UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS – ÁREA DE CONCENTRAÇÃO: BIOLOGIA CELULAR E MOLECULAR

DOUGLAS LOPES DE ALMEIDA

PROGRAMAÇÃO METABÓLICA POR SUPERALIMENTAÇÃO NO INÍCIO DA VIDA, EFEITOS SOBRE O METABOLISMO E O TECIDO ADIPOSO MARROM: PODE O EXERCÍCIO FÍSICO MODERADO SER UM AGENTE PARA DESPROGRAMAÇÃO?

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.
Orientador: Pror. Dr. Paulo Cézar de Freitas Mathias.

Maringá
Fevereiro – 2017

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

Almeida, Douglas Lopes de
A447p Programação metabólica por superalimentação no início da vida, efeitos sobre o metabolismo e o tecido adiposo marrom: pode o exercício físico moderado ser um agente para desprogramação?/. Douglas Lopes de Almeida. -- Maringá, 2017.
52 f. : il. color., figs., tabs.

Orientador: Prof. Dr. Paulo Cezar de Freitas Mathias.
Coorientadora: Dr.a. Kesia Palma-Rigo.
Tese (doutorado) – Universidade Estadual de Maringá, Centro de Ciências Biológicas, Programa de Pós-Graduação em Biologia Celular, Área de Concentração Biologia Celular e Molecular, 2017.

1. Fisiologia animal. 2. Metabolismo Energético - Termogenese. 3. Obesidade - Programação metabólica. 4. Tecido adiposo marrom. 5. Biologia experimental. 6. Exercício Físico. I. Mathias, Paulo Cezar de Freitas, orient. II. Palma-Rigo, Késia, coorient. III. Universidade Estadual de Maringá. Centro de Ciências Biológicas. Programa de Pós-Graduação em Biologia Celular, Área de Concentração Biologia Celular e Molecular. IV. Título.

CDD 22. ed.571.57

JLM-001928

BIOGRAFIA

Douglas Lopes de Almeida nasceu em Maringá – PR em 24/10/1984. Bacharel em Educação Física pela Universidade Estadual de Maringá (colação de grau – Janeiro de 2010). Obeteve o título de Mestre em Biologia Celular pelo Programa de Pós-Graduação em Biologia Celular (área de concentração: Biologia Celular e Molecular) da Universidade Estadual de Maringá – PBC/UEM. Programa no qual atualmente cursa o Doutorado. Com experiência na área de fisiologia e biologia experimental, atuando principalmente nos temas: biologia celular da secreção, fisiologia do exercício, metabolismo energético e obesidade.

AGRADECIMENTOS

À Universidade Estadual Maringá pela estrutura que favoreceu a realização deste trabalho, ao Programa de Pós-Graduação em Biologia Celular pela oportunidade e ao CNPq pelo apoio financeiro.

Aos professores membros da banca, que prontamente aceitaram o convite: Prof. Dr. James A. Armitage (Deakin University - AUS) o qual também abriu as portas de seu laboratório, seu país e mesmo sua casa para nos receber na Austrália; Prof. Dr. Sidney Barnabé Peres (Departamento de Fisiologia – Universidade Estadual de Maringá); Prof. Dr. Antonio Marcos (Universidade Federal de São Luís do Maranhão -MA) e Prof. Dr. Rodrigo Mello Gomes (Universidade Federal de Goiânia - GO).

A todos aqueles que compartilharam comigo esta jornada: família, amigos, colaboradores no Brasil e na Austrália. Em especial agradeço ao Professor Dr. Paulo Cezar de Freitas Mathias e a Dra. Kesia Palma-Rigo, pela oportunidade de trabalhar e aprender com os mesmos. E por fim, a minha esposa, Letícia Xander, por todo o seu amor, encorajamento e suporte.

DEDICATÓRIAS

Dedico esta tese a minha mãe, dona Eurides Lopes da Silva Almeida, e a memória de meu pai, Diógenes de Almeida.

APRESENTAÇÃO

Esta tese é composta de dois artigos científicos experimentais. O primeiro, de título "*Maternal high fat diet alters morphology and gene expression of adipose tissue at early stage of life*", foi desenvolvido durante meu período de doutorado-sanduíche na Deakin University (Waurn Ponds – Vic – AUS) em colaboração com Dr. James A. Armitage. Neste estudo, observamos os efeitos da programação por hiperalimentação no período inicial da vida. Os resultados demonstram alterações morfológicas e da expressão dos genes no tecido adiposo ainda em fase inicial de seu desenvolvimento. Estas dialogam com as mudanças relatadas previamente por nosso grupo em animais adultos, submetidos a um tratamento similar (hiperalimentação) no início da vida. Assim, podemos dizer que os efeitos observados na vida adulta têm início ainda no estágio primário de formação do tecido adiposo marrom, sendo então resultado de 'programação metabólica'. O segundo artigo desta tese, entitulado "*Moderate physical exercise deprogram brown adipose hypoactivity by postnatal early overfeed in male rats*", aponta para a eficácia de um programa de exercício físico de intensidade moderada e de baixa frequência como método de intervenção não-farmacológico, capaz de reverter, ou 'desprogramar', o efeito da hiperalimentação na infância sobre a função do tecido adiposo marrom, aumentando a atividade termogênica do mesmo. Estes resultados colocam ambos, exercício físico moderado e tecido adiposo marrom, como potentes aliados no combate a obesidade e síndrome metabólica programada pela hipernutrição no início da vida. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, os artigos foram redigidos de acordo com as revistas a serem submetidos: *Journal of Endocrinology* e *Cellular Physiology and Biochemistry*, respectivamente.

Maternal high fat diet alters morphology and gene expression of adipose tissue at early stage of life. Douglas Lopes de Almeida[1], Sanna Barrand[2], Kesia Palma-Rigo[1], Paulo César de Freitas Mathias[1], James Andrew Armitage[2]. [1]Laboratory of Secretion Cell Biology, Department of Biotechnology, Genetics and Cell Biology, State University of Maringá – Maringá/PR, Brazil; [2]Deakin University, School of Medicine, Optometry Waurn Ponds, Victoria, Australia.

Moderate physical exercise deprogram brown adipose hypoactivity by postnatal early overfeed in male rats. Douglas Lopes de Almeida[1], Lucas Eduardo Cardoso[1], Audrei Pavanelo[1], Tatiane Aparecida Ribeiro[1], Claudinéia Conationi da Silva Franco[1], Kesia Palma-Rigo[1], Paulo César de Freitas Mathias[1]. [1]Laboratory of Secretion Cell Biology, Department of Biotechnology, Genetics and Cell Biology, State University of Maringá – Maringá/PR, Brazil.

RESUMO GERAL

INTRODUÇÃO

A epidemia de obesidade é uma preocupação de elevada prioridade, estando altamente associada a doenças cardiometabólicas e diabetes tipo 2, com impacto nas taxas de mortalidade e nos custos para a saúde a pública. Dados epidemiológicos apontam que, considerando a alta prevalência de obesidade em *cohorts* de crianças e adolescentes, este quadro tende a aumentar em um futuro próximo. Ainda que de fato a etiologia da obesidade seja ampla, dados substanciais suportam o papel do ambiente perinatal no desenvolvimento futuro da obesidade e síndrome metabólica. A hiperalimentação no inicio da vida, seja por alteração da dieta materna ou por exposição da prole a um quadro de abundância nutritiva, induz mamíferos a obesidade precoce, tornando-os mais susceptíveis a obesidade na vida adulta. Nosso grupo demonstrou em um estudo prévio que a obesidade induzida por hiperalimentação no ínicio da vida prejudica a função do tecido adiposo marrom (TAM), diminuindo sua termogênese. Presente em frações significativas em adultos humanos, o TAM desempenha um importante papel no metabolismo energético dos mamíferos, liberarando energia na forma de calor através da ação da proteína desacopladora-1 (UCP1). Dado o possível impacto do TAM na balança energética, cresce o interesse por encontrar meios de aumentar a atividade termogênica deste tecido. Neste âmbito, o exercício físico começa a ser testado como um método não-farmacológico de ativação do TAM. Considerando a importância do TAM no metabolismo energético e os efeitos duradouros da obesidade induzida no início da vida sobre este tecido, bem como sobre outros parâmetros biométricos e metabólicos. Considerando os possíveis efeitos do exercício físico sobre o metabolismo e sobre o TAM. Nesta tese investigamos os efeitos da hiperalimentação no início da vida sobre o tecido adiposo em estágios iniciais de seu desenvolvimento, e ainda o impacto de um protocolo de exercício físico de intensidade moderada e baixa frequência na função termogênica do TAM de ratos adultos, que foram metabolicamente programados por hiperalimentação no início da vida.

OBJETIVOS

Manuscrito 1: Investigar a morfologia e expressão gênica no tecido adiposo branco (TAB) e tecido adiposo marrom (TAM) interescapular em filhotes expostos a hiperalimentação por dieta materna rica em gordura em estágio inicial de desenvolvimento, aos 10 dias de idade.

Manuscrito 2: Observar os efeitos de um programa de exercício moderado e de baixa frequência

na ‘desprogramação’ da função do TAM em ratos adultos machos programados por hiperalimentação no inicio da vida.

MÉTODOS

Manuscrito 1: Ratos Spreague Dawley fêmeas foram divididas em 2 grupos: dieta controle (*NFD* – n=6) e dieta hiperlipídica (*HFD* – n=10). Os animais foram mantidos na mesma dieta durante todo o período experimental, desde 4 semanas antes da concepção até o dia pós natal 10, quando a prole foi pesada e eutanisiada. Amostras de tecido adiposo foram coletadas dos coxins subcutâneo e interescapular para análises histológicas e da expressão genética.

Manuscrito 2: Ratos Wistar prênhes foram colocadas em gaiolas individuais, o nascimento foi considerado dia 0. No dia 2 as ninhadas foram padronizadas para 9 (grupo controle, NL) ou 3 (grupo experimental, SL) por mãe. Após o desmame (dia 21) os animais foram então randomicamente divididos entre sedentários (NL SED/ SL SED) e exercitados (NL EXE, SL EXE). O protocolo teve início no dia 30 com um teste de esforço para determinação da carga de trabalho. As sessões de treino foram realizadas 3 vezes por semana, a 60% da carga de trabalho (velocidade final) atingida no teste de esforço, com duração de 40 minutos. Os testes foram repetidos a cada 15 dias para ajuste da carga até o final do protocolo no dia 80. NO dia 81 os animais foram submetidos a uma cirurgia para implante de um transponder de temperatura sob o TAM interescapular (TAMi). A temperatura do TAMi foi aferida no ciclo claro e escuro dos dias 87 a 90. No dia 91 os animais foram eutanasiados, amostras de sangue e tecido adiposo marrom interescapular foram armazenadas para futuras análises. Um *set* separado de animais de cada grupo, que não teve transponder implantado, foi usado para registro da atividade da inervação simpática do TAMi.

RESULTADOS E DISCUSSÃO

Manuscrito 1: Os adipócitos do tecido adiposo branco subcutâneo de ratos do grupo *HFD* apresentaram maior área quando comparados ao controle ($p<0.01$). Embora os adipócitos do TAMi de ratos do grupo HFD não diferiu dos controles em tamanho, a incidência de adipócitos uniloculares e de maior tamanho dentro do TAM foi maior comparados ao controle($p<0.05$). A dieta materna rica em gordura aumentou a expressão do gene para Leptina ($p<0.05$) no tecido adiposo subcutâneo; e diminuiu a expressão dos genes ObR, PPAR gama, PPAR alfa e PRDM16 no TAM ($p<0.05$). Enquanto em humanos o desenvolvimento do tecido adiposo se da no 3° trimestre do período intrauterino, em ratos o tecido adiposo se desenvolve principalmente ao

final do período de gestação e na primeira metade da lactação. As alterações histológicas encontradas no tecido adiposo subcutâneo e no TAM demonstram uma descaracterização estrutural do tecido adiposo pela hiperalimentação no inicio da vida, e são semelhantes as previamente descritas na literatura para animais adultos metabolicamente programados. As alterações de expressão de RNAm no tecido adiposo subcutâneo e, principalmente, no TAM; afetam fatores de transcrição chaves para a diferenciação do tecido e sua função, podendo estar envolvidos na hipoatividade observada no mesmo em ratos adultos.

