rat and human islets of Langerhans. The Journal of endocrinology. 2002;173:73-80.
[25] Scott AM, Atwater I, Rojas E. A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. Diabetologia. 1981;21:470-5.
[26] Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. Journal of clinical pathology. 1969;22:158-61.
[27] Simoes FC, Marques RG, Diestel CF, Caetano CE, Dinis AP, Horst NL, et al. Lipidic profile among rats submitted to total splenectomy isolated or combined with splenic autotransplant. Acta cirurgica brasileira / Sociedade Brasileira para Desenvolvimento Pesquisa em Cirurgia. 2007;22 Suppl 1:46-51.
[28] Tofolo LP, da Silva Ribeiro TA, Malta A, Miranda RA, Gomes RM, de Oliveira JC, et al. Short-term moderate exercise provides long-lasting protective effects against metabolic dysfunction in rats fed a high-fat diet. European journal of nutrition. 2015;54:1353-62.
[29] Barella LF, Miranda RA, Franco CC, Alves VS, Malta A, Ribeiro TA, et al. Vagus nerve contributes to metabolic syndrome in high-fat diet-fed young and adult rats. Experimental physiology. 2015;100:57-68.
[30] Fagundes AT, Moura EG, Passos MC, Santos-Silva AP, de Oliveira E, Trevenzoli IH, et al. Temporal evaluation of body composition, glucose homeostasis and lipid profile of male rats

programmed by maternal protein restriction during lactation. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2009;41:866-73.
[31] Bautista CJ, Boeck L, Larrea F, Nathanielsz PW, Zambrano E. Effects of a maternal low protein isocaloric diet on milk leptin and progeny serum leptin concentration and appetitive behavior in the first 21 days of neonatal life in the rat. Pediatric research. 2008;63:358-63.
[32] Martin Agnoux A, Antignac JP, Boquien CY, David A, Desnots E, Ferchaud-Roucher V, et al. Perinatal protein restriction affects milk free amino acid and fatty acid profile in lactating rats: potential role on pup growth and metabolic status. The Journal of nutritional biochemistry. 2015;26:784-95.
[33] Orozco-Solis R, Matos RJ, Lopes de Souza S, Grit I, Kaeffer B, Manhaes de Castro R, et al. Perinatal nutrient restriction induces long-lasting alterations in the circadian expression pattern of genes regulating food intake and energy metabolism. International journal of obesity. 2011;35:990-1000.
[34] Lopes de Souza S, Orozco-Solis R, Grit I, Manhaes de Castro R, Bolanos-Jimenez F. Perinatal protein restriction reduces the inhibitory action of serotonin on food intake. The European journal of neuroscience. 2008;27:1400-8.
[35] Palou M, Torrens JM, Priego T, Sanchez J, Palou A, Pico C. Moderate caloric restriction in lactating rats programs their offspring for a better response to HF diet feeding in a sex-dependent manner. The Journal of nutritional biochemistry. 2011;22:574-84.
[36] Bol VV, Delattre AI, Reusens B, Raes M, Remacle C. Forced catch-up growth after fetal protein restriction alters the adipose tissue gene expression program leading to obesity in adult mice. American journal of physiology Regulatory, integrative and comparative physiology. 2009;297:R291-9.
[37] Bol VV, Reusens BM, Remacle CA. Postnatal catch-up growth after fetal protein restriction programs proliferation of rat preadipocytes. Obesity. 2008;16:2760-3.
[38] Zheng J, Xiao X, Zhang Q, Yu M, Xu J, Qi C, et al. The programming effects of nutrition-induced catch-up growth on gut microbiota and metabolic diseases in adult mice. MicrobiologyOpen. 2016;5:296-306.
[39] Lim K, Armitage JA, Stefanidis A, Oldfield BJ, Black MJ. IUGR in the absence of postnatal "catch-up" growth leads to improved whole body insulin sensitivity in rat offspring. Pediatric research. 2011;70:339-44.
[40] Higa TS, Spinola AV, Fonseca-Alaniz MH, Evangelista FS. Comparison between cafeteria and high-fat diets in the induction of metabolic dysfunction in mice. International journal of physiology, pathophysiology and pharmacology. 2014;6:47-54.
[41] Salimi M, Zardooz H, Khodagholi F, Rostamkhani F, Shaerzadeh F. High-fat diet with stress impaired islets' insulin secretion by reducing plasma estradiol and pancreatic GLUT2 protein levels in rats' proestrus phase. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2016;67:653-66.
[42] Priego T, Sanchez J, Pico C, Palou A. Sex-differential expression of metabolism-related genes in response to a high-fat diet. Obesity. 2008;16:819-26.
[43] Vignini A, Raffaelli F, Cester A, Iannilli A, Cherubini V, Mazzanti L, et al. Environmental and genetical aspects of the link between pregnancy, birth size, and type 2 diabetes. Current diabetes reviews. 2012;8:155-61.
[44] Wells JC. The thrifty phenotype: An adaptation in growth or metabolism? American journal of human biology : the official journal of the Human Biology Council. 2011;23:65-75.
[45] Jousse C, Muranishi Y, Parry L, Montaurier C, Even P, Launay JM, et al. Perinatal protein malnutrition affects mitochondrial function in adult and results in a resistance to high fat diet-induced obesity. PLoS One. 2014;9:e104896.

