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# ESTRESSE NO PERÍODO PRÉ-PUBERE E EXPOSIÇÃO CRÔNICA A DIETAS HIPERPALATÁVEIS: AVALIAÇÃO DO COMPORTAMENTO DO TIPO ANSIOSO E DE ALTERAÇÕES BIOQUÍMICAS EM RATOS MACHOS ADULTOS

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Porto Alegre – RS

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**SUMÁRIO**

## RESUMO

A pré-puberdade é um período crítico para a maturação final dos circuitos neuronais que controlam a homeostase energética e as respostas ao estresse. Exposição a estressores e a dietas hiperpalatáveis neste período de desenvolvimento podem modificar o processo de maturação e causar mudanças comportamentais e neuroquímicas na idade adulta. Desta forma, o objetivo deste estudo é verificar os efeitos da exposição ao estresse por isolamento social no período pré-pubere e o acesso crônico a dietas hiperpalatáveis sobre comportamento do tipo ansioso e alterações metabólicas e neuroquímicas, em ratos machos adultos. Os animais que foram isolados apresentaram um comportamento do tipo ansiolítico no Labirinto em Cruz Elevado, e o mesmo foi observado nos animais isolados com acesso a dieta hiperlipídica. Já os animais estressados com acesso a dieta rica em carboidratos apresentaram comportamento oposto, ou seja, do tipo ansiogênico. Foram observadas mudanças metabólicas principalmente nos animais que receberam dieta rica em gordura (aumento do peso corporal, gordura abdominal, assim como aumento da glicose plasmática e da atividade da colinestarese plasmática) e a maioria desses efeitos foram aumentados com a exposição ao estresse. A dieta hiperlipídica associado ao estresse também afetou o perfil lipídico aumentando LDL-colesterol nesses animais. Além disso, exposição ao estresse levou a um desequilíbrio oxidativo no fígado, com aumento da produção de espécies reativas, assim como aumento da atividade de enzimas antioxidantes (Superóxido dismutase e Catalase), e esses efeitos foram acentuados com o acesso à dieta hiperlipídica (que também causou uma grave redução na atividade da Glutationa peroxidase). No hipocampo, o estresse levou a uma diminuição da atividade das enzimas antioxidantes, do conteúdo de tióis totais e da atividade dos complexos II e IV da cadeira respiratória mitocondrial. A dieta hiperlipídica quando associada ao estresse reverteu esses efeitos. A partir dos resultados encontrados, concluiu-se que o período pré-pubere representa um período crítico para intervenções durante o desenvolvimento, e o estresse nesse período leva a alterações comportamentais, metabólicas e neuroquímicas na idade adulta, diminuindo o comportamento do tipo ansioso e aumentando a suscetibilidade ao estresse oxidativo tanto no fígado como hipocampo. Esse desfecho, porém dependem do tipo de dieta a que o animal tem acesso.

## ABSTRACT

The pre-puberty period is critical for the final maturation of neural circuits that control energy homeostasis and stress responses. Exposure to stressors and palatable diets in this period of development may alter the maturation process and cause behavioral and neurochemical changes in adulthood. Thus, the objective of this study is to investigate the effects of exposure to stress by social isolation in the prepubertal period and of chronic access to palatable diets on anxious-like behavior, and on metabolic and neurochemical changes in liver and hippocampus of adult male rats. The animals that were isolated showed an anxiolytic-like behavior in the Plus Maze test, and the same was observed in isolated animals with access to high-fat diet. The stressed animals with access to high-carbohydrate diet showed opposite behavior; in other words, they presented anxiogenic-like behavior. Metabolic changes were observed mainly in animals fed high-fat diet (increased body weight, abdominal fat, as well as increased plasma glucose and plasma cholinesterase activity), and most of these effects were further increased by exposure to stress. The high-fat diet associated with stress also affected the lipid profile by increasing LDL-cholesterol in these animals. Furthermore, exposure to stress led to an oxidative imbalance in the liver, by increasing production of reactive species, and the activity of antioxidant enzymes (Catalase and Superoxide dismutase), and these effects were accentuated with access to high-fat diet (which also caused a severe reduction in Glutathione peroxidase activity). In the hippocampus, stress led to decrease in antioxidant enzymes activities, total thiol content and activity of complexes II and IV of the mitochondrial respiratory chair. However, high-fat diet, when associated with stress, reversed these effects. Taken together, we concluded that the prepubertal period is a critical period for interventions during development, and stress during this period leads to behavioral, metabolic and neurochemical changes in adulthood, decreasing anxious-like behavior and increasing the susceptibility to oxidative stress in liver and hippocampus, and these effects will differ depending on the type of diet to which the animal has access.

## LISTA DE ABREVIATURAS

ACTH = Hormônio adrenocorticotrópico

CAT = Catalase

CRH = Hormônio liberador de corticotropina

DCFH = diclorodiidrofluoresceína

DNA = Ácido desoxirribonucleico

ERO= Espécies reativas do oxigênio

GPx = Glutationa peroxidase

HDL = Lipoproteína de alta densidade

HHA= Hipotálamo-hipófise-adrenal

LDL = Lipoproteína de baixa densidade

NADPH = Nicotinamida adenina dinucleotídeo fosfato reduzido

RG = Receptor de glicocorticoides

SNC = Sistema nervoso central

SOD = Superóxido dismutase

# 1. INTRODUÇÃO

## 1.1 Estresse

Um estressor é definido como um desafio ao individuo que potencialmente pode perturbar a homeostase, e assim, requer uma reposta fisiológica. A exposição ao estresse induz uma variedade de respostas no organismo, incluindo respostas neurovegetativas, imunológicas e comportamentais (Tsigos & Chrousos, 2002), além da ativação neuroendócrina do sistema simpato-adrenomedular, levando à liberação de catecolaminas e à ativação do eixo hipotálamo-hipófise-adrenal (HHA). Esse eixo, uma vez ativado, inicialmente libera o hormônio liberador de corticotropina (CRH) do hipotálamo, que irá estimular a hipófise a secretar o hormônio adrenocorticotrópico (ACTH). O ACTH, por sua vez, age no córtex da adrenal estimulando a liberação de glicocorticoides (cortisol em humanos e corticosterona em roedores) para a circulação (Lupien et al., 2005).

Os glicocorticoides possuem várias ações, como o aumento da disponibilidade de substratos energéticos no organismo e melhora do fluxo sanguíneo para órgãos-alvo (Tsigos & Chrousos, 2002). Quando a liberação dos glicocorticoides se torna prolongada, essa condição pode levar a alterações metabólicas e neuroquímicas indesejáveis, inclusive dano neuronal (McIntosh & Sapolsky, 1996). Portanto, é importante que haja uma regulação inibitória do eixo HHA, que é feita, em grande parte, por um sistema de retroalimentação negativa dos glicocorticoides sobre estruturas do sistema nervoso central (SNC), dentre elas o hipocampo, aumentando ou diminuindo sua atividade de acordo com as necessidades fisiológicas (Marti et al., 1994).