Manuscrito 2: Corroborando com os dados previamente demonstrados, a hiperalimentação no inicio da vida diminuiu a termogênese em ratos machos adultos no período claro ($p<0.0001$) e escuro ($p<0.01$). O exercício físico foi eficaz em promover o aumento da termogênese no período claro ($p<0.0001$), mas não no escuro ($p=0.2236$). Adicionalmente, a hiperalimentação infantil diminuiu a atividade da inervação simpática do TAMi ($p<0.0001$), enquanto o exercício físico foi eficiente para aumenta-la ($p<0.05$). Por outro lado, o exercício físico não afetou a quantidade de receptores adrenérgicos β3 no TAMi, que foi reduzida pela hiperalimentação infantil ($p<0.05$). Como previamente demonstrado, a hiperalimentação no período infantil reduz a atividade termogênica do TAMi, adicionalmente, neste estudo demonstramos que o exercício é capaz de reverter esta hipoatividade, aumentando assim o gasto energético do animal em período de descanso. A literatura demonstra que exercícios físicos de endurance praticados com regularidade estimulam o TAMi *in vitro* e em humanos magros e animais controle. Contudo, pelo nosso conhecimento, nosso estudo é o primeiro a demonstrar que um protocolo de exercício físico moderado e de baixa frequência é um método viável para a 'desprogramação' da hipoatividade do TAMi em ratos programados para a obesidade por hiperalimentação no início da vida.

CONCLUSÕES

Manuscrito 1: A prole induzida a hiperalimentação por dieta materna rica em gordura apresentou alterações na morfologia e na expressão dos genes do tecido adiposo subcutâneo e marrom interescapular, as quais podem estar implicadas em disfunções de longo prazo no tecido adiposo.

Manuscrito 2: O protocolo de exercício de intensidade moderada e de baixa frequência foi eficiente em 'desprogramar' os efeitos da hiperalimentação infantil em ratos machos na fase adulta. Melhorando parâmetros biométricos e metabólicos e bloqueando a hipoatividade do TAMi nestes animais.

GENERAL ABSTRACT

INTRODUCTION

The obesity epidemic is a priority concern, as its relationship with cardiovascular diseases and type 2 diabetes, is associated with mortality and with elevated costs for public health systems across the globe. Epidemiological data higlights the fact that the obesity epidemic is likely to increase in the near future, as younger cohorts present with, even increasing, high rates of early obesity onset and prevalence. Although there is a broadly understood adult etiology for obesity, substantial data from epidemiological and experimental studies indicate the role of the perinatal environment in promoting obesity and related metabolic syndrome. Postnatal early overfeeding, induced by maternal high fat consumption or by early exposure to overfeeding during the suckling period, lead mammals to demonstrate a precocious onset of obesity. Infants and childrens who are obese are highly susceptible to remain obese into adult life. In a previous study, our group demonstrated that postnatal early overfeeding resulted in rats becoming obese and presenting an impaired interscapular brown adipose tissue (iBAT) thermogenic function, producing less heat than the control animals both, during day and night-time periods. Brown adipose tissue (BAT) is present in adult humans and BAT thermogenesis increase energy expenditure through uncoupling-protein-1 (UCP1) activity, which disrupts the normal mitochondrial energy system, resulting in a lower production of ATP and diversion of energy as heat. Given the potential of BAT to impact upon energy balance, revert metabolic program and augment energy expenditure by BAT to reduce fat storage and counter obesity, is a desirable target. The regular practice of physical exercise is one physiological approach that results in increased BAT activity. Few studies have considered the important role of BAT on the energetic metabolism, as a mechanism to reverse the long-term programmed effects of the early overfeeding on animals. The possible effects of physical exercise upon metabolism and BAT in programmed animals have also received scant attention. In this thesis we investigated the effects of early overfeeding by maternal high fat consumption on the adipose tissue at early stages of life and tissue development, moreover, we tested the effectiveness of a moderate and low-frequency exercise protocol on the thermogenesis function of iBAT of adult rats, which were metabolic programmed by early overnutrition.

AIMS

Manuscript 1: To investigate, at postnatal day 10, the morphology and gene expression in subcutaneous WAT and interscapular BAT from pups born to High Fat Fed (HFD) dams.

Manuscript 2: To investigate the potential of a moderate and low-frequency exercise protocol to 'deprogram' the function of iBAT in adult male rats made obese by exposre to overfeeding during lactation.

METHODS

Manuscript 1: Nine-week-old female Sprague Dawley rats were divided into 2 groups: control normal fat diet (NFD – N=6), or high-fat diet (HFD – N=10). Rats were kept on their respective diet for 4 weeks prior to conception, through gestation and after birth, until postnatal day 10 (PN10), when pups were weighed and euthanized. Adipose tissue was collected from subcutaneous and interscapular depots for histology and gene expression studies.

Manuscript 2: Wistar rats litters were delivered normally but at PN2, litters were adjusted to 9 pups per dam (normal litters - NL) for control, or 3 pups (small litter – SL) per dam to induce overfeeding during early life. At PN21, all offspring were weaned and randomly divided into exercise or sedentary groups (NL SED, NL EXE, SL SED, and SL EXE). An exercise protocol commenced at PN30. Animals were initially subjected to an effort test on a treadmill. Training sessions (45minutes) were then performed 3 times per week, at 60% of the final workload achieved in the effort test. The effort test was repeated each 15 days to adjust the training workload. At PN80, animals ceased training and at PN81, underwent surgery to implant a temperature transponder underneath interscapular BAT (iBAT). The iBAT temperature was measured during light and dark periods from PN87 to PN90. At PN91 animals were euthanized, blood samples and fat pads collected for further analysis. IBAT nerve activity was recorded from a separated set of (non operated) animals at PN90.

RESULTS AND DISCUSSION

Manuscript 1: Subcutaneous white adipose tissue (sWAT) adipocytes from HFD offspring were larger compared to controls ($p<0.01$). Although iBAT adipocytes did not change in size, the presence of unilocular adipocytes found within iBAT were larger in HFD pups than in NFD ($p<0.05$). Maternal HFD increased the expression of Leptin in sWAT ($p<0.05$). Reduced gene expression of LeptinR, PPAR gamma, PPAR alpha and PRDM16 were observed in iBAT of HFD offspring compared with control offspring ($p<0.05$). These morphological changes in WAT and BAT, points to the structural abnormalities within adipose tissue during they earliest days of postnatal life being programmed by early overnutrition. These findings are similar to those observed in the literature for adult rats that were metabolic programmed by early overnutrition,

indicating that the obesity seen inadulthood may be, in fart, caused by abnormal development of these tissues. Moreover, the mRNA altered expression in BAT of transcriptor facts involved in differentiation and function of BAT, may underlie the lower thermogenesic capacity observed in adult animals.

Manuscript 2: Early postnatal overfeed impaired iBAT activity during their inactive (lights-on) ($p<0.0001$) and inactive (lights-off) periods ($p<0.01$). Exercise improved iBAT function in the lights-on ($p<0.0001$), but not on lights-off ($p=0.2236$) perior. Exercise was associated with increased sympathetic iBAT nerve firing rate ($p<0.05$), while postnatal overfeeding was assoiated with a reduction in sympathetic nerve activity ($p<0.0001$) that did not respond to exercise training. In control animals, exercise resulted in a reduction of β3-AR content in iBAT ($p<0.01$), consistent with the increase nerve activity. Consistent with the nerve activity data, early overfeeding did not affect β3-AR content in iBAT ($p=0.2172$. We have presviously shown that postnatal early overfeeding induced iBAT thermogenesis hypoactivity, which was confirmed on this study. Additionally, this study shows that the exercise is able to improve the thermogenesis in lean and early obese programmed rats, which may indicate an elevation on the basal metabolical rate on these animals. Regular endurance exercise is shown to positively affect BAT in vitro differentiation and BAT activity in lean humans and control animals. This study is the first, of our knowledge, to show that a moderate intensity/low-frequency exercise protocol is a viable non-pharmacological method to 'deprogram' the postnatal early overfeeding effects on BAT.

CONCLUSIONS

Manuscript 1: Offspring of fat fed dams demonstrate altered morphology and gene expression in sWAT and iBAT during earliest stages of tissue development, which may have implications for long-lasting dysfunctional adipose tissue.

Manuscript 2: A moderate intensity – low frequency exercise protocol was able to deprogram postnatal early overfeeding effects in adult male rats, improving biometrical and metabolical parameters and reversing iBAT hypoactivity.

TEXTOS REFERENTES AOS ARTIGOS

MANUSCRITO 1

Maternal high fat diet consumption alters morphology and gene expression of adipose tissue at early stage of life.

Douglas Lopes de Almeida[1], Sanna Barrand[2], Kesia Palma-Rigo[1], Paulo César de Freitas Mathias[1], James Andrew Armitage[2]

[1]Laboratory of Secretion Cell Biology, Department of Biotechnology, Genetics and Cell Biology, State University of Maringá – Maringá/PR, Brazil;

[2]Deakin University, School of Medicine, Optometry Waurn Ponds, Victoria, Australia.

Short Running Title: Perinatal overfeeding affects adipose tissue.

Corresponding Author: Dr. Douglas Lopes de Almeida - Department of Biotechnology, Cell Biology and Genetics, State University of Maringá, 5790 Av Colombo, Sala 19, Maringá, PR, 87020-900, Brazil. Phone/fax: +55 44 3011-4892; e-mail: dougalmeida84@gmail.com.

Abstract

Background/Aims: In the current scenario of obesity epidemic and its related comorbities, the adipose tissue has received wide attention. Adipose tissue exhibit great plasticity, participating on several physiological process, including thermogenesis by brown adipose tissue. Perinatal nutrition may lead to dysfunctional adipose tissue in later life. In this study, we investigated the early effects of high-fat-diet fed dams on offspring' developing adipose tissue. **Methods:** Nine-week-old female Sprague Dawley rats were divided into 2 groups: control normal fat diet (6% fat - NFD – N=6), or high-fat diet (21% fat - HFD – N=10). Rats were kept in their respective diet for 4 weeks prior to conception, through gestation and after birth, until postnatal day 10 (PN10), when pups were weighed and euthanized, adipose tissue was collected from subcutaneous and interscapular depots for histology and gene expression. **Main Results:** Subcutaneous white adipose tissue (sWAT) adipocytes from HFD offspring were larger compared to the control ($p<0.01$). Although iBAT adipocytes did not change in size, the presence of unilocular adipocytes found within iBAT were larger in HFD pups than in NFD ($p<0.05$). Maternal HFD increased the expression of Leptin in sWAT ($p<0.05$) and lowered the gene expression of LeptinR, PPAR gamma, PPAR alpha and PRDM16 on iBAT compared to control rats ($p<0.05$). **Conclusions:** Offspring of fat fed dams demonstrate altered morphology and gene expression in sWAT and iBAT during earliest stages of tissue development, which may be imply on long-lasting dysfunctional adipose tissue.

Key words: Maternal high-fat diet, early life, adipose tissue, morphology, gene expression.

Introduction

Obesity is a well-recognized problem for individuals' health, being highly associated to cardiometabolic diseases and several types of cancer, therefore having a great impact on mortality levels and elevated costs for public health (Masters, et al. 2013; Sturm and An 2014). Although there is a broad etiology for obesity, substantial data from epidemiological and experimental studies has supported the role for perinatal environment on obesity and related comorbities onset (Hales and Barker 1992; Nielsen, et al. 2015; Plagemann, et al. 2009). There is a wide range of early life insults with later metabolic implications, among which, maternal diet is a strong factor that may lead offspring to obesity, metabolic syndrome and cardiovascular disease (Lamb, et al. 2010; Wakana, et al. 2015).

The excessive fat accumulation in adipose tissue is a common feature in obesity. Nevertheless, due to its endocrine, immune and thermogenesis capacity, actively participating in whole-body homeostasis, adipose tissue plays a role in mammals far beyond of simple calories storage. Exhibiting a complex plasticity, the adipose organ is classically divided into unilocular white adipose tissue (WAT) and multilocular brown adipose tissue (BAT), which is rich in mitochondrial uncoupling-protein-1 (UCP1) that dissipate chemical energy as heat. Besides, there is a third brown-like, or '*brite*', adipocytes, which are adipocytes that express UCP1 and other characteristics similar to BAT, within some WAT depots (Fenzl and Kiefer 2014; Mraz and Haluzik 2014).

Differently of humans, which adipose tissue development is mainly during late gestation, adipose tissue formation in rodents occur mostly postnatal (Symonds, et al. 2012). At birth, although interscapular BAT (iBAT) in rats is almost fully developed, subcutaneous WAT (sWAT) is organized, but present in a small ratio, and other depots of WAT remain visually undetectable for the first weeks of life (Mori, et al. 2014). Metabolic programming by nutrition on perinatal period has long lasting effects on adipose tissue, affecting the size of the adipocytes, gene and protein expression, inflammation and thermogenesis on adulthood (de Almeida, et al. 2013; Figueiredo, et al. 2012; Lukaszewski, et al. 2013).

Maternal high-fat-diet (HFD) consumption is a well-stablished model for metabolic program studies and may affect different tissues plasticity during the fetal development (Hokke, et al. 2016). Murabayashy and colleagues showed that pregnant mice HFD feed induced adipocyte hypertrophy, increased TNF alpha and decreased GLUT-4 expression in intrauterine WAT, leading the fetus to insulin resistance (Murabayashi, et al. 2013). However, because the early effects of maternal HFD exposure on offspring developing WAT and BAT are not completely elucidate and may imply in later tissue

malfunction. The present study aims to investigate the morphology and gene expression on sWAT and iBAT of pups from HFD fed dams at half of the lactation period. This may contribute to a better understanding of the mechanisms underlying metabolic program effects on the adipose organ, which impairs its physiological functions in later life.