[46] Luque V, Closa-Monasterolo R, Escribano J, Ferre N. Early Programming by Protein Intake: The Effect of Protein on Adiposity Development and the Growth and Functionality of Vital Organs. Nutrition and metabolic insights. 2015;8:49-56.
[47] Lecoutre S, Breton C. Maternal nutritional manipulations program adipose tissue dysfunction in offspring. Frontiers in physiology. 2015;6:158.
[48] Niswender KD, Schwartz MW. Insulin and leptin revisited: adiposity signals with overlapping physiological and intracellular signaling capabilities. Frontiers in neuroendocrinology. 2003;24:1-10.
[49] Zheng J, Xiao X, Zhang Q, Yu M, Xu J, Wang Z. Maternal protein restriction induces early-onset glucose intolerance and alters hepatic genes expression in the peroxisome proliferator-activated receptor pathway in offspring. Journal of diabetes investigation. 2015;6:269-79.
[50] Holness MJ, Sugden MC. Antecedent protein restriction exacerbates development of impaired insulin action after high-fat feeding. The American journal of physiology. 1999;276:E85-93.
[51] Garcia-Souza EP, da Silva SV, Felix GB, Rodrigues AL, de Freitas MS, Moura AS, et al. Maternal protein restriction during early lactation induces GLUT4 translocation and mTOR/Akt activation in adipocytes of adult rats. American journal of physiology Endocrinology and metabolism. 2008;295:E626-36.
[52] Sampaio de Freitas M, Garcia De Souza EP, Vargas da Silva S, da Rocha Kaezer A, da Silva Vieira R, Sanchez Moura A, et al. Up-regulation of phosphatidylinositol 3-kinase and glucose transporter 4 in muscle of rats subjected to maternal undernutrition. Biochimica et biophysica acta. 2003;1639:8-16.
[53] Rodriguez-Trejo A, Ortiz-Lopez MG, Zambrano E, Granados-Silvestre Mde L, Mendez C, Blondeau B, et al. Developmental programming of neonatal pancreatic beta-cells by a maternal low-protein diet in rats involves a switch from proliferation to differentiation. American journal of physiology Endocrinology and metabolism. 2012;302:E1431-9.
[54] Dumortier O, Hinault C, Gautier N, Patouraux S, Casamento V, Van Obberghen E. Maternal protein restriction leads to pancreatic failure in offspring: role of misexpressed microRNA-375. Diabetes. 2014;63:3416-27.
[55] Balbo SL, Grassiolli S, Ribeiro RA, Bonfleur ML, Gravena C, Brito Mdo N, et al. Fat storage is partially dependent on vagal activity and insulin secretion of hypothalamic obese rat. Endocrine. 2007;31:142-8.
[56] Valensi P, Doare L, Perret G, Germack R, Paries J, Mesangeau D. Cardiovascular vagosympathetic activity in rats with ventromedial hypothalamic obesity. Obesity research. 2003;11:54-64.
[57] Gravena C, Andreazzi AE, Mecabo FT, Grassiolli S, Scantamburlo VM, Mathias PC. Protein restriction during lactation alters the autonomic nervous system control on glucose-induced insulin secretion in adult rats. Nutritional neuroscience. 2007;10:79-87.

**Table 1** – Effect of a high fat diet consumption on body weight and biochemical parameters of adult animals programmed by protein restriction in lactation.

| Parameters | NP | | LP | | | | |
|---|---|---|---|---|---|---|---|
| **Body weight (g) at 21 days old** | 50.34±0.5 | | 22.1±0.55$^{\Omega\Omega\Omega\Omega}$ | | Factors | | |
| | **NP/NF** | **NP/HF** | **LP/NF** | **LP/HF** | **LP** | **HF** | **I** |
| **Body weight (g) at 90 days old** | 368.9±5.2 | 446.2±11.4$^{\varphi\varphi\varphi\varphi}$ | 282.3±8.4$^{\#\#\#\#}$ | 326±4.2$^{\varphi\varphi\#\#\#\#}$ | **** | **** | ns |
| **Lee index** | 314.8±1.2 | 334±2.3 $^{\varphi\varphi\varphi\varphi}$ | 316±3.4 | 317.8±1.5$^{\#\#\#}$ | ** | **** | *** |
| **Fasting glycemia (mg/dl)** | 100.2±3.7 | 124.3±2.4 $^{\varphi\varphi\varphi\varphi}$ | 120.8±3.2$^{\#\#\#}$ | 112.6±2.8$^{\#}$ | ns | * | **** |
| **Fasting insulinemia (ng/ml)** | 0.45±0.04 | 0.8±0.06$^{\varphi\varphi\varphi}$ | 0.19±0.04$^{\#}$ | 0.58±0.11$^{\varphi\#\#}$ | ** | **** | ns |
| **Fasting leptinemia (pg/ml)** | 779.6±201 | 4776.2±810 $^{\varphi\varphi\varphi\varphi}$ | 601.7±108 | 2674.2±559 $^{\varphi\#}$ | * | **** | * |
| **Total cholesterol (mg/dl)** | 87.6±3.85 | 119.6±3.75 $^{\varphi\varphi\varphi\varphi}$ | 77.26±1.35$^{\#}$ | 95±2.68$^{\varphi\varphi\varphi\varphi\#\#\#\#}$ | **** | **** | * |
| **Triglycerides (mg/dl)** | 66.06±2.89 | 86.11±6.45$^{\varphi\varphi}$ | 52.71±4.83$^{\#}$ | 61.92±3.28$^{\#\#\#}$ | **** | *** | ns |
| **HDL-cholesterol (mg/dl)** | 56.25±6.31 | 66.4±2.82 | 32.35±2.95$^{\#\#}$ | 43.38±5.43$^{\#}$ | **** | * | ns |
| **LDL-cholesterol (mg/dl)** | 21.24±1.49 | 31.29±1.67$^{\varphi}$ | 44.56±1.72$^{\#\#\#\#}$ | 37.88±2.83 | **** | ns | *** |
| **VLDL-cholesterol (mg/dl)** | 13.21±0.57 | 15.65±1.19 | 10.17±0.76$^{\#\#}$ | 12.57±0.47$^{\#}$ | **** | ** | ns |

All data are expressed as the mean ± SEM of 24-30 rats from at least 3 different litters. NP/NF: control offspring fed with normal fat diet; LP/NF: low-protein offspring fed with normal fat diet; NP/HF: control offspring fed with high-fat diet and LP/HF: low-protein offspring fed with high-fat diet. $^{\Omega\Omega\Omega\Omega}$P<0.0001 for Student's t-test; $^{\#}$P<0.05,$^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001,$^{\#\#\#\#}$P<0.0001 to NP vs LP in the same conditions; $^{\phi}$P<0.05, $^{\phi\phi}$P<0.01,$^{\phi\phi\phi}$P<0.001,$^{\phi\phi\phi\phi}$P<0.0001 to NF vs HF in the same group for the probability based on the Tukey's *post hoc* analysis. LP, low-protein diet factor; HF, high-fat diet factor; and I, interaction between low-protein and high-fat factors. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on a two-way analysis of variance.