## 1.2 Estresse e período pré-pubere

Durante períodos de desenvolvimento a plasticidade cerebral é alta e intervenções durante esses períodos podem ter efeitos permanentes. Uma dessas fases críticas do desenvolvimento é a pré-puberdade. Nesse período ocorre a maturação final de circuitos neuronais que controlam a homeostase energética e as respostas ao estresse (McCormick & Mathews, 2007). Assim, exposição a estressores nessa fase do desenvolvimento pode influenciar respostas comportamentais e neuroendócrinas na idade adulta (Paus et al., 2008).

Um potente estressor, nessa fase de desenvolvimento, é o isolamento social (Weiss et al., 2004). Caracterizado como um tipo de estresse psicológico em roedores (Serra et al., 2005), pode causar mudanças comportamentais, anatômicas e neuroquímicas que persistem até a idade adulta (Weiss et al., 2004; Ferdman et al., 2007).

## 1.3 Estresse e Comportamento Alimentar

O comportamento alimentar pode ser alterado por diferentes fatores, como a disponibilidade de nutrientes e o estresse. A influência do estresse no comportamento alimentar, intensificando ou atenuando o apetite ou ainda aumentando o consumo de macronutrientes ou sabores específicos varia conforme a intensidade e a duração do agente estressor. (Marti et al., 1994; Silveira et al., 2000; Ortolani et al., 2011). Quando o estresse está associado com um aumento do consumo de alimentos, pode levar à obesidade e aos problemas de saúde associados a essa doença (Klump et al., 2007). Essa suscetibilidade à obesidade induzida pelo estresse pode estar ligada ao fato de que o

estresse possui também um efeito sobre a escolha do tipo de alimento. Estudos mostram que pessoas preferem alimentos palatáveis mais calóricos, ricos em açúcar e gorduras, quando estão estressadas (Wardle et al., 2000; Cartwright et al., 2003; Zellner et al., 2006). Essa preferência por alimentos palatáveis mais calóricos durante a exposição ao estresse também é observada em modelos animais (Ely et al., 1997; Pecoraro et al., 2004; Silveira et al., 2010). O consumo de alimento palatável estimulado pelo estresse é proposto como uma alimentação baseada na recompensa, a qual indiretamente diminui as respostas ao estresse (Adam & Epel, 2007). É interessante observar que o tipo de alimento palatável consumido vai ter diferentes ações sobre a atividade do eixo HHA: dietas ricas em calorias e açúcar reduzem a resposta do eixo ao estresse (Pecoraro et al., 2004), enquanto que dietas ricas em gorduras realçam os níveis de glicocorticoides basais e induzidos pelo estresse, podendo agir como um tipo de estressor (Tannenbaum et al., 1997; Kamara et al., 1998).

Além disso, muitos fatores estão envolvidos na relação do estresse com o comportamento alimentar, incluindo ativação do sistema nervoso autônomo, ou neurovegetativo (Torres & Nowson, 2007; Berthoud & Morrison, 2008), efeitos dos hormônios relacionados às respostas ao estresse, como CRH e glicocorticoides (Tsigos & Chrousos, 2002; Adam & Epel, 2007), leptina (Gamaro et al., 2003; Baranowska et al., 2008) e ativação de sistemas neuronais envolvidos em aspectos cognitivos, de recompensa e emocionais relacionados ao comportamento alimentar (Torres & Nowson, 2007).

### 1.4 Exposição ao estresse e dietas palatáveis e sua relação com a ansiedade

O estresse pode induzir mudanças no comportamento emocional, como por exemplo, nos níveis de ansiedade (McEwen et al., 2012). Isso pode ser observado em estudos que utilizam fármacos ansiolíticos, como o diazepam, onde ocorre reversão das alterações comportamentais induzidas pelo estresse no teste de labirinto em cruz elevado e no campo aberto (Mechan et al., 2002; Boufleur et al., 2012a), que são testes utilizados para avaliar comportamento do tipo ansioso em roedores. Essas alterações, porém, também irão depender da intensidade e da duração do estressor. Além disso, distintas respostas comportamentais podem ocorrer em função do período de desenvolvimento em que há a exposição a estressores. Por exemplo, alguns estudos mostram que manipulação no período neonatal pode levar a uma diminuição do comportamento do tipo ansioso (McIntosh et al., 1999; Silveira et al., 2005; Boufleur et al., 2012b), enquanto que isolamento social no período pré-pubere mostrou um efeito do tipo ansiogênico nos animais (Marcolin Mde et al., 2012).

Adicionalmente, existe uma relação da ansiedade com comportamento alimentar em animais submetidos a estressores. Estudos mostram que o estresse crônico aumenta o consumo de alimento doce (Ely et al., 1997; Silveira et al., 2000). Esses resultados sustentam a hipótese de que o estresse promove um aumento no consumo de alimentos hiperpalatáveis, ricos em açúcares e gorduras, usados como forma compensatória para reduzir as respostas ao estresse (chamados "*comfort foods*") (Dallman et al., 2005; Tomiyama et al., 2011).

## 1.5 Exposição ao estresse e dietas palatáveis estão associados ao desequilíbrio oxidativo celular

Exposição ao estresse e a níveis elevados de glicocorticóides podem levar a um aumento na produção de espécies reativas e consequentemente causar um desequilíbrio oxidativo no organismo (Liu & Mori, 1999; Radak et al., 2001; Costantini et al., 2011), que pode estar envolvido na patogênese de diversas doenças cerebrais (Oishi et al., 1999; Gutteridge & Halliwell, 2000; Radak et al., 2001; Mitra et al., 2005).

As espécies reativas do oxigênio (ERO) são produzidas constantemente em baixos níveis pelas células dos mamíferos em vários processos metabólicos, como a cadeia respiratória na mitocôndria, a atividade da NADPH oxidase e o metabolismo oxidativo do ácido araquidônico. ERO são espécies muito reativas, oxidantes, capazes de mudar a conformação, a estrutura e/ou função de diferentes componentes celulares, como proteínas, fosfolipídios de membrana, proteoglicanos, ácidos nucléicos e outros. Para combater essas espécies, o organismo apresenta sistemas de defesa antioxidante enzimático e não-enzimático (Halliwell & Cross, 1994). O sistema antioxidante enzimático é composto pelas enzimas Superóxido Dismutase (SOD), que faz a conversão do radical superóxido em peróxido de hidrogênio; Catalase (CAT), responsável pela neutralização do peróxido de hidrogênio; e Glutationa Peroxidase (GPx), outra enzima que promove a degradação de peróxidos, especialmente derivados da oxidação dos fosfolipídeos de membrana (Kehrer, 2000). Quando a célula perde a capacidade de destoxificar o excesso de ERO, causado por uma produção aumentada de espécies reativas sem o aumento proporcional da atividade dessas enzimas antioxidantes, haverá um quadro de estresse oxidativo (Halliwell, 1996). Esse por sua

vez, pode levar a danos a moléculas como lipídeos, proteínas e ácidos nucleicos (Cochrane, 1991; Valko et al., 2007), podendo até causar morte celular.