Methods

All animal handling and experimental procedures were conducted in accordance with the *Australian Code for the Care and Use of Animals for Scientific Purposes*, National Health and Medical Research Council of Australia. The Animal Ethical Committee of Deakin University approved the protocol. Nine-week-old female Sprague Dawley rats were housed (at Deakin Waurn Ponds Animals Facility) in a controlled environment (12-hour light-dark cycle, room temperature 22 ±2°C) with *ad libitum* access to food and water. After an adaptation week, rats were randomly assigned to either, normal-fat (6% fat - NFD – n 6) or high-fat diet (21% fat - HFD – n 10) group (Table 1). Rats were maintained in their diet for 3 weeks prior to breeding week, throughout gestation and the first half of lactation, until post-natal-day (PN) 10, when the experiments were carried out.

Offspring from NFD and HFD fed dams were weighed at PN 01, 05 and 10. Prior to euthanasia, PN10 rats body composition was assessed by dual-energy x-ray absorptiometry technique. Animals were euthanized by decapitation, blood samples were taken for further biochemical assays and fat depots from sWAT and iBAT were excised and collected for histologic and gene expression evaluations.

Histology

Subcutaneous WAT and iBAT were dissected and fixed in PBS -4% paraformaldehyde for 24 hours. The tissues were processed as described in the literature (Berry et al, 2014, p56) prior to paraffin embedding. Five (5) µm serial sections were made using a microtome (Microtec cut 4060) and placed on glass slides, four (4) slices per slide with an interval of 100 µm between slides. The slices were stained with hematoxylin and eosin following standard lab protocols. The images were obtained using a light microscope coupled to a camera (HCR) and software (Axivision) at an increase of 40X. To analyze the images was used the ImageJ analysis software. For cells counting and size just whole cells with nuclei, which membrane does not touch the borders of the optic zone were considered (N = 4 animals per group, 300 cells per animal for sWAT and iBAT, 100 cells per animal for white-like adipocytes in iBAT).

Biochemical measurements

The collected blood samples were centrifuged and the plasma collected. The glucose and leptin plasma concentration were assessed by ELISA method, using specific commercial kits (IBL-America, USA), and a microplate counter (Multi-Mode Microplate Reader, FlexStation® 3 Benchtop, Molecular Devices, Sunnyvale/CA, USA).

RNA isolation and gene expression assay

Total RNA was extracted from frozen sWAT and iBAT using a homogenizer and RNeasy columns (QIAGEM®) following manufacturer's protocol. For cDNA synthesis, a Thermo Scientific (Thermo Fisher Scientific, DE, USA) kit was used. Quantitative real-time PCR was performed at TwoSteps Instrument (Applied Biosystems, CA, USA) following standard laboratory protocol. Key genes were analyzed for sWAT, Leptin, Leptin Receptor (OB-R), Peroxisome Proliferator activated receptor (PPAR) alpha, Peroxisome Proliferator activated receptor (PPAR) gamma. For iBAT, the UCP1, OB-R, PPAR gamma, PR domain containing 16 (PRDM16) and PPAR alpha were assessed. Primers were designed for rats. GAPDH was used as internal control and gene expression was determined by relative expression deltaCt method. Relative expression normalised to [cDNA] per well = deltaCt/[cDNA per well], n=6 for both dietary groups.

Statistical Analysis

Data are expressed as mean ± SEM and *p* value for significant differences, except for histogram of adipocytes, where the frequency of distribution by cell area is showed as percentage (%) of the total cells analysed per group. Statistical analysis were performed using GraphPad Prism® 6.01 software (San Diego, CA, USA) and IBM SPSS 20 (Chicago, IL, USA). Data were subjected to Student's *t-test* or One-way ANOVA, followed by Bonferroni's post-test. $P<0.05$ was considered statistically significant.

Results

HFD dams lead infants to early postnatal obesity

The maternal high fat diet was able to promote higher body weight in the offspring compared to control litters at PN01 (NFD vs HFD, $p<0.05$), PN05 (NFD vs HFD, $p<0.05$) and PN10 (NFD vs HFD, $p<0.05$). The body mass assessment shows that at PN10, compared to NFD pups, HFD rats present different body composition, with similar ratio to lean mass (NFD vs HFD) but greater ratio for fat mass (NFD vs HFD, $p<0,05$). Plasma analysis reveals that HFD group exhibit higher concentrations of blood glucose (NFD vs HFD, $p<0.05$) and leptin (NFD vs HFD, $p<0.05$) than the control group (Fig 1).

Altered adipose tissue morphology in HFD offspring

The morphological analysis of sWAT shows that the mean area of adipocytes from HFD group was larger than in NFD rats (NFD 147,6 ± 17,62 vs HFD 284,8 ± 30,03, $p<0.01$) (Fig2A). In the same way, the distribution frequency of the adipocytes by cell area shows that while HFD 75% of sWAT adipocytes area were at maximum of 385 μm^2, 75% of sWAT adipocytes cell area from NFD group was at maximum of 215 μm^2, (fig 2.B).

No difference was noted in iBAT adipocytes area (NFD 27,48 ± 2,344 vs HFD 28,70 ± 2,570, ns) (Fig3A), and the frequency of distribution per cell area is similar between groups (75% NFD cell area <33,7 μm^2; 75% HFD cell area < 34,3 μm^2) (Fig.3B). Nevertheless, HFD offspring presented larger white-like adipocytes in IBAT than NFD offspring (NFD 10,45 ± 1,590 vs HFD 25,13 ± 5,740, $p<0.05$), with a different distribution (75% NFD cell area <7.9 μm^2; 75% HFD cell area <18.05 μm^2).

HFD changes adipose tissue genes expression at early life

The morphology results may suggest that maternal HFD is able to program adipose tissue development, to assess whether these changes would also carry molecular implications; we test sWAT and iBAT expression of some key genes. In sWAT, we note that Leptin gene expression was higher in HFD than in NFD group (NFD 17.5 ± 5 vs HFD 32.7 ± 5, $p<0.05$). No difference was observed for the expression of OB-R (NFD 2.6 ± 1 vs HFD 2.2 ± 1, ns), PPAR gamma (NFD 40.1 ± 9.8 vs HFD 40.6 ± 10, ns), or PPAR alpha (NFD 14.3 ± 2 vs HFD 13.7 ± 2.3, ns) (Fig 4).

On iBAT, no difference was noted on UCP1 gene expression (NFD 205 ± 20 vs HFD 165 ± 22.5, ns). Nevertheless, HFD group exhibited lower gene expression for OB-R (NFD 3 ± 0.25 vs HFD 1.9 ± 0.3, $p<0.05$), PPAR gamma (NFD 9.2 ± 0.8 vs HFD 6.9 ± 1, $p<0.05$), PRDM16 (NFD 0.525 ± 0.025 vs HFD 0.378 ± 0.03, $p<0.05$) and PPAR alpha (NFD 1.1 ± 0.1 vs HFD 0.7 ± 0.13, $p<0.05$) (Fig.5).

Discussion

The perinatal environment may shapes mammals' metabolism and behavior for lifetime (Hales and Barker 2013; Vickers, et al. 2003). With obesity raising to epidemic proportions and the Western diet based on food with high fat content, address the effects of elevated levels of maternal fat consumption on offspring developing organism it is an important step on the way to understand the adverse physiological and metabolical outcomes that they may face in later life. In this study, the HFD maternal consumption changes the morphology and alters the gene expression of sWAT and even more of iBAT at an early phase of life.

The present data shows that early HFD exposed rats were obese and metabolically different from NFD group in the first half of the lactation period. HFD offspring were significantly heavier than their NFD counterparts from PN01 to PN10. This increased body weight was accompanied by greater adiposity, as showed by the higher fat mass ratio, when the lean mass was not different of the control. Similar effects of maternal HFD were observed on rats weaned from HFD fed dams (White, et al. 2009).

At PN10, HFD pups were glucose intolerant, corroborating with the reduced GLUT4 expression in the adipose tissue and insulin resistance of fetus from HFD mothers documented on the cited literature (Murabayashi et al. 2013). Additionally, the elevated fat mass ratio on HFD animals can be related to their higher plasma leptin levels. This elevated leptin plasma levels on HFD group is driven by the presence of larger adipocytes and the elevated leptin gene expression on sWAT. The exacerbation of the hormone secretion leads to its central and peripheral resistance. Besides, the literature reports a greater leptin content on HFD dams' breastmilk. Leptin actions reduces the size of WAT depots by increasing sympathetic tone or inducing circulating factors that suppress adipocyte proliferation. Nevertheless, prenatal HFD decreases rats leptin sensitivity at PN10 (Harris 2014; Sun, et al. 2012), inhibiting its actions on the obese animals. In this way, the previous literature and the present data confirm the early effects of maternal HFD consumption over the offspring metabolism and the role of adipose tissue in this scenario.

The response of WAT to HFD consumption in adult animals was depot-specific reported, being the visceral fat pad more susceptible to develop hypertrophied and dysfunctional adipocytes (Joe, et al. 2009). Differently, in the early life, as sWAT is the more developed white-adipose depot, it carries the response to the early HFD challenge, as showed by the larger adipocytes found in a higher frequency than in the controls. BAT adipocytes did not differ in size; however, its worth of note the presence of larger unilocular adipocytes within BAT of HFD animals compared to NFD group, a similar phenomenon was

observed previously in adult animals reared on small litters (de Almeida et al. 2013). These morphological changes in WAT and BAT, points to the structural decharacterization of the adipose tissue programmed by early overnutrition.

The observed effects of the HFD maternal feeding on adipose tissue were not limited to the morphology; moreover, they lead to gene expression changes, which at this stage occurred mainly on the BAT. While the only difference observed here on mRNA expression in sWAT was for leptin, in the BAT the changes reach the expression of marker genes related to its differentiation and metabolism. In this study, UCP1 expression at PN10 was not different between NFD and HFD groups; nevertheless, the gene expression of PPARα, PPARγ, and PRDM16 were downregulated. Maternal HFD consumption impaired BAT thermogenic function of mice offspring at weaning, despite of local elevated UCP1 expression at PN10 and PN21, no changes were observed in PRDM16 or PPARα expression (Liang, et al. 2016). Nevertheless, the expression of marker genes in BAT seems controversial in the literature, presenting variations according to strain and age.

PPARα and PPARγ signaling was reported as part of the mechanism involved in perinatal HFD long-term obesity (Zheng, et al. 2014). PPARγ is an adipogenic factor and the early HFD expose upregulates PPARγ expression on adipose tissue later in life (Desai, et al. 2015). On the other hand, PPARα agonists enhances BAT thermogenesis in adult rodents (Rachid, et al. 2015), denoting its role in thermogenesis. PRDM16 is a transcription factor involved in the BAT differentiation, besides, it is involved on the expression of UCP1 on sWAT (Seale, et al. 2011). In addition, PPARγ, PPARα and PRDM16 are involved in the cascade of transcription factors that drives BAT and brite adipogenesis (Peirce, et al. 2014). The downregulation of these transcription factors at PN10 and the morphological changes found here may underlies the mechanisms for later BAT dysfunction, including hypo thermogenesis observed in early overnutrition models.

In conclusion, our study confirms the early effects of maternal HFD consumption on offspring tissue formation and metabolism. Furthermore, our results add new insights for perinatal adipose tissue formation exposed to HFD, showing the evidences for sWAT hypertrophy, iBAT fat accumulation and gene expression alterations, which may be implied on long-term adipose tissue dysfunction. Although these abnormalities need further research to the fully understanding of the underlying mechanisms, leading to novels treatment possibilities, the evidence showed here reinforce the importance of the mothers diet and health in the future metabolism of their offspring.

Acknowledgments

This work was supported by the Brazilian Federal Foundation, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflict of interest

None of the authors has potential conflicts of interest, including any financial, personal or other relationships with other people or organizations.

References

de Almeida DL, Fabricio GS, Trombini AB, Pavanello A, Tofolo LP, da Silva Ribeiro TA, de Freitas Mathias PC & Palma-Rigo K 2013 Early overfeed-induced obesity leads to brown adipose tissue hypoactivity in rats. *Cell Physiol Biochem* **32** 1621-1630.

Desai M, Jellyman JK, Han G, Lane RH & Ross MG 2015 Programmed regulation of rat offspring adipogenic transcription factor (PPARgamma) by maternal nutrition. *J Dev Orig Health Dis* **6** 530-538.

Fenzl A & Kiefer FW 2014 Brown adipose tissue and thermogenesis. *Horm Mol Biol Clin Investig* **19** 25-37.