633 **Figure legends**

634

635 **Figure 1– Body weight gain and caloric intake.** Body weight gain (A) and caloric intake (B)
636 from 21 to 60 days of age; body weight gain (C) and caloric intake (D) from 60 to 90 days of
637 age. The data are expressed as the means ± SEM and were obtained from 32 rats/4 litters of each
638 group (A and C) and from 4 experimental group litters (B and D). The inset represents the area
639 under the curve (AUC). ΩΩP<0.01, ΩΩΩΩP<0.0001 for Student's t-test. #P<0.05, ####P<0.0001 for
640 NP vs LP in the same conditions; ϕϕP<0.01, ϕϕϕϕP<0.0001 for NF vs HF in the same group based
641 on Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet factor and I: interaction
642 between LP and HF factors for the probability based on two-way analysis of variance, *P<0.05
643 and ****P<0.0001. NP: offspring of dams fed a normal-protein diet; LP: offspring of dams fed a
644 low-protein diet; NF: normal-fat diet and HF: high-fat diet.

645

646 **Figure 2– White adipose tissue accumulation.** Periepididymal (A), retroperitoneal (B) and
647 mesenteric (C) fat pad stores. The data are expressed as the means ± SEM and were obtained
648 from 24 rats/3 litters of each group. #P<0.05, ##P<0.01 and ####P<0.0001 for NP vs LP in the
649 same conditions; ϕϕϕϕP<0.0001 for NF vs HF in the same group based on Tukey's *post hoc*
650 analysis. LP: low-protein diet factor; HF: high-fat diet factor and I: interaction between LP and HF
651 factors for the probability based on two-way analysis of variance. **P<0.01, ***P<0.001 and
652 ****P<0.0001. NP: offspring of dams fed a normal-protein diet; LP: offspring of dams fed a
653 low-protein diet; NF: normal-fat diet and HF: high-fat diet.

654

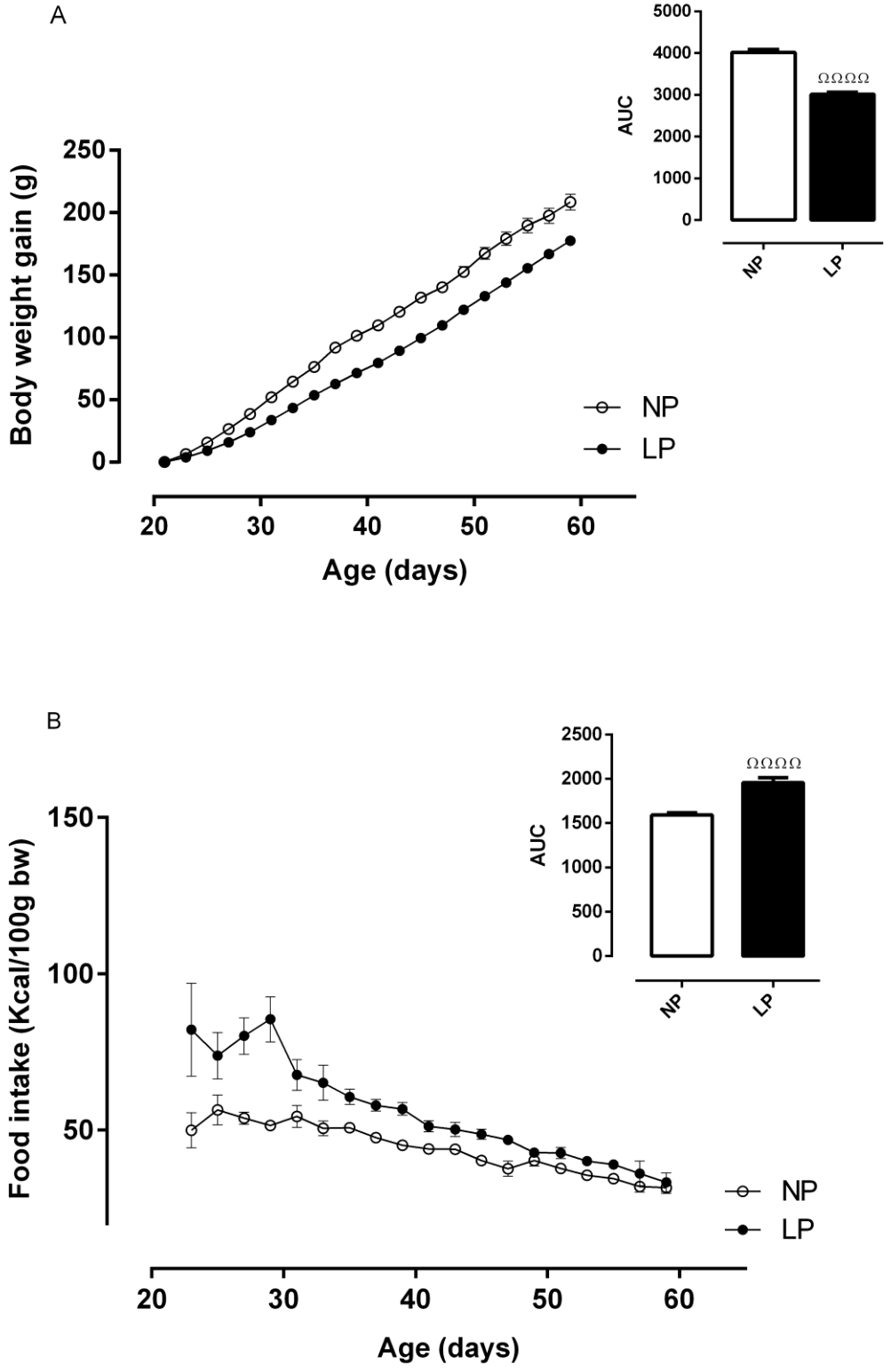
655 **Figure 3– Plasma glucose during ivGTT (A) and $K_{itt}$ (B).** The data are expressed as the means
656 ± SEM and were obtained from 12 rats of each group (from 3 different litters). The inset

represents the area under the curve (AUC). $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#\#}P<0.0001$ for NP vs LP in the same conditions; $^{\phi\phi}P< 0.01$, $^{\phi\phi\phi\phi}P<0.0001$ for NF vs HF in the same group based on Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet factor and I: interaction between LP and HF factors based on two-way analysis of variance. *P<0.05, **P<0.01 and ****P<0.0001. NP: offspring of dams fed a normal-protein diet; LP: offspring of dams fed a low-protein diet; NF: normal-fat diet and HF: high-fat diet.