As respostas adaptativas ao estresse envolvem mudanças importantes nas funções mitocondriais, permitindo um ajuste bioenergético, termogênese oxidativa, e/ou respostas apoptóticas (Manoli et al., 2007). ERO podem induzir disfunção mitocondrial, interrupção de vias de energia (Papadopoulos et al., 1997), danos a precursores neuronais, além de prejuízos na neurogênese (Kroemer, 1997).

Além disso, o tipo de dieta consumida também parece influenciar o estresse oxidativo. Alguns estudos demonstraram uma associação entre a presença de ERO e uma excessiva ingestão de alimentos ricos em gordura levando a quebras no DNA (Olivo-Marston et al., 2008; de Assis et al., 2009; Higashimoto et al., 2009; Krolow et al., 2010; Du et al., 2012). Por outro lado, uma dieta rica em carboidratos oferecida no período pré-pubere foi capaz de reverter o desequilíbrio oxidativo causado pela manipulação neonatal no córtex cerebral de ratos machos jovens, enquanto tal efeito não foi observado no hipocampo desses animais (Marcolin Mde et al., 2012). Também foi observado que o isolamento no período pré-pubere induziu apoptose, aumento do potencial mitocondrial, desequilíbrio oxidativo e dano ao DNA e que uma dieta palatável foi capaz de prevenir somente os efeitos associados a apoptose no hipocampo de ratos jovens (Krolow et al., 2013).

## 2. OBJETIVOS

## 2.1 Objetivo geral

Analisar a influência dos efeitos da exposição a um estressor ao longo do desenvolvimento (período pré-pubere) associado ao acesso crônico a distintas dietas hiperpalatáveis, ricas em açúcar e gorduras, sobre o comportamento do tipo ansioso, alterações metabólicas e parâmetros relacionados ao desequilíbrio oxidativo em ratos machos adultos.

## 2.2 Objetivos específicos

- Verificar os efeitos da exposição a um estressor sub-agudo no período pré-pubere associado ao consumo crônico de alimento hiperpalatável sobre tarefas comportamentais na idade adulta, avaliando comportamento do tipo ansioso.
- Avaliar a relação da exposição a um estressor sub-agudo no período pré-pubere sobre o peso corporal e consumo crônico de dietas hiperpalatáveis e de ração padrão desde o período pré-pubere até a idade adulta.
- Analisar os efeitos e possíveis interações do estresse por isolamento no período pré-pubere e o acesso crônico a dietas hiperpalatáveis sobre o perfil metabólico dos animais na idade adulta, analisando glicose e lipídeos plasmáticos, níveis de leptina e atividade da colinesterase.
- Verificar os efeitos do estresse por isolamento no período pré-pubere e o acesso crônico a dietas hiperpalatáveis sobre parâmetros de estresse oxidativo no fígado e hipocampo de ratos machos adultos [enzimas antioxidantes (SOD, CAT e GPx), conteúdo total de tióis, produção de espécies reativas (DCFH)].

- Avaliar os efeitos do estresse por isolamento no período pré-pubere e o acesso crônico a dietas hiperpalatáveis sobre a atividade mitocondrial medindo-se a atividade dos complexos da cadeira respiratória no hipocampo de ratos machos adultos.

## 3. MATERIAIS, MÉTODOS E RESULTADOS

O material e métodos e resultados dessa dissertação estão apresentados a seguir, da seguinte forma:

- Capítulo 1: Artigo submetido para publicação na revista *Physiology & Behavior*

- Capítulo 2: Artigo a ser submetido para publicação na revista *Neurochemical Research*

## 3.1 Capítulo 1

**Isolation during the prepubertal period associated with chronic access to palatable diets: effects on plasma lipid profile and liver oxidative stress.**

Artigo submetido para publicação na revista *Physiology & Behavior*.

# ISOLATION DURING THE PREPUBERTAL PERIOD ASSOCIATED WITH CHRONIC ACESS TO PALATABLE DIETS: EFFECTS ON PLASMA LIPID PROFILE AND LIVER OXIDATIVE STRESS

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**Abstract**

Pre-puberty is a critical period for the final maturation of the neural circuits that control energy homeostasis, as external stimuli such as exposure to diets and stress may influence the adaptive responses with long-term repercussions. Our aim is to investigate the effects of isolation stress during early life and of chronic access to palatable diets, rich in sugar or fat, on the metabolic profile (glycemia, plasma lipids, leptin and cholinesterase activity) and oxidative stress parameters in the livers of adult male rats. We observed changes mainly in animals that received the high-fat diet (increased body weight and abdominal fat in adults, as well as increased plasma glucose, and cholinesterase activity), and most of these effects were further increased by exposure to stress. High-fat diet also affected the rats' lipid profile (increased cholesterol, LDL-cholesterol and triglycerides); these effects were more marked in stressed animals. Additionally, exposure to stress led to an oxidative imbalance in the liver, by increasing production of reactive species, as well as the activity of antioxidant enzymes (superoxide dismutase and catalase); these effects were accentuated with the high-fat diet (which also caused a severe reduction in glutathione peroxidase activity). Taken together, these results show that the pre-pubertal period constitutes a critical window for stressful interventions during development, leading to alterations in metabolic parameters and increased oxidative stress during adulthood that may be more pronounced in animals that receive a high-fat diet.

Key-words: High-fat diet, high-carbohydrate diet; isolation stress; pre-pubertal period; oxidative stress; liver.

## 1. Introduction

The consumption of diets rich in sugar and fat, along with a sedentary lifestyle, is associated with increased obesity prevalence [1]. Levels of obesity have been increasing in children and adolescents, creating concern, as exposure to diets rich in calories during this period of development could modify the maturation of neuronal circuits and lead to dysfunction or diseases during adulthood [2]. In addition, it has been suggested that environmental factors, such as exposure to stress, are strongly implicated in this higher prevalence of obesity [3].

A stressor is defined as a challenge to the organism that can potentially disrupt homeostasis and, therefore, requires a physiological response. During development, when the plastic capacity is maximal, these adjustments become more important. Moreover, it is known that the prepubertal period is critical for the maturation of the neural circuits that control energy homeostasis and stress responses [4]. In this period, one of the most potent stressors is social isolation [5], which can lead to behavioral, anatomical and neurochemical changes that may remain during adulthood, when these animals are compared to their socially-reared litter mates [5-6].