Figueiredo MS, da Fonseca Passos MC, Trevenzoli IH, Troina AA, Carlos AS, Alves Nascimento-Saba CC, Fraga MC, Manhaes AC, de Oliveira E, Lisboa PC, et al. 2012 Adipocyte morphology and leptin signaling in rat offspring from mothers supplemented with flaxseed during lactation. *Nutrition* **28** 307-315.

Hales CN & Barker DJ 1992 Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* **35** 595-601.

Hales CN & Barker DJ 2013 Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. 1992. *Int J Epidemiol* **42** 1215-1222.

Harris RB 2014 Direct and indirect effects of leptin on adipocyte metabolism. *Biochim Biophys Acta* **1842** 414-423.

Hokke S, Puelles VG, Armitage JA, Fong K, Bertram JF & Cullen-McEwen LA 2016 Maternal Fat Feeding Augments Offspring Nephron Endowment in Mice. *PLoS One* **11** e0161578.

Joe AW, Yi L, Even Y, Vogl AW & Rossi FM 2009 Depot-specific differences in adipogenic progenitor abundance and proliferative response to high-fat diet. *Stem Cells* **27** 2563-2570.

Lamb MM, Dabelea D, Yin X, Ogden LG, Klingensmith GJ, Rewers M & Norris JM 2010 Early-life predictors of higher body mass index in healthy children. *Ann Nutr Metab* **56** 16-22.

Liang X, Yang Q, Zhang L, Maricelli JW, Rodgers BD, Zhu MJ & Du M 2016 Maternal high-fat diet during lactation impairs thermogenic function of brown adipose tissue in offspring mice. *Sci Rep* **6** 34345.

Lukaszewski MA, Eberle D, Vieau D & Breton C 2013 Nutritional manipulations in the perinatal period program adipose tissue in offspring. *Am J Physiol Endocrinol Metab* **305** E1195-1207.

Masters RK, Reither EN, Powers DA, Yang YC, Burger AE & Link BG 2013 The impact of obesity on US mortality levels: the importance of age and cohort factors in population estimates. *Am J Public Health* **103** 1895-1901.

Mori S, Kiuchi S, Ouchi A, Hase T & Murase T 2014 Characteristic expression of extracellular matrix in subcutaneous adipose tissue development and adipogenesis; comparison with visceral adipose tissue. *Int J Biol Sci* **10** 825-833.

Mraz M & Haluzik M 2014 The role of adipose tissue immune cells in obesity and low-grade inflammation. *J Endocrinol* **222** R113-127.

Murabayashi N, Sugiyama T, Zhang L, Kamimoto Y, Umekawa T, Ma N & Sagawa N 2013 Maternal high-fat diets cause insulin resistance through inflammatory changes in fetal adipose tissue. *Eur J Obstet Gynecol Reprod Biol* **169** 39-44.

Nielsen LA, Nielsen TR & Holm JC 2015 The Impact of Familial Predisposition to Obesity and Cardiovascular Disease on Childhood Obesity. *Obes Facts* **8** 319-328.

Peirce V, Carobbio S & Vidal-Puig A 2014 The different shades of fat. *Nature* **510** 76-83.

Plagemann A, Harder T, Brunn M, Harder A, Roepke K, Wittrock-Staar M, Ziska T, Schellong K, Rodekamp E, Melchior K, et al. 2009 Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome. *J Physiol* **587** 4963-4976.

Rachid TL, Penna-de-Carvalho A, Bringhenti I, Aguila MB, Mandarim-de-Lacerda CA & Souza-Mello V 2015 PPAR-alpha agonist elicits metabolically active brown adipocytes and weight loss in diet-induced obese mice. *Cell Biochem Funct* **33** 249-256.

Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, Cohen P, Cinti S & Spiegelman BM 2011 Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J Clin Invest* **121** 96-105.

Sturm R & An R 2014 Obesity and economic environments. *CA Cancer J Clin* **64** 337-350.

Sun B, Purcell RH, Terrillion CE, Yan J, Moran TH & Tamashiro KL 2012 Maternal high-fat diet during gestation or suckling differentially affects offspring leptin sensitivity and obesity. *Diabetes* **61** 2833-2841.

Symonds ME, Pope M, Sharkey D & Budge H 2012 Adipose tissue and fetal programming. *Diabetologia* **55** 1597-1606.

Vickers MH, Breier BH, McCarthy D & Gluckman PD 2003 Sedentary behavior during postnatal life is determined by the prenatal environment and exacerbated by postnatal hypercaloric nutrition. *Am J Physiol Regul Integr Comp Physiol* **285** R271-273.

Wakana N, Irie D, Kikai M, Terada K, Yamamoto K, Kawahito H, Kato T, Ogata T, Ueyama T, Matoba S, et al. 2015 Maternal high-fat diet exaggerates atherosclerosis in adult offspring by augmenting periaortic adipose tissue-specific proinflammatory response. *Arterioscler Thromb Vasc Biol* **35** 558-569.

White CL, Purpera MN & Morrison CD 2009 Maternal obesity is necessary for programming effect of high-fat diet on offspring. *Am J Physiol Regul Integr Comp Physiol* **296** R1464-1472.

Zheng J, Xiao X, Zhang Q, Yu M, Xu J & Wang Z 2014 Maternal high-fat diet modulates hepatic glucose, lipid homeostasis and gene expression in the PPAR pathway in the early life of offspring. *Int J Mol Sci* **15** 14967-14983.

Table and Figures List

Tables

Table 1.

Calculated Nutritional Parameters	NFD	HFD
Protein	19%	19%
Fat	6%	21%
Crude Fibre	4.70%	4.70%
Adequate Dietary Fibre	4.70%	4.70%
Digestible Energy	16.1 MJ/kg	19.4 MJ/kg
% Total digestible energy from lipids	14%	40%
% Total digestible energy from protein	21%	17%