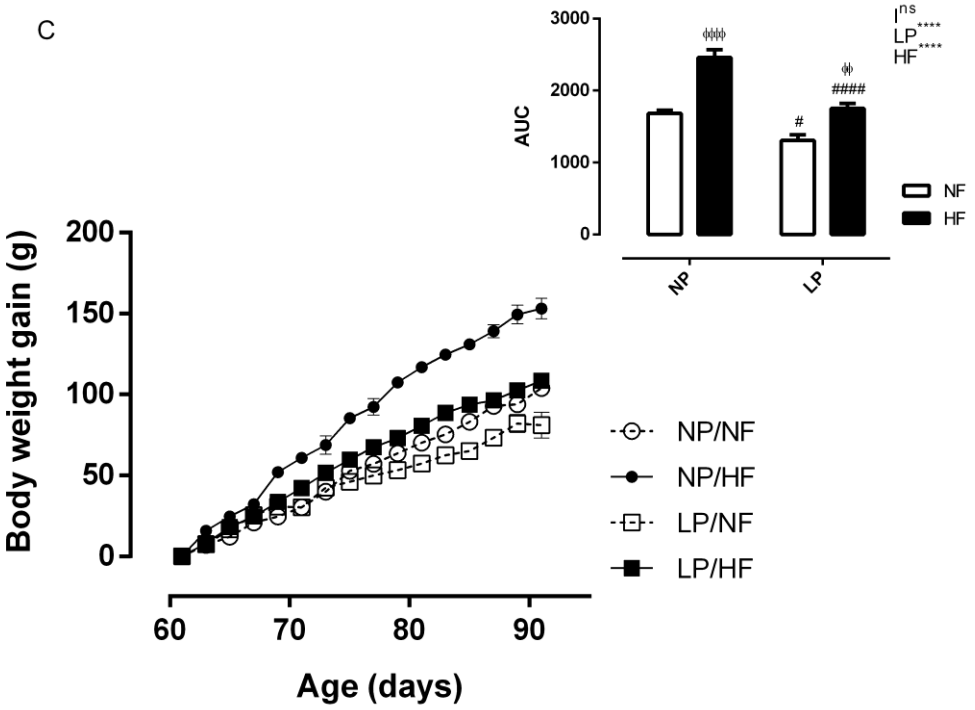
**Figure 4– Pancreatic islet insulin secretion.** Insulin secretion stimulated by different glucose concentrations (A). Percentage of insulin secretion stimulated by 8.3 mmol/l glucose and potentiated by 10 µmol/l ACh (B). Inhibition or activation of the insulinotropic effect of ACh (8.3 mmol/l glucose + 10 µmol/l ACh in the presence of 10 µmol/l neostigmine) by the nonselective antagonist Atr (10 µmol/l) (C), the selective M3 mAChR antagonist 4-DAMP (100 µmol/l) (D)and the selective M2 mAChR antagonist MTT (1 µmol/l) (E). Percentage of insulin secretion stimulated by 16.7 mmol/l glucose and inhibited by Adr (1 µmol/l) (F); insulin secretion (16.7 mmol/l glucose and 1 µmol/l Adr) in the presence of Yoh (100 µmol/l) (G). The line at 0 (B) represents 100% glucose insulinotropic action, (C, D and E) represents 100% ACh insulinotropic action and (F and G) represents 100% Adr insulinostatic action. The data were obtained from three different litters of each experimental group. $^{\Omega}P<0.05$ for Student's t-test; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#\#}P<0.0001$ for NP vs LP in the same conditions; $^{\phi}P<0.05$, $^{\phi\phi}P<0.01$, $^{\phi\phi\phi\phi}P<0.0001$ for NF vs HF in the same group based on Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet factor and I: interaction between LP and HF factors based on two-way analysis of variance. **P<0.01, ***P<0.001 and ****P<0.0001. NP: offspring of dams fed a normal-protein diet; LP: offspring of dams fed a low-protein diet; NF: normal-fat diet and HF: high-fat diet.

**Figure 5– Parasympathetic (A) and sympathetic (B) electrical nerve activity.** The data are expressed as the means ± SEM and were obtained from 24 rats/3 litters of each group. Lower panel graphs are representative records of nerve discharges of each group. $^{\Omega}P<0.05$ for Student's t-test; $^{\#}P<0.05$ for NP vs LP in the same conditions; $^{\phi}P<0.05$, $^{\phi\phi\phi}P<0.001$ for NF vs HF in the same group based on Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet factor and I: interaction between LP and HF factors based on two-way analysis of variance. *P< 0.05, **P< 0.01 and ****P< 0.0001. NP: offspring of dams fed a normal-protein diet; LP: offspring of dams fed a low-protein diet; NF: normal-fat diet and HF: high-fat diet.