Exposure to stress induces a variety of responses, including activation of the sympatho-adrenomedullar system, release of catecholamines, and activation of the hypothalamic-pituitary-adrenal (HPA) axis, culminating in the release of glucocorticoids (GCs) [7]. The metabolic effects of the GCs include increased plasma glucose due to gluconeogenesis and glycogen degradation, as well as inhibition of glucose uptake in some tissues, mobilization of amino acids from extrahepatic tissues, stimulation of lipolysis in adipose tissue and increased metabolic rate [8-9]. Animal studies show that stress may both increase and decrease food ingestion

depending on the duration and intensity of the stress [10-14]. Many factors may be involved in these effects related to stress and feeding behavior, including autonomic nervous system activation [8, 15], effects of hormones related to the stress response, such as CRH and glucocorticoids [1, 16], leptin [17-18], and/or stress activation of neural systems involved in the cognitive, rewarding, and emotional aspects of ingestive behavior [8]. Stress exposure may increase food intake and insulin levels, facilitating the development of obesity and the metabolic syndrome [19-20]. Conversely, the regulation of the HPA axis will depend on the type of palatable food consumed: Diets high in calories and sugar reduce this axis response to stress [21], while diets rich in fat enhance stress-induced levels of glucocorticoids [22-23].

Additionally, stress exposure and elevated GCs levels have been reported to increase the generation of reactive oxygen species (ROS) [24-27]. When there is an imbalance between antioxidant defenses and oxidative species, oxidative stress occurs [28], which may lead to damage to cell structures like proteins, lipids, membranes and DNA [29-30]. Moreover, some studies have presented evidence that the presence of ROS and excessive intake of fatty foods can lead to breaks in cellular DNA [31-35]. In this context, the consequences of stress exposure in animals with ad libitum access to palatable diets require a better understanding.

Since the pre-pubertal period is critical for development, being important to the stress response and for the emergence of eating disorders, the aim of our study is to verify whether stress by social isolation during the pre-puberty period in animals with chronic access to palatable diets until adulthood may alter oxidative stress parameters in the liver, and metabolic profiles such as plasma lipids, plasma glucose and leptin. Serum cholinesterase activity was also measured, since relationships

between the activity of this enzyme and hyperlipidemia, diabetes, and obesity have been reported [36].

## 2. Material and Methods

### *2. 1. Experimental subjects*

All animal proceedings were performed in strict accordance to the recommendations of the Brazilian Society for Neurosciences (SBNeC) and Brazilian Law on the use of animals (Federal Law 11.794/2008), and were approved by the Institutional Ethical Committee. All efforts were made to minimize animal suffering, as well as to reduce the number of animals used.

Animals were housed in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust, and were maintained on a standard 12h dark/light cycle (lights on between 7:00h and 19:00h), temperature of 22 ± 2°C. On postnatal day (PND) 21, sixty-three Wistar rats were weaned. Only male pups were used from each litter, and these pups were divided into six groups, in such a way that only one animal per litter was used in each group. Male pups were weighed at PND 21 and distributed into 3 groups, according to the diet that they received: (1) receiving standard lab chow (50% carbohydrate, 22% protein and 4% fat); (2) receiving both standard chow and a diet with a high content of simple carbohydrate [37] and (3) receiving standard chow and a high-fat diet (25% carbohydrate, 28% protein and 42% fat). Therefore, animals from these last two groups could choose the diet they consumed from the two diets available. Half of the animals on each diet were housed in groups of 4; the other half were stressed by isolation (one animal in a smaller home cage, 27x17x12 cm) [38], in such a way that six groups were obtained; controls receiving standard chow (CC),

controls receiving standard chow and high-carbohydrate diet (HCC), controls receiving standard chow and high-fat diet (HFC), isolated animals receiving standard chow (IC), isolated animals receiving standard chow and high-carbohydrate diet (HCI), and isolated animals receiving standard chow and high-fat diet (HFI). The isolation stress occurred between postnatal days 21-28. On PND 28, isolated animals were returned to regular home cages (65 x 25 x 15 cm) in groups of four. During 40 days, beginning on PND 21, amounts of palatable diets and standard lab chow were offered *ad libitum*. At postnatal day 60, the animals were killed by decapitation and biochemical evaluations were performed.

*2.2 Diets*

The nutritional compositions of each diet used are displayed in Table 1. The high-carbohydrate diet (HCD) used in this study was enriched in simple carbohydrates, and made with condensed milk, sucrose, vitamins and a salt mix, powder standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet is similar to that of a standard lab chow (including 22% protein and 4-6% fat), however most carbohydrates in the palatable diet were sucrose [37]; in contrast, the standard lab chow contained carbohydrates obtained mainly from starch.

The high fat diet (HFD) used in the study was enriched with fat (42%) from lard and soy oil. In addition, this diet contained vitamins and a salt mixture, purified soy protein, methionine, lysine and starch [adapted from39].

*2.3 Food consumption*

Previously weighed quantities of standard lab chow and palatable diets were offered and the remaining amounts of pellets were measured each day to evaluate

consumption. The food consumption was measured per cage and the amount of food consumed was divided by the number of animals per cage to determine mean consumption per animal. To verify the amount of kilocalories consumed per animal, we multiplied the amount of food ingested by the caloric content per gram of standard chow or diets. The standard lab chow has a caloric content of 3.24 kcal/g, whereas the high-carbohydrate diet has a caloric content of 4.5 kcal/g and the high-fat diet has a caloric content of 5.8 kcal/g (being 38% and 79%, respectively, more caloric than standard chow).

*2.4 Abdominal fat dissection and preparation of the samples for biochemical measurements*

Forty days after receiving these diets, the animals were killed by decapitation (following 6h of fasting). The two major portions of abdominal fat (gonadal and retroperitoneal adipose tissue depots) and adrenal glands were carefully dissected and weighed. Trunk blood was collected into tubes with EDTA for glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL), leptin, and cholinesterase activity determination. Plasma was separated and frozen until the day of analysis. The liver was perfused with cold saline, dissected out and stored at –80$^{o}$ C until analysis, when it was homogenized in 50 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged at 1000 x g for 10 min at 4$^{o}$ C and the supernatant was assayed for oxidative stress parameters using the chemical oxidation of dichlorodihydrofluorescein (DCFH), the determination of total thiol content and the evaluation of antioxidant enzyme activity.

*2.5 Biochemical analysis*

Leptin was measured by ELISA (Abnova Corporation, Jhongli City, Taoyuan County, Taiwan). Plasma glucose, total cholesterol, HDL-cholesterol and triglycerides were measured with commercial kits; glucose, total cholesterol, and triglycerides were measured using kits from Wiener Laboratorios (Rosario, Argentina), and HDL-cholesterol was measured using a kit from Labtest Diagnóstica S.A. (Minas Gerais, Brazil). LDL-cholesterol was evaluated using the Friedewald formula [40].