Figures

Figure 1

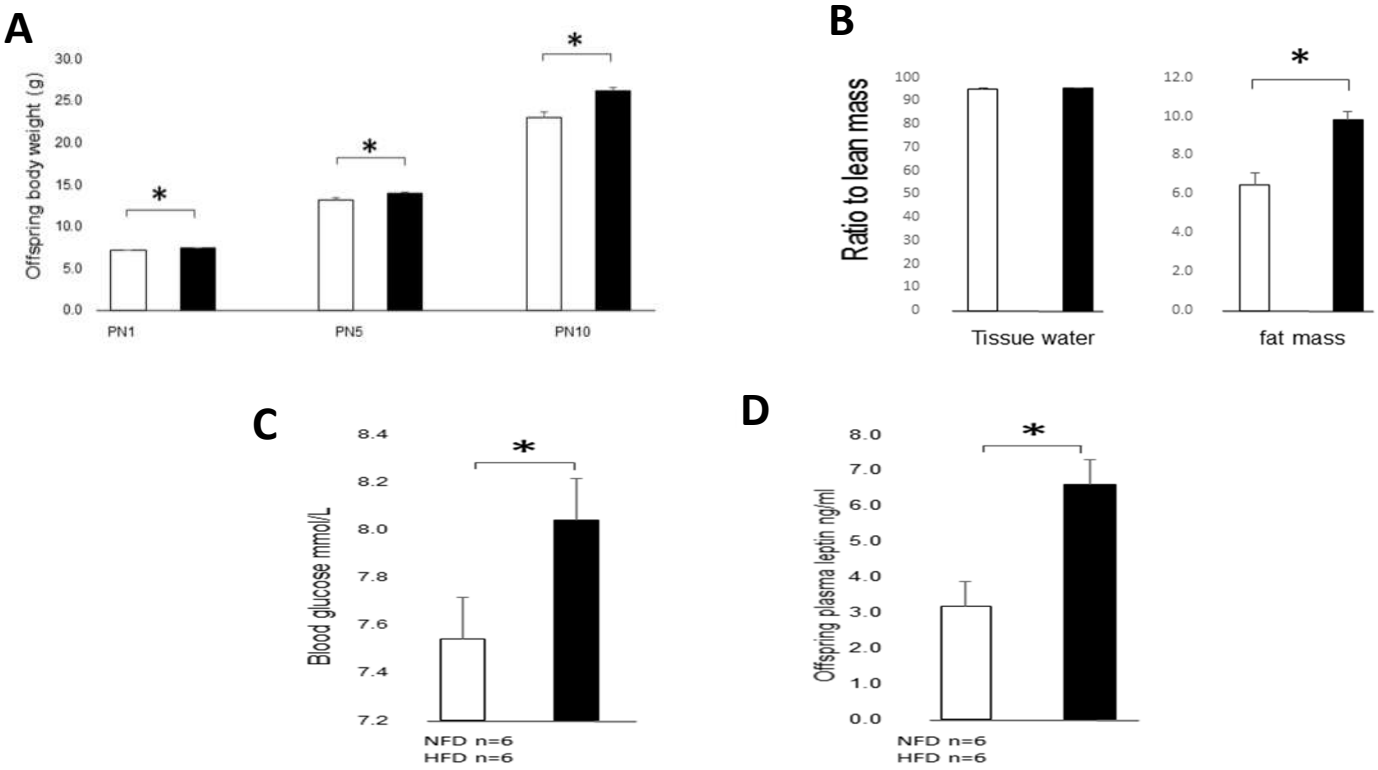


Figure 2

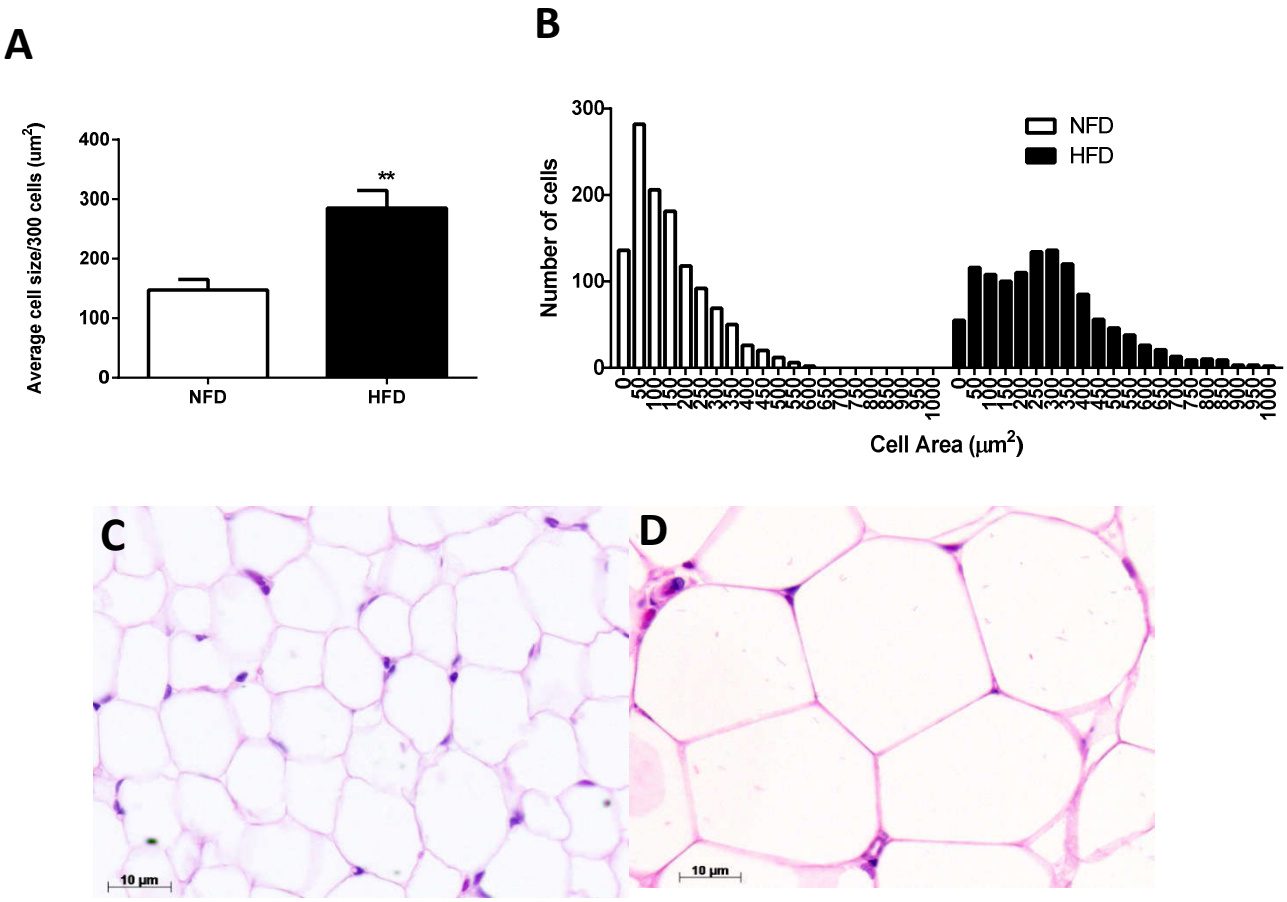
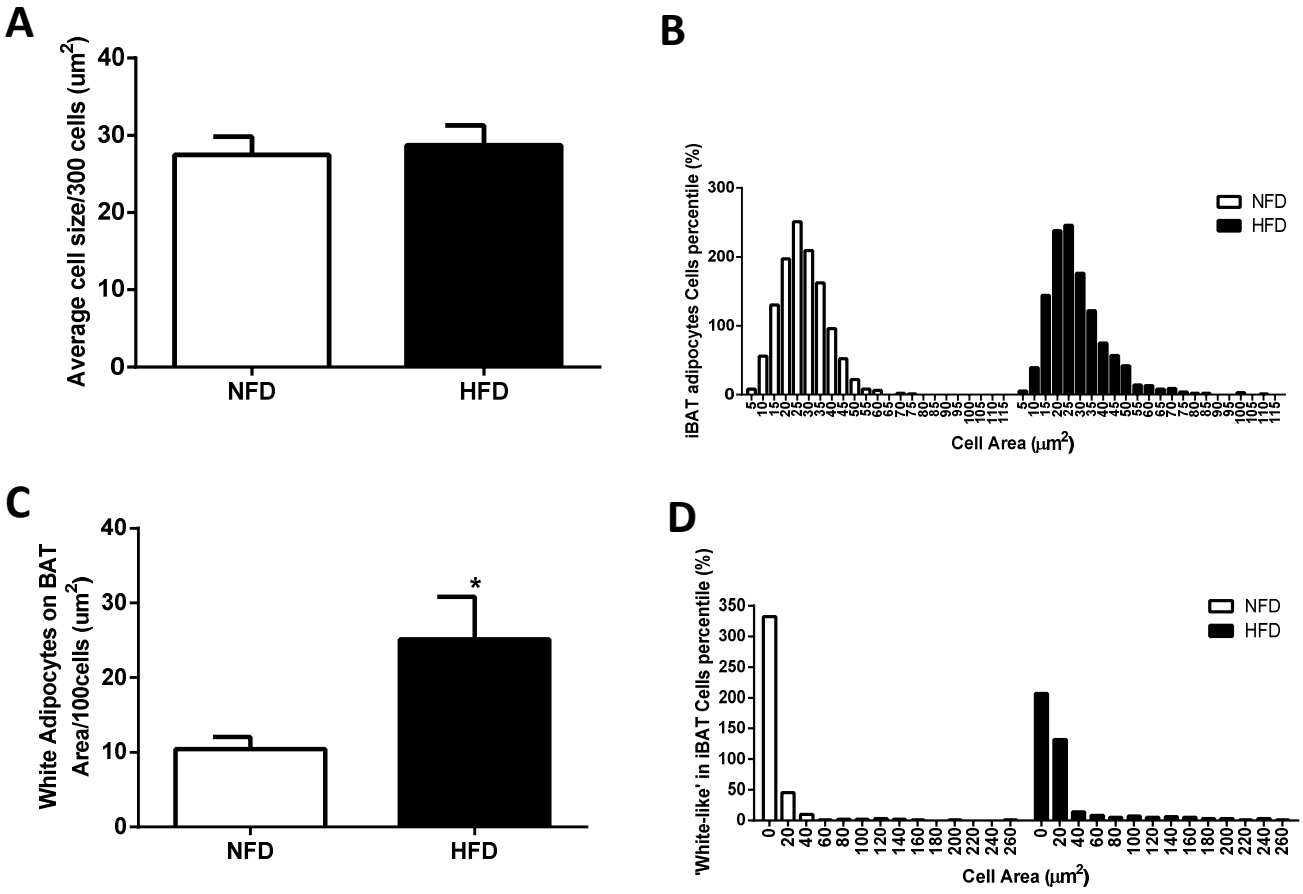

A
Average cell size/300 cells (um²)
400
300
200
100
0
**
NFD
HFD
B
Number of cells
300
200
100
0
NFD
HFD
Cell Area (μm²)
C
10 μm
D
10 μm

Figure 3


A
Average cell size/300 cells (um2)
40
30
20
10
0
NFD
HFD
B
iBAT adipocytes Cells percentile (%)
300
200
100
0
NFD
HFD
Cell Area (μm2)
C
White Adipocytes on BAT Area/100cells (um2)
40
30
20
10
0
*
NFD
HFD
D
'White-like' in iBAT Cells percentile (%)
350
300
250
200
150
100
50
0
NFD
HFD
Cell Area (μm2)

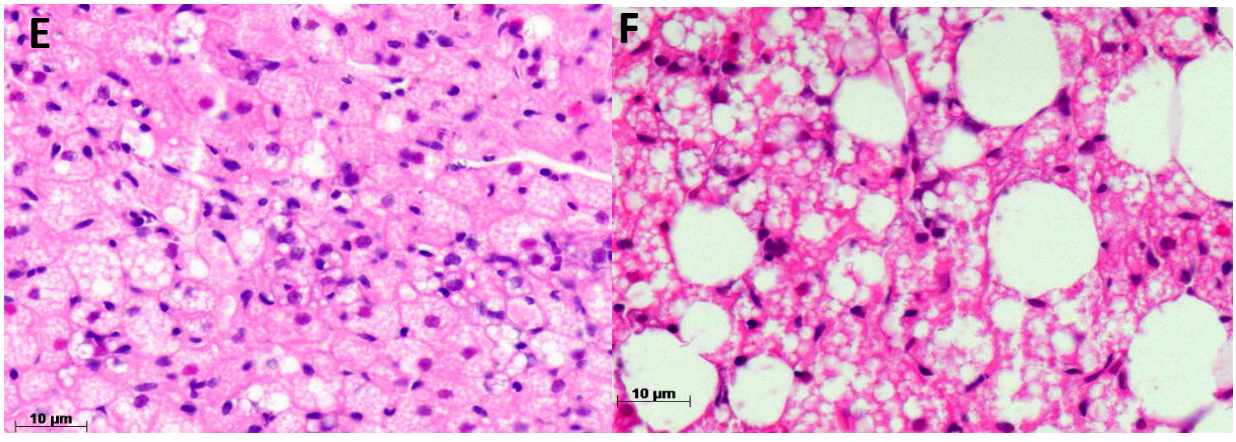

E
10 μm
F
10 μm

Figure 4

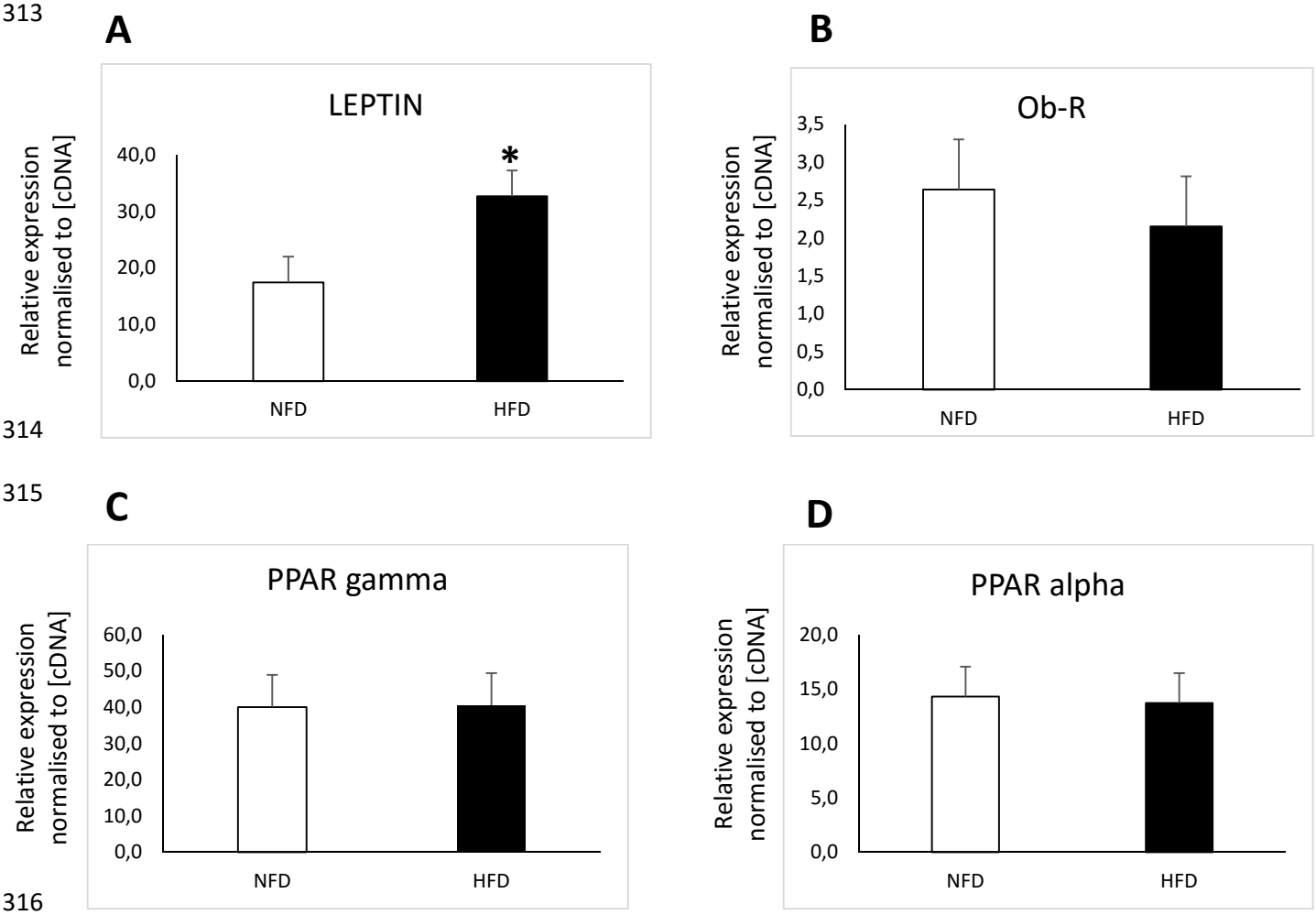
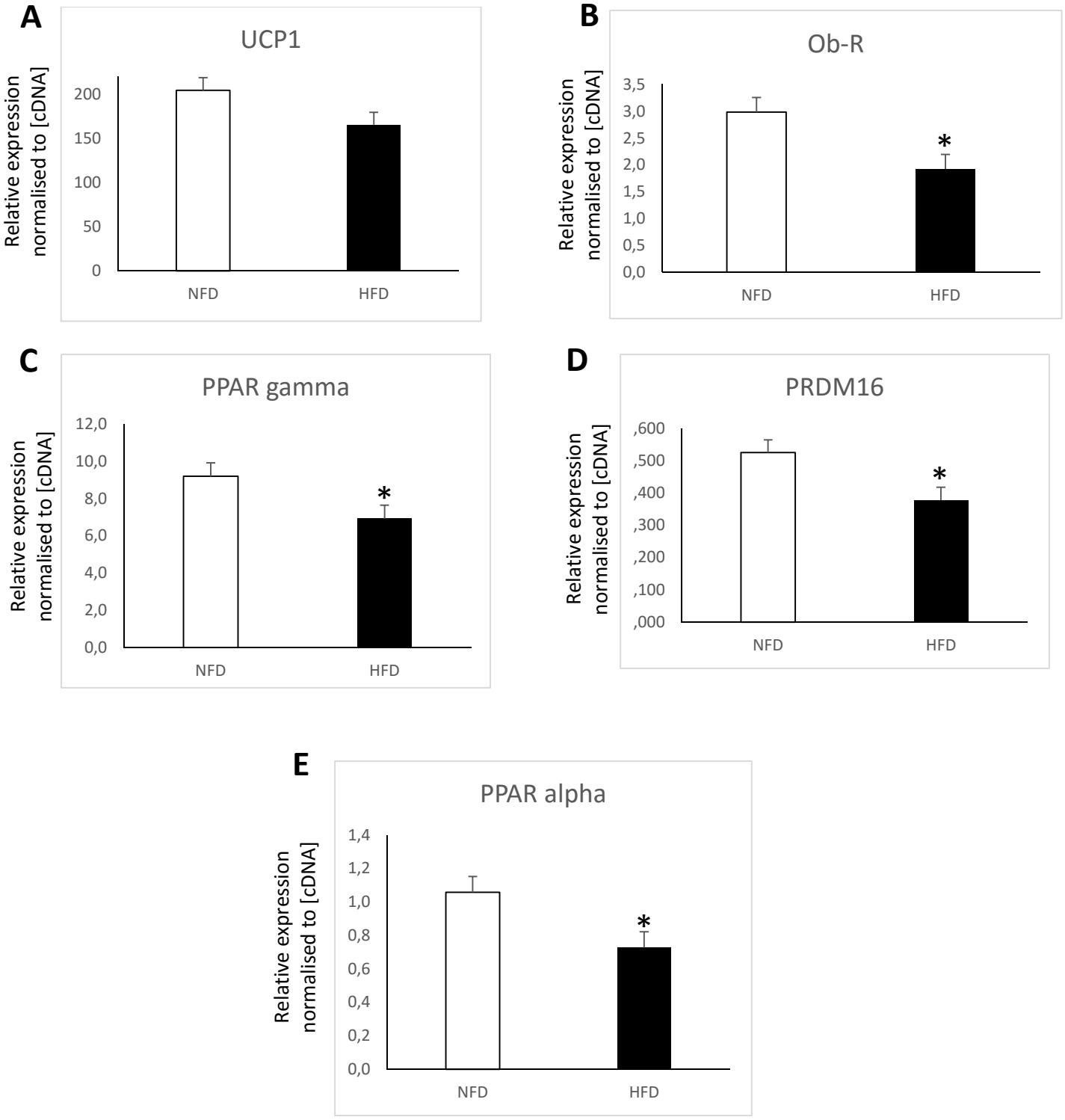