Figure 1


A
Body weight gain (g)
250
200
150
100
50
0
20
30
40
50
60
Age (days)
NP
LP
AUC
5000
4000
3000
2000
1000
0
ΩΩΩΩ
NP
LP
B
Food intake (Kcal/100g bw)
150
100
50
20
30
40
50
60
Age (days)
NP
LP
AUC
2500
2000
1500
1000
500
0
ΩΩΩΩ
NP
LP

C


Body weight gain (g)
200
150
100
50
0
60
70
80
90
Age (days)
NP/NF
NP/HF
LP/NF
LP/HF
AUC
3000
2000
1000
0
NP
LP
NF
HF
I ns
LP ****
HF ****

D

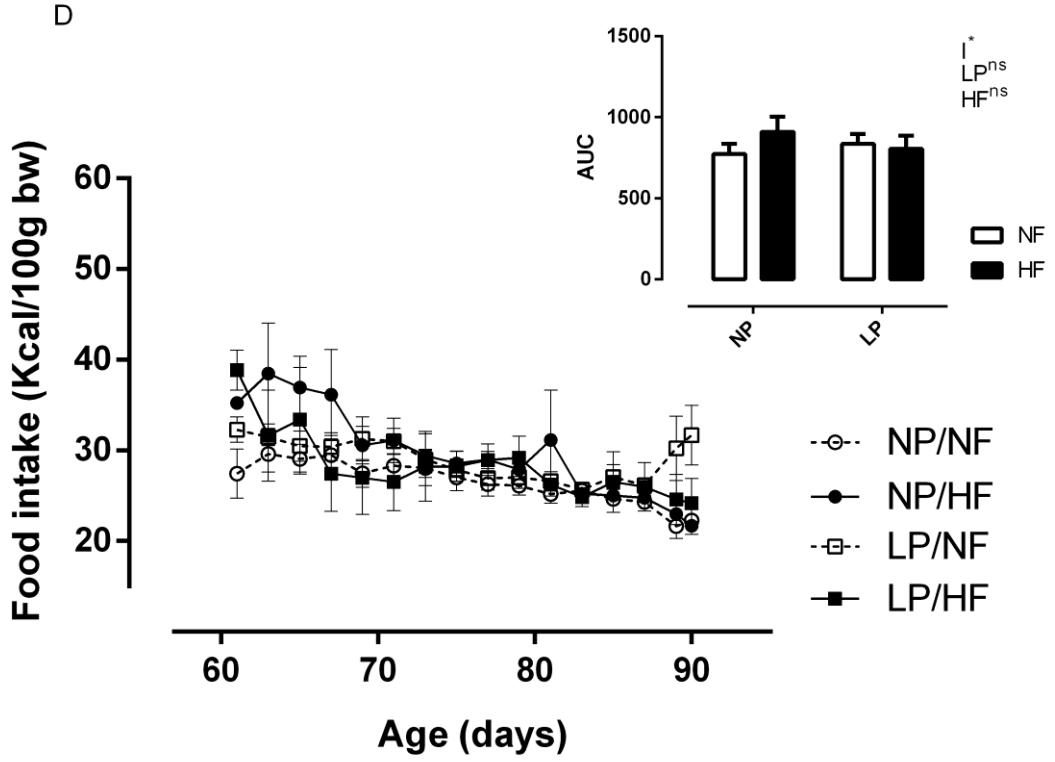

Food intake (Kcal/100g bw)
60
50
40
30
20
60
70
80
90
Age (days)
NP/NF
NP/HF
LP/NF
LP/HF
AUC
1500
1000
500
0
NP
LP
NF
HF
I *
LP ns
HF ns

Figure 2

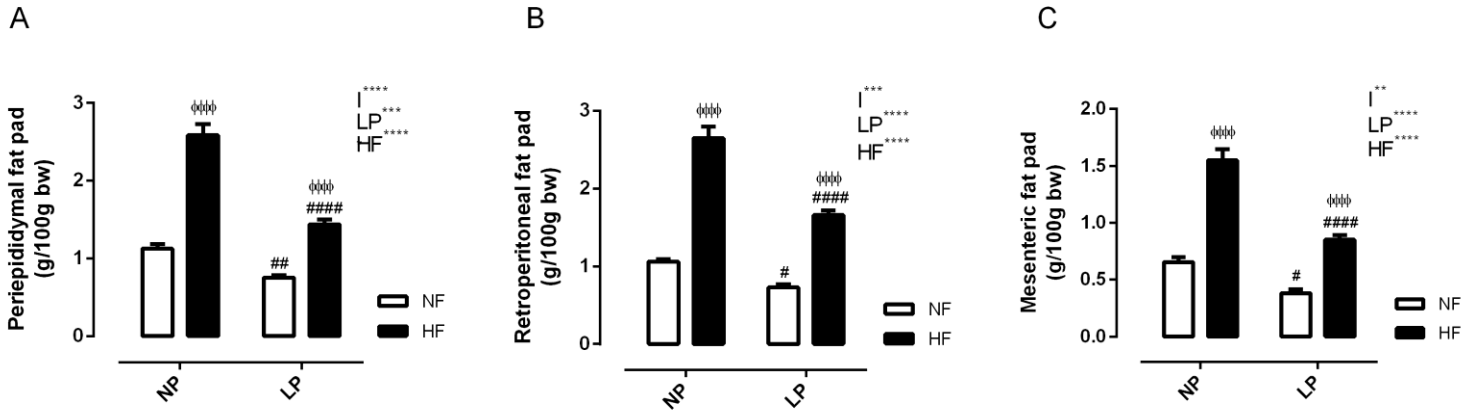
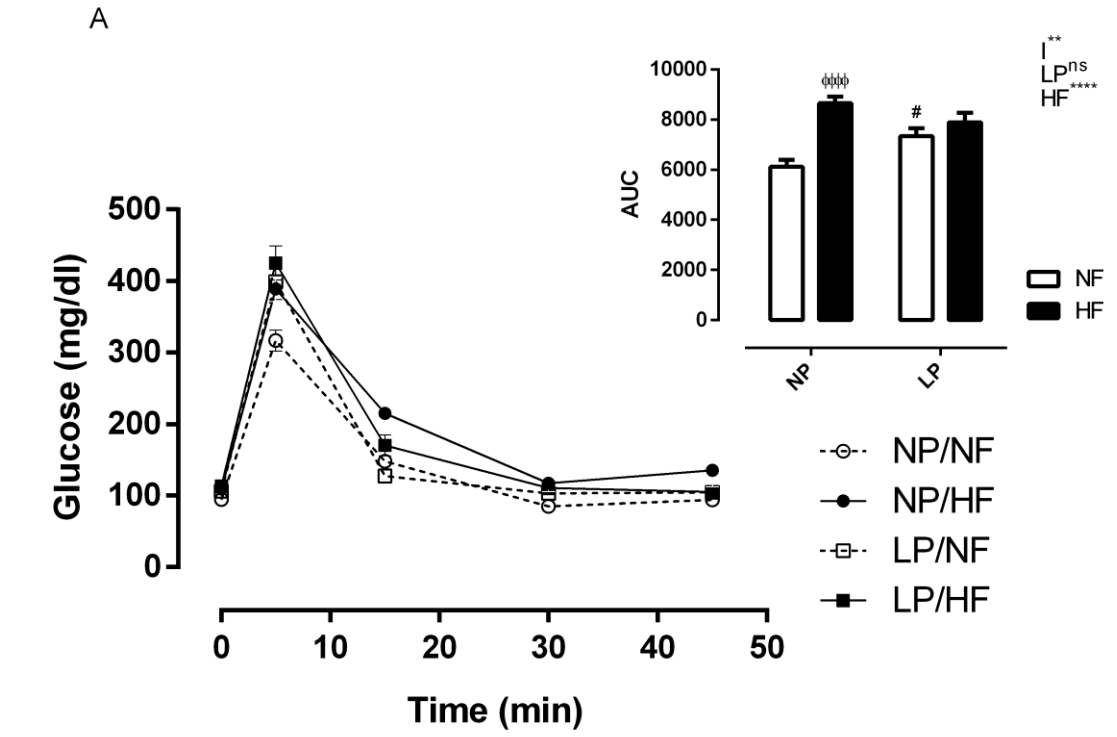