*2.5.1 Cholinesterase activity evaluation*

Plasma cholinesterase (ChE) activity was determined by the method of Ellman et. al., [41] with some modifications. Hydrolysis rate was measured at an acetylthiocholine (AcSCh) concentration of 0.8 mM in 1 mL assay solutions with 100 mM potassium phosphate buffer, pH 7.5, and 1.0 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). Fifty microliters of diluted rat serum was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2 min (intervals of 30 s) at 25$^{o}$ C. All samples were run in duplicate. Specific enzyme activity was expressed as mmol acetylthiocholine hydrolyzed per hour per milligram of protein.

*2.5.2 Superoxide Dismutase Activity*

Superoxide dismutase activity was determined using a RANSOD kit (Randox Labs., USA), which is based on the procedure described by Delmas-Beauvieux, et al. [42] This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37$^{o}$ C. The inhibition of the production of the chromogen is proportional to the activity of

SOD present in the sample; one unit of SOD causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

*2.5.3 Glutathione Peroxidase Activity*

Glutathione peroxidase activity was determined according to Wendel [43], with modifications. The reaction was carried out at 37$^{o}$C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione, and 0.4 U glutathione reductase. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

*2.5.4 Catalase Activity*

Catalase activity assessment is based upon the spectrophotometric establishment of the rate of $H_2O_2$ degradation at 240 nm at 25$^{o}$C [44]. CAT activity was calculated in micromoles of $H_2O_2$ consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 $M^{-1}$ $cm^{-1}$.

*2.5.5 Evaluation of free radical production by the chemical oxidation of dichlorodihydrofluorescein (DCFH)*

The samples were incubated with 2',7'-dichlorodihydrofluorescein diacetate (100 μM) at 37°C for 30 minutes. DCFH is released by cellular esterases and oxidized reactive oxygen/nitrogen species. The formation of the fluorescent derivative dichlorofluorescein (DCF) was monitored by excitation and emission wavelengths of 488 and 525nm, respectively, using a spectrum photometer. The formation of oxidized reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmol of DCF formed per mg of protein [45].

*2.5.6 Determination of total thiol content*

This assay is based on the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by thiol groups, which become oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm [46].

*2.5.7 Protein Assay*

The protein concentration was determined in the samples using the method described by Lowry et al., [47], with bovine serum albumin as the standard.

*2.6 Statistical Analysis*

Data are expressed as mean ± SE of the mean, and analyzed using two-way ANOVA, with isolation stress and diet as factors. For body weight and caloric intake, Repeated Measures ANOVA was used (the within subjects factor was time; the between subjects factors were stress and diet). With regard to Repeated Measures ANOVA, the Greenhouse-Greisser correction was applied when necessary, considering the violation of the sphericity assumption, as shown by the Mauchly test. ANOVA tests were followed by the Tukey multiple range test, when indicated. All analyses were performed using SPSS software and a $P \leq 0.05$ was considered significant.

## 3. Results

*3.1 Body weight and caloric consumption*

Body weight gain and caloric consumption were analyzed during the period of stress and until 60 days of life (Figure 1 for body weight and Figures 2 and 3 for caloric consumption). During the first week (period of isolation), animals receiving the high-fat diet gained less weight than the other groups [two-way ANOVA, $F(2,44)=12.145$, $P<0.001$, followed by Tukey post-hoc]. There was also an interaction between stress and diet [$F(2,44)=3.407$, $P<0.05$], since stressed animals did not present a reduced

weight gain when receiving the high-fat diet (Figure 1A). With regard to the caloric consumption during this first week, the animals with access to a high-carbohydrate diet had a higher caloric consumption, compared to the other groups [two-way ANOVA, $F(2,22)=52.92$, $P<0.001$, followed by Tukey post-hoc], as displayed in Figure 2A. There was a marginally-significant interaction between isolation stress and diet on caloric consumption during this period [$F(2,22)=2.87$, $P=0.07$], since isolated animals receiving the high-fat diet had an increased caloric consumption. The caloric consumption evaluated considered both palatable diets and standard chow consumed. When evaluating just the consumption of high-carbohydrate or high-fat diets, we observed an interaction between isolation stress and diet [repeated measures ANOVA, $F(1,16)=10.87$, $P=0.005$]; while both groups (isolated or controls) receiving the high-carbohydrate diet had a marked increase in consumption, only stressed animals receiving the high-fat diet showed an increased consumption with time; diet consumption also increased as the days passed by [$F(6,96)=8.47$, $P<0.001$] (Figure 2B). Additionally, there was an interaction between diet and time [$F(6,96)=4.37$, $P=0.001$], since animals with access to the high-carbohydrate diet consumed more food than those receiving the high-fat diet and increased this consumption as the time passed by. Accordingly, there was a main effect of diet [$F(1,16)=212,45$, $P<0.001$], with the high-carbohydrate diet being more consumed. When considering body weight until 60 days of age, animals gained weight [repeated measures ANOVA, $F(5,220)=3142.48$, $P<0.001$] and there were interactions between body weight along time and with stress [$F(5,220)=2.59$, $P<0.05$] and between time and diet [$F(10,220)=5.49$, $P<0.001$]. There was also a main effect of diet [$F(2,44)=4.97$, $P=0.01$], since animals receiving the high-carbohydrate diet presented a higher weight gain that animals receiving regular standard chow (Tukey post-hoc, $P<0.05$) (Figure 1B). Accordingly, as observed in Figure 3A,

caloric consumption was higher in animals with access to the high-carbohydrate diet [$F(2,7)=148.56$, $P<0.001$, followed by Tukey post-hoc]. In addition, there was an effect of time [$F(4,28)=30.31$, $P<0.001$], since caloric consumption increased over time. The higher caloric consumption was due to increased ingestion of the diet and not the standard chow (Figure 3B) [$F(1.5,20)=20.57$, $P<0.001$ for time; $F(1.5,20)=5.81$, $P<0.05$ for the interaction between time and diet; $F(1,5)=461.67$, $P<0.001$ for diet].

*3.2. Abdominal fat and adrenal gland weight*

Fat deposition and adrenal gland weight were analyzed in adults and are shown in Table 2. Significant differences were observed both on retroperitoneal fat [Two-way ANOVA; $F(2,45)=23.88$, $P<0.001$] and gonadal fat [$F(2,45)=32.35$, $P<0.001$]. The groups that received palatable diets had increased abdominal fat, when compared to the group receiving regular standard chow, and the group receiving the high-fat diet had the highest fat accumulation (Tukey post-hoc, $P<0.05$). Furthermore, a significant interaction between stress and diet was found in gonadal fat [$F(2,45)=3.44$, $P<0.05$] and this interaction was almost significant in the retroperitoneal fat [$F(2,45)=2.79$, $P=0.07$], where stress decreased the fat weight in the HCD and increased it in the HFD. With regard to adrenal gland weight, there was a marginally significant effect of exposure to stress during the prepubertal period [$F(1,45)=3.83$, $P=0.057$], where stress increased adrenal gland weight. If the adrenal gland weight is expressed as the adrenal/body weight ratio, the result remains the same.