A
LEPTIN
Relative expression normalised to [cDNA]
40,0
30,0
20,0
10,0
0,0
*
NFD
HFD
B
Ob-R
Relative expression normalised to [cDNA]
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
NFD
HFD
C
PPAR gamma
Relative expression normalised to [cDNA]
60,0
50,0
40,0
30,0
20,0
10,0
0,0
NFD
HFD
D
PPAR alpha
Relative expression normalised to [cDNA]
20,0
15,0
10,0
5,0
0,0
NFD
HFD

Figure 5


A
UCP1
Relative expression normalised to [cDNA]
200
150
100
50
0
NFD
HFD
B
Ob-R
Relative expression normalised to [cDNA]
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
NFD
HFD
C
PPAR gamma
Relative expression normalised to [cDNA]
12,0
10,0
8,0
6,0
4,0
2,0
0,0
*
NFD
HFD
D
PRDM16
Relative expression normalised to [cDNA]
,600
,500
,400
,300
,200
,100
,000
*
NFD
HFD
E
PPAR alpha
Relative expression normalised to [cDNA]
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
NFD
HFD

MANUSCRITO 2

Moderate physical exercise deprogram brown adipose hypoactivity by postnatal early overfeed in male rats.

Douglas Lopes de Almeida[1], Lucas Eduardo Cardoso[1], Audrei Pavanelo[1], Veridiana Mota Moreira[1], Tatiane Aparecida Ribeiro[1], Claudinéia Conationi da Silva Franco[1], Kesia Palma-Rigo[1], Paulo César de Freitas Mathias[1].

[1] Laboratory of Secretion Cell Biology, Department of Biotechnology, Cell Biology and Genetics, State University of Maringá - Maringá/PR, Brazil.

Short Running Title: Exercise deprogram early overfeed iBAT hypoactivity.

Corresponding Author: Dr. Douglas Lopes de Almeida - Department of Biotechnology, Cell Biology and Genetics, State University of Maringá, 5790 Av Colombo, Sala 19, Maringá, PR, 87020-900, Brazil. Phone/fax: +55 44 3011-4892; e-mail: dougalmeida84@gmail.com.

Abstract

Background: Early postnatal overfeed reduces BAT thermogenesis. Physical exercise may be a non-pharmacological approach to improve BAT function. In this study, we investigated the effects of a moderate intensity/low-frequency exercise protocol on BAT function of postnatal early overfeed male rats. **Methods:** Wistar rats litters deliver was PN0, at PN2, the litters were adjusted to 9 pups per dam (control – NL), or 3 pups (postnatal early overfeeding – SL) per dam. At PN21, animals were weaned and randomly divided into exercise or sedentary groups (NL SED, NL EXE, SL SED, and SL EXE). The exercise protocol starts at PN30 with an effort test; training sessions were performed 3 times per week, at 60% of the final workload achieved in tests, repeated each 15 days to adjust the training workload, finished at PN80. At PN81, animals underwent a surgery to implant a temperature transponder underneath interscapular BAT (iBAT). The iBAT temperature was measured at light and dark periods from PN87 to PN90. Animals euthanize, blood samples and fat pads collection for further analysis was at PN91. The iBAT nerve activity was recorded from a separated set of animals at PN90. **Results:** Early postnatal overfeed impairs iBAT activity on lights-on ($p<0.0001$) and lights-off ($p<0.01$) shifts. Exercise improves iBAT function in lights-on ($p<0.0001$), but not on lights-off ($p=0.2236$) turn. Additionally, exercise increases Sympathetic iBAT nerve firing rate ($p<0.05$), while postnatal overfeed reduced it ($p<0.0001$). Early overfeed did not affect b3-AR content on iBAT ($p=0.2172$), which is downregulated by exercise protocol ($p<0.01$). **Conclusion:** The moderate intensity – low frequency exercise protocol was able to deprogram postnatal early overfeeding effects on adult male rats, improving biometrical and metabolical parameters and blocking iBAT hypoactivity.

Key words: Metabolic program, postnatal early overfeed, iBAT activity, moderate/low-frequency exercise, deprogram

Introduction

There is a widespread knowledge that obesity epidemic is a priority concern for public health. Obesity, in the past few decades, accounts for a large share of adults deaths on US. This cumulative effect of obesity epidemic is likely to increase in a near future, considering younger cohorts with high rates of early obesity onset and prevalence [1]. The postnatal early overfeeding leads humans and animals to precocious obesity onset, infants obese are highly susceptible to turn into an adult obese, condition strongly related to metabolic syndrome development [2].

Energy imbalance, favoring fat accumulation, is a major effector on the obesity etiology [3]. The need to address ways of counterattack the positive energy balance has placed brown adipose tissue (BAT) thermogenesis on the line for its potential to increase energy expenditure through uncoupling-protein-1 (UCP1) activity, especially after its detection in a significant fraction on adult humans [4, 5].

Small litter reared rats are a valuable animal model to study metabolic program and early overnutrition effects on life course [6]. In a previous study, our group demonstrate that postnatal early overfeed induced obese rats have impaired interscapular BAT (iBAT) thermogenic function, producing less heat than the control animals at day and night, besides morphology changes that connotes fat accumulation on iBAT [7], which means lower energy dissipation and consequent energy storage.

Revert metabolic program and augment energy expenditure on iBAT, fighting obesity, is a desirable target. Nevertheless, obese subjects were not responsive to a pharmacological approach to activate BAT, as were lean subjects [8]. The regular practice of physical exercise, which is often present on guidelines for lifestyle changes to health promotion, especially for obese and metabolic syndrome individuals [9], has been consider as a non-pharmacological approach to increase BAT activity on humans [10] and animals conducted research [11, 12].

Although there is some evidence supporting endurance training acute positive effects for BAT function, mainly for control animals, little is known whether a moderate low-frequency exercise program, which is more accessible for most of people, may play a role to increase energy expenditure on BAT of obese individuals. In this study, we investigate the

effects of a moderate and low-frequency exercise protocol on the 'deprograming' and function of iBAT of postnatal early overfeed male rats.

Methods

Ethical Approval. All the experimental protocol conducted in this study complies with the norms of the Brazilian Association for Animal Experimentation (COBEA) and with Brazilian Federal Law. Animal Ethics Committee of Maringa State University approved the procedures (protocol number 013/2014).

Animals. Seventy-days-old virgin females and eighty-days-old virgin males Wistar rats were obtained from Central Animals Facility – Parana State University and housed at sectorial laboratory animals house at standard conditions of light (12 hours dark/12 hours light cycle) and temperature (22° ± 2°C). Animals have *ad libitum* access to standard chow and water throughout all the experimental period. After five days of adaptation, animals were placed to breed in a cage proportion of 2 females for 1 male. Pregnant rats were placed at individual cages until delivery (day-zero). On postnatal-day-2 (PN2), the number of pups were adjusted to 9 per dam for normal litters (NL) and to 3 pups per dam for small litters (SL). At PN21, animals were weaned and kept in 3 per cage for both groups and randomly assigned to either, exercise or sedentary groups, originating the four groups presents on this study, NL sedentary (NL SED), NL exercise (NL EXE), SL sedentary (SL SED) and SL exercised (SL EXE), only male animals were used.

Body weight and food intake (Fi). After weaning, animals body weight and chow consumption was assessed at each 5 days. Food consumption value was calculated by determining the difference between the amount of chow remaining (Df) and the total amount of food that was previously placed in the cage (Di), dividing the difference by the number of days and the number of rats per cage: [FI (g) = (Df – Di) ⁄ days⁄ rats]. The area under the curve (AUC) for the food consumption versus time was calculated for the entire observation period (21–90 days).

Training Protocol. Previously to the moderate intensity and low frequency exercise protocol, the rats have a week of adaptation to the treadmill for rodents (Panlab, Harvard Apparatus®, Cornellà- Barcelona - Spain) with constant workload (16 cm/s) and daily progressive time (from 10 min to 20 min, adding 2 minutes per session). Forty-eight hours

after adaptations' last session, on PN30, animals from the four groups were subjected to an effort test for the determination of VO2max, utilizing a gas analyzer coupled to an individual treadmill for rodents (Panlab, Harvard Apparatus®, Cornellà- Barcelona - Spain). The test began with a warm up (5 min/ 10 cm/s / 0º of inclination), followed by a velocity increment of 9 cm/s every 3 minutes until the exhaustion of the animal, determined by their inability to keep the pace. The VO_{2max} was considered as the value achieved when an increase in workload did not affect the consumption of O_2 ± 5%, the final workload (FWL) was considered as the last velocity achieved by the animal on the effort test and used to determine the training workload (TWL). EXE animals performed the effort test at PN30, 45, 60 and PN80 to adjust the TWL and monitoring physical progress, while SED groups were subjected to it at PN30 and PN80 for standard. The training sessions lasted 40 minutes, with a progressive TWL from 55% to 65% of the FWL achieved on the previous test, 3 times a week.

IBAT Temperature. At PN81 animals were anesthetized using ketamine and xylazine (3 and 0.6 mg⁄100 g of body weight, respectively),and a temperature transponder (implantable programmable temperature transponder 300 - IPTT-300, BioMedic Data Systems, Seaford, DE) was implanted under the IBAT pads and secured to the surrounding muscle. Before implantation, each transponder was checked comparing the given transponder temperature and ambient temperature measured with a thermometer. The temperature from both pads was measured [13]. Animals were placed in individual cages and had 6 days to recover from surgery. A recording apparatus (DAS 5002 Notebook System; BioMedic Data Systems, Seaford, DE) was used to measure temperature. Measurements were made twice a day between days 87 and 90, around one hour after the lights were turned on or off (7:00 am and 7:00 pm).

Sympathetic iBAT Nerve Activity. From each group, a separated set of 90-day-old rats with no transponder implantation was anesthetized using thiopental (45 mg⁄kg of body weight) after 12h of fasting. A sympathetic branch nerve from iBAT innervation was surgically exposed and placed on a pair of platinum electrodes. Recordings were made with a Bio-Amplificator (Insight®, Ribeirão Preto, Brazil) in the 1–80 kHz range and amplified 10000-

fold, as previously described [14]. The average numbers of spikes/5s (spk/5s) were used to calculate the nerve-firing rate from 5 to 7 sections of 15s recordings for each rat.

Blood analysis and Tissue collection. At PN91, overnight fasted animals were weighed, anesthetized (thiopental (45 mg／kg of body weight) and euthanized via quick decapitation. Blood samples were collected and centrifuged, and the plasma was stored at -20°C for subsequent analysis. The glycemia levels was assessed using the glucose oxidase method [15] with a commercial kit (Gold Analisa®, Minas Gerais, Brazil), while the fast insulinemia evaluation was through radioimmunoassay [16]. Insulin variation coefficients was 9.8%, the inferior and superior detection limits was, respectively, 0.006 ng/ml and 100 ng/ml. Fat from the retroperitoneal, epididymal and IBAT pads was dissected and weighted. An IBAT lobe was snap frozen with liquid nitrogen and stored at -80°C for further Western Blotting analysis.