A
B
C
Periepididymal fat pad (g/100g bw)
Retroperitoneal fat pad (g/100g bw)
Mesenteric fat pad (g/100g bw)
I
LP
HF
NF
HF
NP
LP
##
####
#

Figure 3


A
Glucose (mg/dl)
500
400
300
200
100
0
0
10
20
30
40
50
Time (min)
AUC
10000
8000
6000
4000
2000
0
NP
LP
NF
HF
I**
LPns
HF****
NP/NF
NP/HF
LP/NF
LP/HF

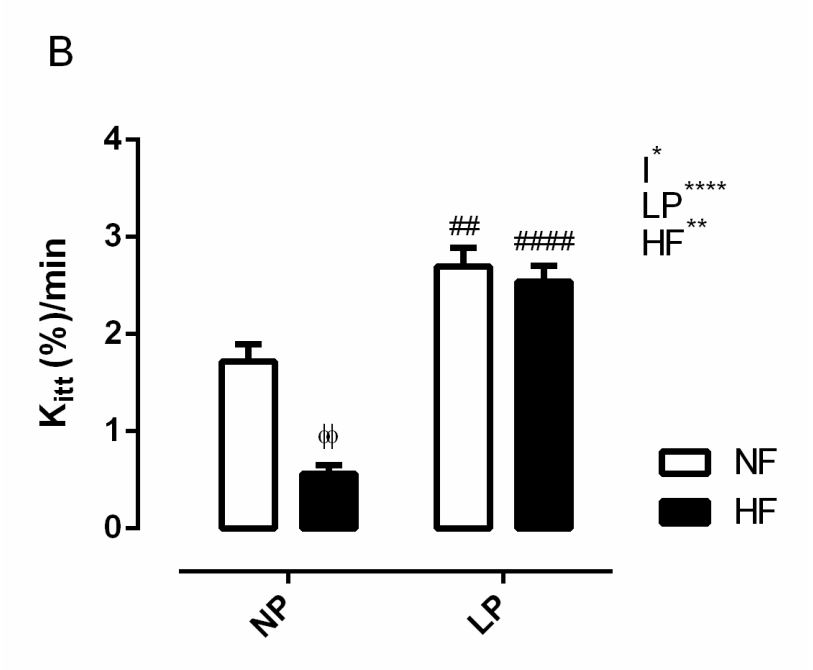
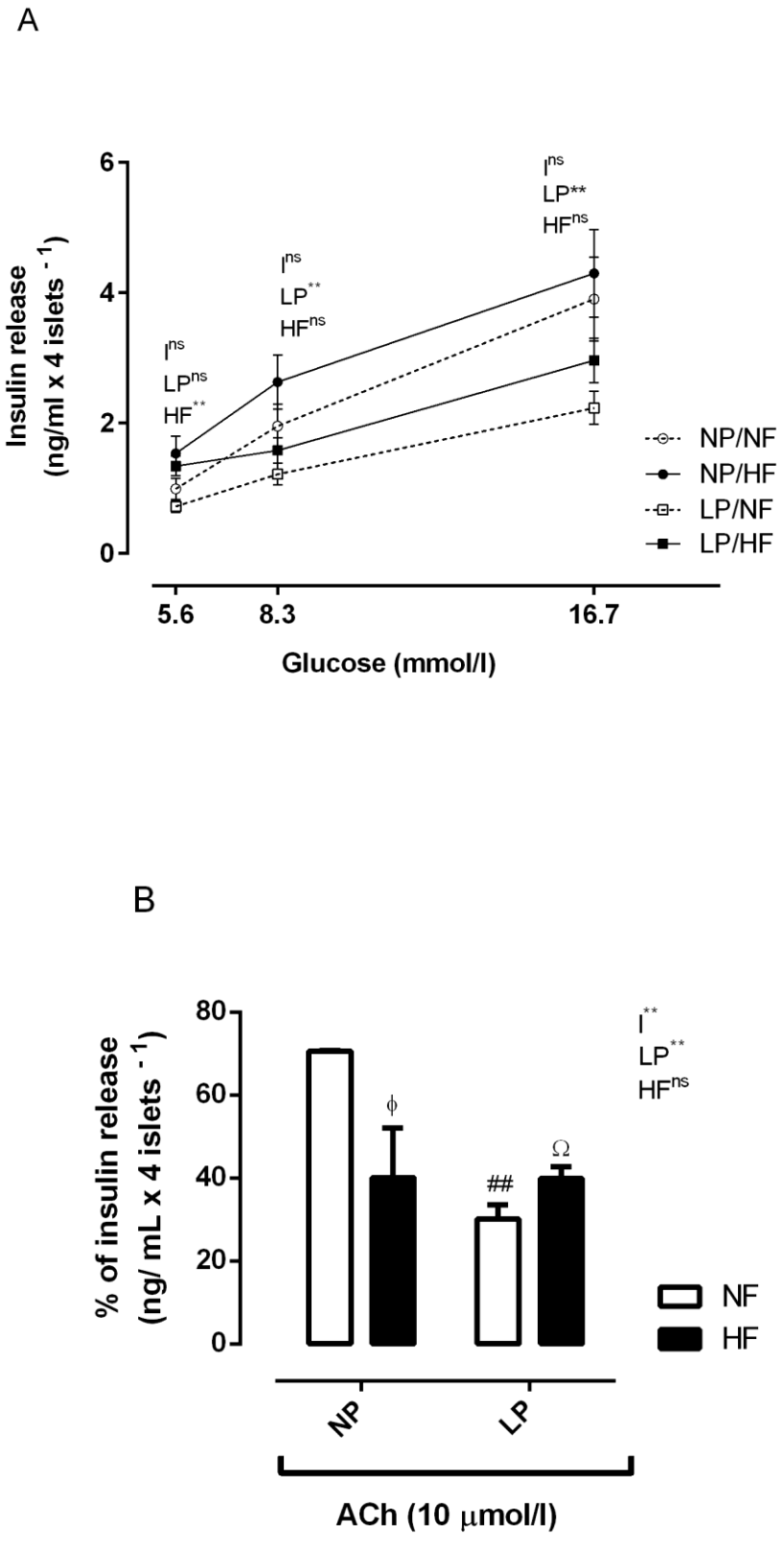