*3.3 Plasma lipid levels and serum cholinesterase activity*

Results from plasma lipid measurements, as well as serum cholinesterase activity, were analyzed using a Two-way ANOVA and are shown in Table 3. The levels

of total cholesterol showed a marginally significant effect of diet [$F(2,27)=3.11$, $P=0.06$]. No differences were found in HDL-cholesterol levels ($P>0.05$). However, a significant interaction between stress and diet was verified on LDL-cholesterol levels [$F(2,27)=3.54$, $P<0.05$], as well as a main effect of stress [$F(1,27)=4.08$, $P=0.05$], where stress during the prepubertal period was found to reduce LDL-cholesterol during adulthood, but not in those animals receiving the high-fat diet. Additionally, there was an almost significant interaction between stress and diet on triacylglicerol levels [$F(2,21)=3.02$, $P=0.07$]. Analyses of cholinesterase activity showed a significant difference between diet groups [$F(2,22)=19.07$, $P<0.001$, followed by Tukey post-hoc], since the animals receiving the HFD presented higher plasma enzyme activity.

*3.4 Plasma glucose and leptin levels*

As displayed in Table 4, rats with access to a HFD had higher glucose plasma levels than the other groups [two-way ANOVA, $F(2,27)=3.77$, $P<0.05$, followed by Tukey post-hoc]. Exposure to stress caused a reduction in leptin levels [$F(1,21)=8.07$, $P<0.01$], whereas both palatable diets increased these levels [$F(2,21)= 12.15$, $P<0.001$].

3.5 *Antioxidant enzyme activities, total thiol content and free radical production in the liver*

Oxidative stress parameters were analyzed to verify whether there was an oxidative imbalance in the liver after exposure to isolation stress during the prepubertal period, when palatable diets were chronically offered (Figure 4). When evaluating SOD activity, an interaction between stress and diet was shown [two-way ANOVA, $F(2,21)=3.48$, $P<0.05$]: isolation stress led to increased SOD activity in the group receiving the HFD. Exposure to stress increased CAT activity [$F(1,21)=34.03$, $P<0.001$]

and there was an interaction between stress and diet [$F(2,21)=9.92$, $P<0.001$], since the increased activity induced by stress exposure was higher in the group receiving standard chow. For GPx activity, there was an expressive effect of diet, with both palatable diets decreasing this activity; however, a really marked decrease was observed in animals receiving HFD [$F(2,21)= 361.51$, $P<0.001$, followed by Tukey post-hoc]. In relation to total thiol content, there was an effect of diet [$F(2,21)=4.83$, $P=0.01$; no significant difference was observed in post-hoc test]. Additionally, free radical production, as evaluated by the DCFH test, showed an effect of diet [$F(2.21)=7.46$, $P<0.005$] and an interaction between stress and diet [$F(2,21)=5.44$, $P=0.01$], since stress increased free radical production in the liver, particularly in the animals receiving palatable diets.

## 4. Discussion

In the present study, we evaluated the effect of chronic access to distinct palatable diets (when the animals could choose between these diets and standard chow), associated with stress by isolation early on in life, in adult male rats. Animals receiving HCD showed a higher weight gain compared to the control and higher calorie consumption when compared to other groups. However, the group receiving HFD presented the highest accumulation of abdominal fat, and this was further increased when associated with stress. The association of stress with HFD increased LDL-cholesterol levels. In the groups receiving the other diets, however, stress appeared to be beneficial, reducing LDL-cholesterol. The high-fat diet also increased plasma glucose levels and cholinesterase activity. In addition, the two palatable diets increased leptin levels, while stress caused a decrease. In the liver, stress induced an oxidative imbalance, and the group that received the high-fat diet was the most affected.

The animals with access to a high-fat diet demonstrated a lower weight gain during the first week, and the caloric consumption of these animals was similar to that of controls, suggesting a lower caloric efficiency in these animals. However, stressed animals that received this diet increased their consumption, and presented a weight gain that was similar to that of the controls. On the other hand, animals that had access to a high-carbohydrate diet had a higher caloric intake during the first week, mainly due to the increased consumption of the palatable diet, even though the weight gain of these animals did not present an equivalent increase. Stress increased consumption of both palatable diets, supporting the hypothesis that palatable food may be used as compensation during periods of stress ("comfort foods") [48].

After one week of isolation stress, the animals were returned to living in groups, but were still given access to palatable diets. Considering body weight until 60 days of age, the group that received the HCD had a higher weight gain, reflecting their increased consumption. The same did not occur in animals that received the HFD. Furthermore, of the stressed groups, those receiving palatable diets presented increased body weight. Access to both diets (HCD and HFD) led to increased abdominal fat depots (both gonadal and retroperitoneal fat), probably reflecting the higher consumption in the HCD. In the case of the HFD, the increased deposition of fat suggests a lower lean body mass, as there was no increase in weight when compared to the controls. Therefore, the HCD appears to be more palatable, inducing higher consumption and leading to a higher weight gain in these animals. In addition, both diets showed increased abdominal fat, which is an important risk factor for metabolic syndrome [49-50]. Moreover, stress exposure further increased abdominal fat in the HFD group, while the opposite was observed in animals receiving the HCD. This may be explained by the fact that high-fat diets enhance stress-induced levels of

glucocorticoids [22-23], and thus may increase fat accumulation, while diets that are high in sugar reduce the HPA axis response to stress [21].

The adrenal glands were only slightly increased in stressed animals. However, since this measurement is performed in adulthood, and isolation stress was applied in childhood, this result suggests a long-lasting effect of isolation on the HPA axis function. In rats, during the prepubertal period, this axis functions in a different way compared with adults, and habituation to stressors is less efficient [51]. This result adds further support to the notion that, during early life, the brain is very susceptible to genetic influences and environmental experiences [52-55], and that early adverse experiences may lead to abnormal behavior associated with alterations of the HPA axis [5,56].