Western Blotting Analysis. The β3-AR protein contents of the iBAT was determined by immunoblotting. Samples of iBAT lobe from each experimental group were collected, frozen, and late macerated in radio immunoprecipitation assay (RIPA) as previously described [17]. Total protein extracts (30µg) separation carried out by 12% SDS-PAGE at 150V for 60min. The proteins were then transferred from the gel to a polyvinylidene difluoride (PVDF) membrane by a Trans-Blot® turbo system (Bio-Rad® Laboratories, Hercules, CA, USA) and were then blocked with 5% BSA in Tween-Tris-buffered saline (TTBS; Tris-HCl, 1mol/l; NaCl, 5mol/l; and Tween 20, 0.05%, v/v) for 90 min under continuous shaking. Sigma Aldrich produced the primary antibodies anti- β3-AR (1:1000) used. After wash PVDF membranes three times with Tween–TBS (0.1%), followed by the incubation for 1h with the appropriate secondary antibodies conjugated to biotin (Santa Cruz Biotechnology, Inc). Then, the membranes were incubated with streptavidin-conjugated HRP (Caltag Laboratories, Burlingame, CA, USA). The immunoreactive proteins were visualized using an ECL Prime kit and Image Quant LAS (GE Healthcare, Buckingham, Shire, UK). The bands were quantified by densitometry using ImageJ 1.4 software (Wayne Rasband, National Institutes of Health, Bethesda, MA, USA). β-Actin protein content (Santa Cruz Biotechnology®, Santa Cruz, CA, USA) was used for data normalization [18]. Results are given in percentage (%) of the control (β-Actin).

Statistical analysis. The obtained data were submitted to D'Agostino & Pearson normality test and then analyzed by two-way ANOVA (factors: postnatal early overfeed and exercise) followed by Tukey's multiple comparisons post-test, except for body weight at weaning where Students' Test T was used. The N of litters are 10 for NL and 10 for SL groups prior of weaning, being divided into 5 litters for each group after training protocol introduction, unless otherwise noted. $P < 0.05$ was considered statistically significant. The tests were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

The litter reduction was effective in promote obesity during the suckling period as indicated by the body weight of SL compared to the NL litters at weaning (NL 42.27 ± 2.00g vs SL 58.35 ± 1.94g, $p<0.0001$). The early postnatal overfeeding leads animals to overweight at adulthood, being heavier at PN80 ($p<0.01$), whilst the training protocol was able to reduce body weight ($p<0.01$). The early overfeeding elevate rats food intake after weaning, as indicated by the AUC values for PN21 to PN90 ($p<0.05$), the exercise reduced food intake on the same period ($p<0.05$) (Table 1).

Regarding body fat, the litter reduction leads to a larger fat accumulation on retroperitoneal ($p<0.001$), perigonadal ($p<0.0001$) and iBAT ($p<0.01$) on later life. In contrast, exercise was effective in decrease fat pads (retroperitoneal - $p<0.05$; perigonadal - $p<0.05$ and iBAT - $p<0.001$). A significant interaction ($p<0.05$) between factors is observed to iBAT weight (Table 1).

The fast glycemia appears not significantly changed by early overfeeding ($p=0.1074$), however, the exercise program was able to decrease it ($p<0.05$). Differently, fast insulinemia was elevated by postnatal overfeed ($p<0.01$), being lowered by exercise ($p<0.01$) (Table 1).

The postnatal early overfeeding impairs physical performance on effort test at PN80 ($p<0.05$ – Fig. 1A), as the oxygen consumption during the tests ($p<0.01$ – Fig. 1B). The moderate training protocol improves animals' physical capacity at PN80 ($p< 0.0001$ – Fig. 1A). The exercise also augments VO_{2max} ($p<0.01$) on the effort test, although the post-tests

show no significant difference between SL groups (SL SED 20.47±0.6 ml/min/kg vs SL EXE 21.79±0.7 ml/min/kg, p=0.3042 – Fig. 1B).

Early obesity reduces iBAT thermogenic activity on adulthood in the lights-on ($p<0.0001$ – Fig. 2A) and lights-off ($p<0.01$ – Fig. 2B) periods. The moderate exercise protocol was able to increases iBAT thermogenesis during the lights-on shift ($p<0.0001$– Fig. 2A); however, there is no significant changes in the lights-off turn (p=0.2236 – Fig. 2B).

The local sympathetic nerve activity on iBAT appears reduced by the postnatal early overfeeding ($p<0.0001$ – Fig. 3), while physical exercise elevate the iBAT nerve firing rate ($p<0.05$ – Fig. 3). A significant interaction ($p<0.05$) between the factors is noted, the post-test shows no significant difference between the SL groups (SL SED 4.98±0.4spk/5s vs SL EXE 5.17±0.3spk/5s, $p>0.9999$ – Fig. 3). The β3-AR content on iBAT appears not be affected by the litter reduction (p=0.2172 – Fig. 4). Nevertheless, the exercise downregulated β3-AR content ($p<0.01$ – Fig. 4), the data analysis shows a significant interaction between factors ($p<0.05$ – Fig. 4), still, the post-test show no significant changes for SL reared rats (p=0.8021 – Fig. 4).

Discussion

The early overfeeding everlasting impact on mammals is well described in the literature [2, 19], besides, its effects on the BAT and thermogenesis starts to be elucidated [20, 21]. In this study, the results show that a physical exercise program, of moderate intensity and low frequency, was able to improve metabolism, physical performance and mainly the iBAT activity during the lights-on shift for both, controls and early postnatal overfeed rats. Interestingly, although exercise does have an effect on iBAT nerve firing rate, this change is not observed on the sympathetic iBAT activation of SL reared rats.

As expected, the litter reduction leads the rats to obesity during the suckling period, followed by post weaning hyperphagia, overweight, fat accumulation and later fast hyperinsulinemia. The literature reports different levels of response to exercise protocols in experimental models, depending on stream, type of stimulus (e.g. aerobic/anaerobic), frequency, intensity and duration [22]. In this study, the physical exercise protocol was effective to reduce body weight, fat accumulation, hyperphagia and insulinemia on the early postnatal overfeed rats. Similar moderate exercise effects are report in control animals [23],

however using a resistance stimulus and a 5 times per week frequency. On another hand, postnatal early overfeeding affects rats' physical performance, including the VO_{2max}, of SL trained rats on the effort test. Early life nutritional status may impairs skeletal muscle development with implications on gene expression in later life [24, 25], which may underlie the reduced response of the SL EXE rats compared to the NL EXE rats to the exercise regarding O_2 consumption on the physical tests, despite of the cited biometric and metabolic changes.

Our group previously discussed the postnatal early overfeeding induced iBAT thermogenesis hypoactivity, as indicated by its lower heat production [7 1]. The data obtained on this study for sedentary animals agree with our previous report on iBAT thermogenesis of SL rats. Additionally, shows that the exercise is able to improve the thermogenesis on lean and early obese programmed rats, mainly on the light-on period, knew as the rodents less active phase, which may indicate an elevation on the rest metabolical rate on these animals. The iBAT thermogenesis and the whole-body metabolic rate seems to be tightly coupled, independently of the thermoregulatory homeostatic process [26], which indicate that this increased thermogenesis during the animals rest period is quite implicated on the metabolic improvements observed on the trained animals.

Regular exercise is shown to affect positively BAT progenitor cells and specific gene expression *in vivo* and enhances *in vitro* differentiation of preadipocytes into brown adipocytes and UCP1 expression [27]. In addition, Dinas and cols found a relation between regular physical exercise and increased BAT activity in humans, been higher on lean than in obese individuals [10]. These findings corroborate with this study results, which is the first, of our knowledge, to point to the implication of the BAT and the exercise acting together on 'deprogramming' of the postnatal early overfeed obese rats. In fact, given that early overfeeding by HFD maternal exposure affects BAT morphology and gene expression since the suckling period (Almeida, Armitage *et al*, unpublished data), the term 'deprogram' for the BAT function seems applicable here.

The iBAT is classically activated *via* the sympathetic nerve (SN) system [4]. A previous work demonstrate the acute effect of the exercise training, which increases splanchnic sympathetic activation on control and obese rats [28]. In the present study, the

chronical effects of the exercise over the SN activation of iBAT was tested, with the iBAT temperature and SN activity record being performed 10 days after the end of the training protocol. The results show that the exercise affect SN activity on iBAT, which is higher on trained rats, however, the early overfeeding reduced this effect on SL rats. Morrison (2006) shows that, although both, splanchnic and iBAT nerves are controlled by the SNS, they are controlled by a different populations of sympathetic neurons [29].

The higher SN outflow for the iBAT on exercised rats is counterbalanced by the downregulation of the β3-AR on the NL EXE rats; once again, the early postnatal overfeeding decreased this exercise effect. The activation of the BAT by β3-AR agonists has been a target for obesity treatment [30]. The present results suggest that on early metabolic programmed mammals, the BAT thermogenesis improvement may require a distinct pathway. We are aware of the study limitations, further studies will be carry to elucidate the mechanisms underlying the iBAT thermogenesis improvement by physical exercise on early overfeed animals.

In conclusion, in this study a moderate intensity – low frequency exercise protocol was able to deprogram postnatal early overfeeding effects on adult male rats, improving biometrical and metabolical parameters and blocking iBAT hypoactivity. These changes contribute to a higher resting metabolic rate, being an important clue on the fighting against early life metabolic program, obesity and metabolic syndrome.

Acknowledgments

This work was supported by the Brazilian Federal Foundation, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflict of interest

None of the authors has potential conflicts of interest, including any financial, personal or other relationships with other people or organizations.

References

1. Masters, R.K., et al., *The impact of obesity on US mortality levels: the importance of age and cohort factors in population estimates.* Am J Public Health, 2013. **103**(10): p. 1895-901.
2. Jansen, P.W., et al., *Children's eating behavior, feeding practices of parents and weight problems in early childhood: results from the population-based Generation R Study.* Int J Behav Nutr Phys Act, 2012. **9**(1): p. 130.
3. Pereira-Lancha, L.O., P.L. Campos-Ferraz, and A.H. Lancha, Jr., *Obesity: considerations about etiology, metabolism, and the use of experimental models.* Diabetes Metab Syndr Obes, 2012. **5**: p. 75-87.
4. Cannon, B. and J. Nedergaard, *Brown adipose tissue: function and physiological significance.* Physiol Rev, 2004. **84**(1): p. 277-359.
5. Nedergaard, J., T. Bengtsson, and B. Cannon, *Unexpected evidence for active brown adipose tissue in adult humans.* Am J Physiol Endocrinol Metab, 2007. **293**(2): p. E444-52.
6. Plagemann, A., et al., *Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome.* J Physiol, 2009. **587**(Pt 20): p. 4963-76.
7. de Almeida, D.L., et al., *Early overfeed-induced obesity leads to brown adipose tissue hypoactivity in rats.* Cell Physiol Biochem, 2013. **32**(6): p. 1621-30.
8. Carey, A.L., et al., *Ephedrine activates brown adipose tissue in lean but not obese humans.* Diabetologia, 2013. **56**(1): p. 147-55.
9. Lin, Y.H. and L.L. Chu, *The health promotion lifestyle of metabolic syndrome individuals with a diet and exercise programme.* Int J Nurs Pract, 2014. **20**(2): p. 142-8.
10. Dinas, P.C., et al., *Association between habitual physical activity and brown adipose tissue activity in individuals undergoing PET-CT scan.* Clin Endocrinol (Oxf), 2015. **82**(1): p. 147-54.
11. De Matteis, R., et al., *Exercise as a new physiological stimulus for brown adipose tissue activity.* Nutr Metab Cardiovasc Dis, 2013. **23**(6): p. 582-90.
12. Slocum, N., et al., *Responses of brown adipose tissue to diet-induced obesity, exercise, dietary restriction and ephedrine treatment.* Exp Toxicol Pathol, 2013. **65**(5): p. 549-57.
13. Brito, M.N., et al., *Differential activation of the sympathetic innervation of adipose tissues by melanocortin receptor stimulation.* Endocrinology, 2007. **148**(11): p. 5339-47.
14. Scomparin, D.X., et al., *Autonomic activity and glycemic homeostasis are maintained by precocious and low intensity training exercises in MSG-programmed obese mice.* Endocrine, 2009. **36**(3): p. 510-7.
15. Trinder, P., *Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen.* J Clin Pathol, 1969. **22**(2): p. 158-61.
16. Hermans, M.P., W. Schmeer, and J.C. Henquin, *Modulation of the effect of acetylcholine on insulin release by the membrane potential of B cells.* Endocrinology, 1987. **120**(5): p. 1765-73.
17. Malta, A., et al., *Neonatal treatment with scopolamine butylbromide prevents metabolic dysfunction in male rats.* Sci Rep, 2016. **6**: p. 30745.
18. Miranda, R.A., et al., *HPA axis and vagus nervous function are involved in impaired insulin secretion of MSG-obese rats.* J Endocrinol, 2016. **230**(1): p. 27-38.
19. Plagemann, A., et al., *Early postnatal life as a critical time window for determination of long-term metabolic health.* Best Pract Res Clin Endocrinol Metab, 2012. **26**(5): p. 641-53.