B
Kitt (%)/min
4
3
2
1
0
NP
LP
NF
HF
I*
LP****
HF**

Figure 4


A
Insulin release
(ng/ml x 4 islets -1)
6
4
2
0
Ins
LPns
HF**
Ins
LP**
HFns
Ins
LP**
HFns
NP/NF
NP/HF
LP/NF
LP/HF
5.6
8.3
16.7
Glucose (mmol/l)
B
% of insulin release
(ng/ mL x 4 islets -1)
80
60
40
20
0
ϕ
##
Ω
I**
LP**
HFns
NF
HF
NP
LP
ACh (10 μmol/l)

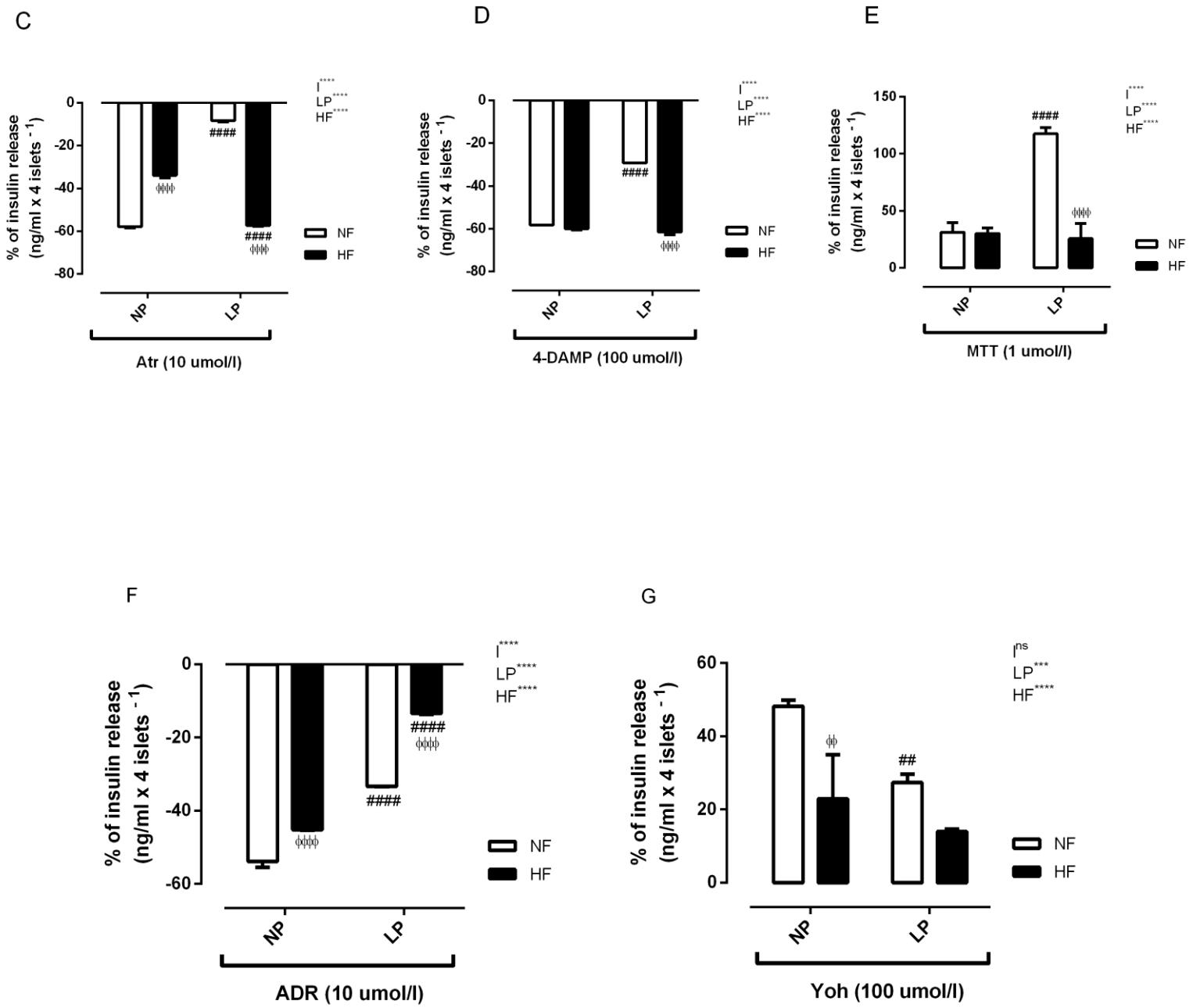
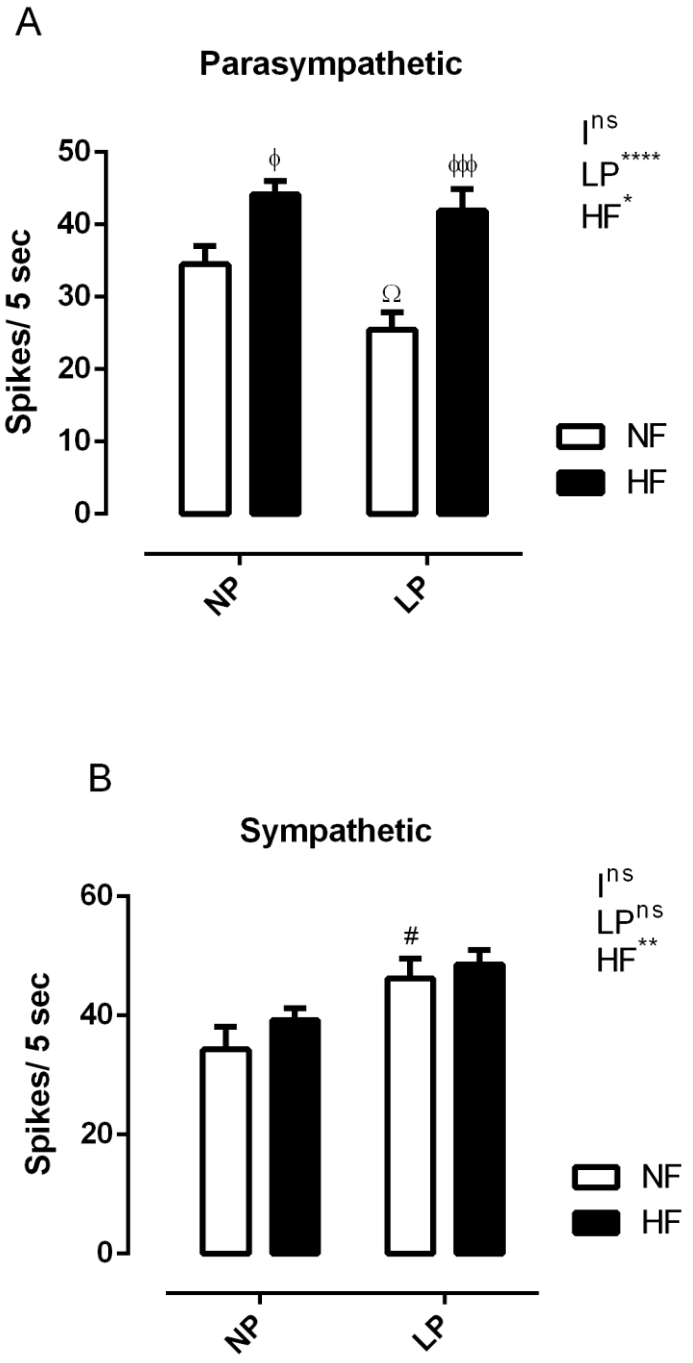
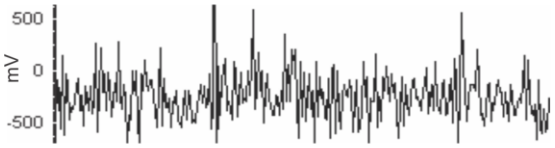