In relation to plasma lipid levels, it is known that there is a relationship between cardiovascular disease and high intake of saturated fat [57-58], as well as increased plasma levels of total cholesterol, triglycerides and LDL-cholesterol, and decreased HDL levels [59-61]. Accordingly, in our study, the high-fat diet animals presented higher levels of LDL-cholesterol, and a tendency towards increased triglycerides and total cholesterol; however these findings were observed only in the animals that had been stressed during the prepubertal period. On the other hand, HDL-cholesterol levels were not significantly different. Stress by social isolation is an aversive event that increases the activity of the HPA axis [4,38]. It is possible that exposure to stress during the prepubertal period permanently programs the organism's metabolism; this effect could be associated with the alterations observed in the lipid profile in the HFD group. Studies show that glucocorticoids can increase circulating fatty acids through an increase in dietary fat intake [62]; these findings support our observations, since HFD consumption increased during isolation stress. This effect, however, did not persist until

adulthood, showing that programming of the ingestion of this type of diet was not modified in the long term, while HCD continued to be more consumed for some weeks after stress. Glucocorticoids are also known to increase lipogenesis and VLDL secretion from liver [62], which could help to explain the tendency to increase plasma triglycerides in the stressed group receiving HFD, and the synergic effect between stress and HFD, since it has been suggested that HFD enhances stress-induced levels of these hormones [22-23]. Depending on the intensity and duration of stress exposure, both lipolytic and antilipolytic effects may be observed in adipocytes, although the mechanisms by which these metabolic effects occur are still unclear [63-66]. Additionally, stressed animals had a decrease in LDL-cholesterol levels (except the group receiving HFD), when compared to the control group receiving regular standard chow, and stress had a tendency to increase cholesterol levels, which was more pronounced in animals that received the HFD. Both the adrenal and non-adrenal (ovarian and testicular) syntheses of steroid hormones employ LDL-cholesterol mainly from the circulation [67-68]. Thus, it is possible that the decrease in LDL-cholesterol levels that occurs as a result of stress is the consequence of the redirection of these lipids to the synthesis of steroids hormones; in other words, stress-induced steroidogenesis (which would be in agreement with the slightly increased adrenals in these animals). In summary, the effects of the consumption of a high-fat diet on serum lipid profile depend on the previous history of the animal, and exposure to stress during the prepubertal period endangers these animals, when this type of diet is associated.

One possible modulator of stress-induced eating is leptin [69-70]. This hormone is secreted from adipose tissue and influences energy homeostasis, immune and neuroendocrine function [71-73]. Production of leptin correlates positively with adipose tissue mass [74], and circulating leptin levels are involved both in the signaling of

energy stores and in food intake. We found that the two palatable diets increased leptin levels, which can be correlated with the increase in abdominal fat found in these animals. Increased leptin levels may act in the hypothalamus to decrease appetite [72]. Interestingly, consumption in animals receiving the high-carbohydrate diet remained high. This may have occurred due to a lower sensitivity to leptin in the hypothalamus, where high plasma leptin concentrations do not induce the reduction in food intake, suggesting resistance to the effects of endogenous leptin [75-76]. Furthermore, stress exposure decreased leptin levels in those animals given palatable diets (non-standard). Previous studies in the literature showed elevated leptin levels in human patients under glucocorticoid therapy [77-78], which could result from an inhibitory role on the action of leptin [79-80], which is suggested to contribute to "leptin resistance". Our finding that stress exposure decreased leptin levels does not agree with the above reports, this may be due to the fact that we used sub-acute stress during the developmental phase.

Animals that received the high-fat diet presented increased levels of blood glucose compared to other groups. As shown in the literature, the high-fat diet contributes to impaired glucose tolerance and insensitivity to the blood-glucose lowering effect of insulin [81]. This has been related to an impaired insulin binding and/or glucose transporters, due to changes in the fatty acid composition of the membrane induced by dietary fat modification [82]. Moreover, plasma cholinesterase activity was also higher in animals fed on the high-fat diet. Although the exact physiological function of plasma cholinesterase is unclear, reports from the literature suggest a relationship between the increased activity of this enzyme and hyperlipidemia, diabetes, and obesity [36], which are all risk factors for coronary artery disease [83] and heart disease [84]. Thus, increased levels of blood glucose and activity of plasma

cholinesterase in animals receiving HFD are possibly risk factors for metabolic syndrome and cardiovascular disease in these animals.

In liver, exposure to isolation stress increased SOD activity in the group receiving HFD; stress also increased CAT activity in all the diet groups. GPx activity decreased with palatable diets, and most markedly in the HFD group. The production of free radicals was increased by stress, especially in the groups fed with the palatable diets, and total thiol content had an effect of diet, but no difference was observed in the post-hoc test. Thus, these results showed that stress during the prepubertal period induced a long-lasting increase in the production of free radicals in the liver, concomitantly with an increased activity of antioxidant enzymes. These increased antioxidant defenses are probably the result of the response to free radical formation, in an effort to protect cells against oxidative damage. This increase seemed to be higher in the HFD group. Moreover, this diet also induced a drastic reduction in GPx activity. It is known that high-fat diet can aggravate oxidative stress [85-86], causing the formation of toxic intermediates that can diminish the activity of antioxidant enzymes [87-88]. One possible explanation for the decreased GPx activity may be the rapid consumption and exhaustion of these enzyme molecules, due to reactions with the free radicals generated. Other studies have also shown a decrease in the activity of antioxidant enzymes in the liver in association with high-fat diets [86, 89-91]. In our study, however, only GPx activity was significantly reduced by the HFD, and further studies are necessary to understand the mechanism for this marked reduction. This decrease in GPx activity, however, may lead to an oxidative imbalance, leaving the liver vulnerable to an overproduction of hydrogen peroxide produced by the superoxide dismutase enzyme, the activity of which is increased in these animals (HFD associated with stress). This is especially important, considering that data suggest that oxidative stress

can lead to various forms of chronic liver injury [92].

## 5. Conclusion

In conclusion, chronic access to palatable diets, especially a HFD, from the pre-pubertal period until adulthood, leads to changes in parameters related to risk factors for cardiovascular diseases and metabolic syndrome (increased body weight, abdominal fat, and leptin levels, as well as increased glycemia and cholinesterase activity); this condition is made worse by exposure to stress during the pre-pubertal period. Additionally, exposure to stress led to an oxidative imbalance in the liver and this was even more marked in the animals fed on the HFD. These data emphasize the importance of considering the previous history of individuals when investigating the effects of diets on metabolic parameters.

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Table 1. Nutritional composition/100 g of the food used in the studies performed. HCD: high carbohydrate diet; HFD: high-fat diet.

| Food | Energy (kcal) | Total protein (g) | Total carbohydrate (g) | Total fat (g) |
|---|---|---|---|---|
| Standard Chow[a] | 324 | 22 | 55 | 4.5 |
| HCD[b] | 450 | 25 | 65 | 10 |
| HFD[c] | 588 | 28 | 25 | 42 |

**a** Nuvilab®

**b** Souza CG, et. al. Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like beharior. Life Sci, 2007, 81:198-203.

**c** Adapted Ziegler DR, et. al. A ketogenic diet increases protein phosphorylation in brain slices of rats. J Nutr 2002, 132:483-487.