20. Liang, X., et al., *Maternal high-fat diet during lactation impairs thermogenic function of brown adipose tissue in offspring mice.* Sci Rep, 2016. **6**: p. 34345.
21. Xiao, X.Q., et al., *Excess weight gain during the early postnatal period is associated with permanent reprogramming of brown adipose tissue adaptive thermogenesis.* Endocrinology, 2007. **148**(9): p. 4150-9.
22. Abreu, P., et al., *Anaerobic threshold employed on exercise training prescription and performance assessment for laboratory rodents: A short review.* Life Sci, 2016. **151**: p. 1-6.
23. Ebal, E., et al., *Effect of a moderate exercise on the regulatory hormones of food intake in rats.* Appetite, 2007. **49**(2): p. 521-4.
24. Liu, H.W., et al., *Developmental programming in skeletal muscle in response to overnourishment in the immediate postnatal life in rats.* J Nutr Biochem, 2013. **24**(11): p. 1859-69.
25. Sharples, A.P., C.E. Stewart, and R.A. Seaborne, *Does skeletal muscle have an 'epi'-memory? The role of epigenetics in nutritional programming, metabolic disease, aging and exercise.* Aging Cell, 2016. **15**(4): p. 603-16.
26. Ootsuka, Y., et al., *Brown adipose tissue thermogenesis heats brain and body as part of the brain-coordinated ultradian basic rest-activity cycle.* Neuroscience, 2009. **164**(2): p. 849-61.
27. Xu, X., et al., *Exercise ameliorates high-fat diet-induced metabolic and vascular dysfunction, and increases adipocyte progenitor cell population in brown adipose tissue.* Am J Physiol Regul Integr Comp Physiol, 2011. **300**(5): p. R1115-25.
28. Rinaldi, W., et al., *Low-intensity and moderate exercise training improves autonomic nervous system activity imbalanced by postnatal early overfeeding in rats.* J Int Soc Sports Nutr, 2014. **11**: p. 25.
29. Morrison, S.F., *Differential regulation of brown adipose and splanchnic sympathetic outflows in rat: roles of raphe and rostral ventrolateral medulla neurons.* Clin Exp Pharmacol Physiol, 2001. **28**(1-2): p. 138-43.
30. Cypess, A.M., et al., *Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist.* Cell Metab, 2015. **21**(1): p. 33-8.

Table and Figures list

Table

Table 1

	groups (Mean ± SEM)				analysis factors (p value)		
Biometric Parameters	**NL SED**	**NL EXE**	**SL SED**	**SL EXE**	**Postnatal Early Overfeeding**	**Exercise**	**Interaction**
Body weight PN21 (g)	42.2 ± 2.0	none	58.3 ± 1.9	none	<0.0001	none	none
Body weight PN80 (g)	346.4 ± 4.95	336.3 ± 6.12	369.2 ± 5.88	349.6 ± 3.52	<0.01	<0.01	ns
AUC Food intake PN21-PN90	304.3 ± 3.79	300.4 ± 10.6	332.5 ± 4.33	302.1 ± 4.43	<0.05	<0.05	ns
Retroperitoneal fat (g)	3.90 ± 0.4	3.78 ± 0.2	5.74 ± 0.3	4.48 ± 0.2	<0.001	<0.05	ns
Perigonadal fat (g)	3.15 ± 0.2	3.11 ± 0.1	4.63 ± 0.2	3.68 ± 0.2	<0.0001	<0.05	ns
iBAT fat (g)	0.20 ± 0.009	0.18 ± 0.006	0.24 ± 0.008	0.19 ± 0.008	<0.01	<0.001	<0.05
Fast glycemia (mg/dL)	146.8 ± 5.52	132.8 ± 5.64	165.6 ± 11.8	139.5 ± 8.05	ns	<0.05	ns
fast insulinemia (ng/dL)	0.355 ± 0.04	0.279 ± 0.02	0.559 ± 0.05	0.349 ± 0.03	<0.01	<0.01	ns

Figures

Figure 1.

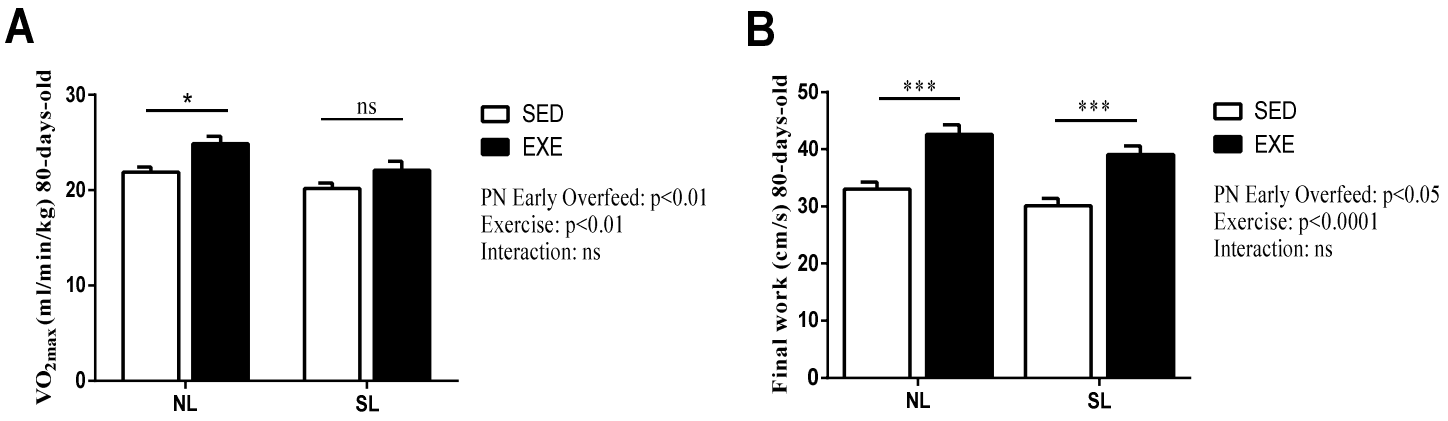


Figure 2

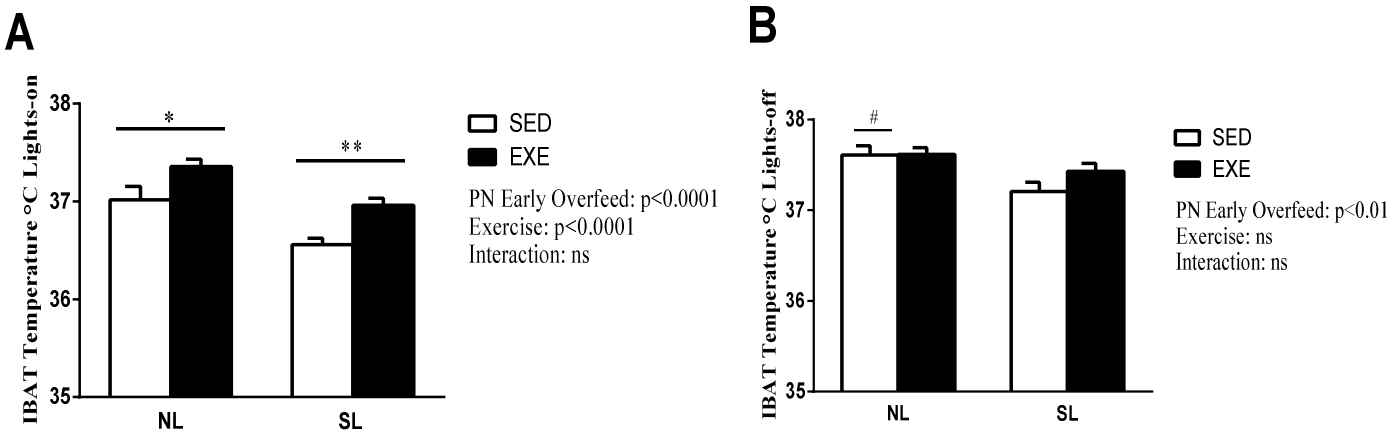
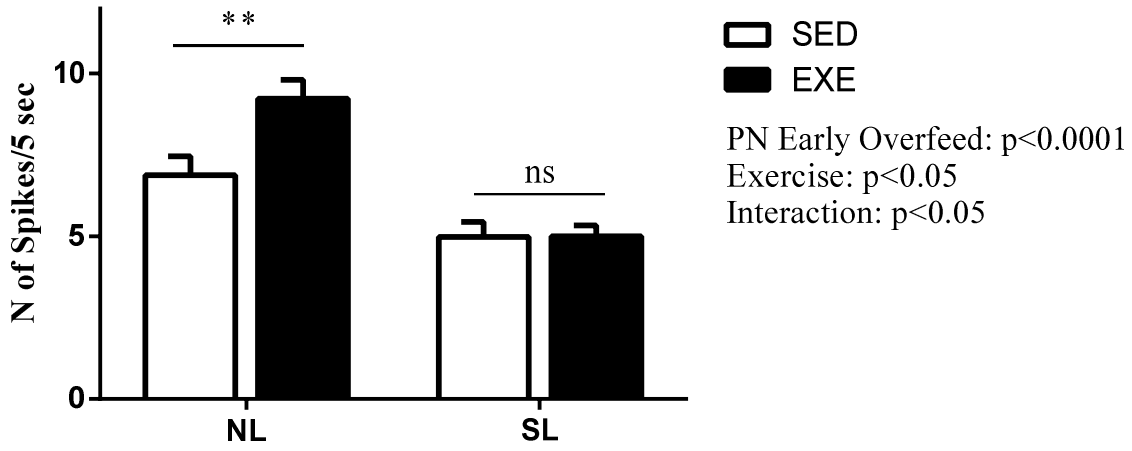

A
IBAT Temperature °C Lights-on
38
37
36
35
*
**
NL
SL
SED
EXE
PN Early Overfeed: p<0.0001
Exercise: p<0.0001
Interaction: ns
B
IBAT Temperature °C Lights-off
38
37
36
35
#
NL
SL
SED
EXE
PN Early Overfeed: p<0.01
Exercise: ns
Interaction: ns

Figure 3.


**
ns
10
5
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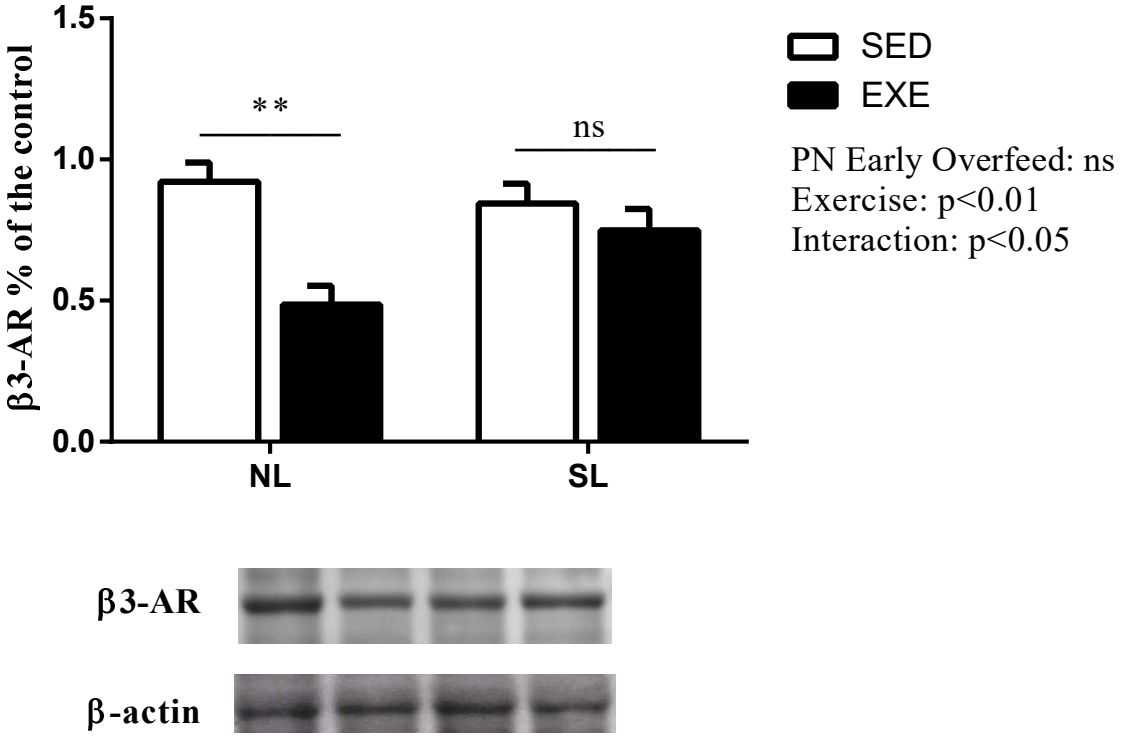
N of Spikes/5 sec
NL
SL
SED
EXE
PN Early Overfeed: p<0.0001
Exercise: p<0.05
Interaction: p<0.05

Figure 4.


β3-AR % of the control
1.5
1.0
0.5
0.0
**
ns
NL
SL
SED
EXE
PN Early Overfeed: ns
Exercise: p<0.01
Interaction: p<0.05
β3-AR
β-actin