C
D
E
% of insulin release (ng/ml x 4 islets -1)
0
-20
-40
-60
-80
I****
LP****
HF****
NF
HF
NP
LP
Atr (10 umol/l)
4-DAMP (100 umol/l)
150
100
50
0
MTT (1 umol/l)
F
G
ADR (10 umol/l)
Yoh (100 umol/l)
Ins
LP***
60
40
20

Figure 5

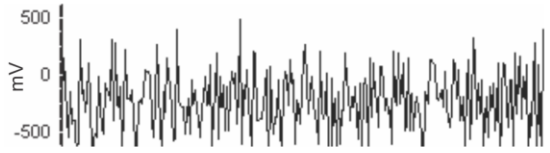

A
Parasympathetic
Spikes/ 5 sec
50
40
30
20
10
0
ϕ
ϕϕϕ
Ω
I ns
LP ****
HF *
NF
HF
NP
LP
B
Sympathetic
Spikes/ 5 sec
60
40
20
0
#
I ns
LP ns
HF **
NF
HF
NP
LP

## Parasympathetic

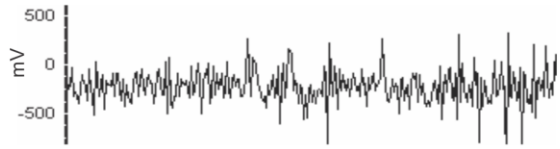
NP/NF



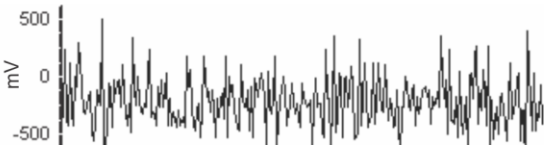

NP/HF




LP/NF

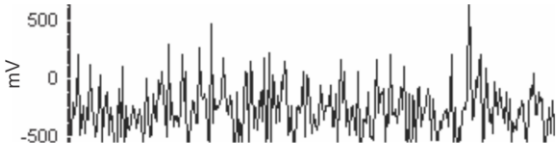



LP/HF

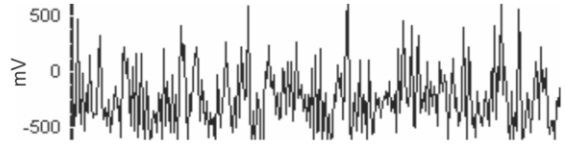



## Sympathetic

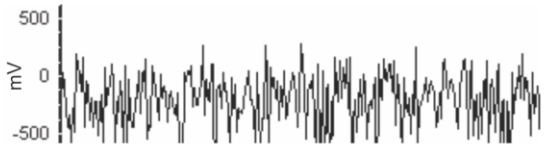
NP/NF




NP/HF




LP/NF




LP/HF