Table 2. Effect of isolation stress during the prepubertal period and chronic access to palatable diets on weights of retroperitoneal and gonadal fat and adrenal glands in adult rats.

| | Control | | | Stress | | |
|---|---|---|---|---|---|---|
| | Standard Chow | HCD | HFD | Standard Chow | HCD | HFD |
| Retroperitoneal fat | 4.16±0.43 | 7.70±0.75* | 7.91±0.58** | 4.21±0.53 | 5.66±0.65* | 8.73±0.99** |
| Gonadal fat | 3.31±0.24 | 5.87±0.54* | 6.45±0.55** | 3.16±0.35 | 4.12±0.47* | 7.04±0.97** |
| Adrenal glands | 0.061±0.005 | 0.056±0.005 | 0.061±0.005 | 0.062±0.003 | 0.071±0.004 | 0.068±0.007 |

Fat deposition and adrenal gland weight were evaluated and expressed in mg tissue. Data are expressed as mean ± SEM, N=10-12/group. There was an effect of diet on retroperitoneal fat (two-way ANOVA, $P<0.001$) and gonadal fat ($P<0.001$); interaction between stress and diet on retroperitoneal fat ($P<0.05$) and gonadal fat ($P=0.07$); effect of stress ($P=0.057$) on adrenal glands.

* Significantly different from standard chow and HFD. ** Significantly different from standard chow and HCD (Tukey post-hoc, $P<0.05$).

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Table 3. Effect of isolation stress during the prepubertal period and chronic access to palatable diets on total plasma cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride levels, and plasma cholinesterase (ChE) activity in adult rats.

| | Control | | | Stress | | |
|---|---|---|---|---|---|---|
| | Standard Chow | HCD | HFD | Standard Chow | HCD | HFD |
| Total cholesterol | 54.23+5.45 | 46.43+4.45 | 52.89+3.38 | 43.18+4.62 | 47.67+3.05 | 61.65+5.11 |
| HDL-Cholesterol | 24.10+3.00 | 28.63+4.56 | 26.07+1.13 | 27.67+5.94 | 29.28+4.98 | 33.57+3.23 |
| LDL-cholesterol | 47.49+6.82 | 39.74+4.60 | 39.73+4.75 | 26.26+5.15 | 29.12+2.87 | 45.60+5.30 |
| Triglycerides | 86.84+17.61 | 109.71+18.38 | 64.56+10.11 | 53.77+11.14 | 53.68+1.50 | 87.61+15.27 |
| ChE activity | 0.412±0.02 | 0.417±0.01 | 0.592±0.04* | 0.380±0.02 | 0.462±0.04 | 0.530±0.02* |

Plasma lipids are expressed as mg/dL of plasma (mean ± SEM; N=5-7/group). Cholinesterase activity is expressed as µmol AcSCh/h . mg protein (N=4-5/group). Samples were collected from animals with 6h of fasting. Two-way ANOVA showed an interaction between stress and diet ($P<0.05$), and an effect of stress ($P=0.05$) on LDL-cholesterol; and an effect of diet ($P<0.001$) on cholinesterase activity.

* Significantly different from standard chow and HCD (Tukey post-hoc, $P<0.001$).

Table 4. Effect of isolation stress during the prepubertal period and chronic access to palatable diets on plasma glucose and leptin levels in adult rats.

| | Control | | | Stress | | |
|---|---|---|---|---|---|---|
| | Standard Chow | HCD | HFD | Standard Chow | HCD | HFD |
| Glucose | 132.3±9.4 | 128.8±2.7 | 144.1±10.5* | 120.7±5.8 | 131.7±4.5 | 147.9±2.8* |
| Leptin | 4.98±0.44 | 9.32±1.35** | 10.92±0.89** | 4.83±0.22 | 6.46±1.15** | 7.57±1.12** |

Data are expressed as mean ± SEM, N=5-7/group for plasma glucose; N=4-5/group for leptin. Plasma glucose is expressed as mg/dL; Leptin is expressed as ng/mL. Samples were collected from animals with 6h of fasting. Two-way ANOVA showed an effect of diet ($P<0.05$) on plasma glucose levels; an effect of stress ($P<0.01$) and diet ($P<0.001$) on leptin levels.

* Significantly different from the other groups (Tukey post-hoc, $P<0.05$). ** Significantly different from standard chow (Tukey post-hoc, $P<0.001$).

**Legends to Figures**

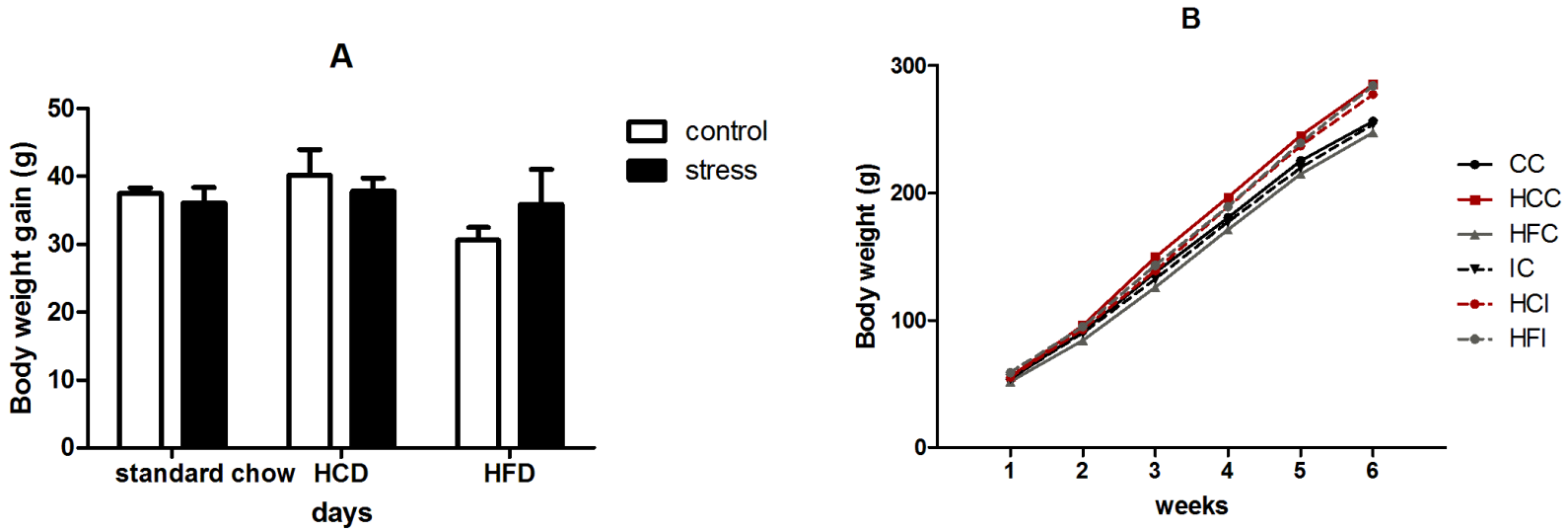
**Figure1**. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on body weight gain. **A.** Body weight gain during the period of stress isolation. Two-way ANOVA showed a significant effect of diet ($P<0.001$) and an interaction between stress and diet ($P<0.05$). **B.** Body weight until 60 days of age. Repeated measures ANOVA showed an effect of diet ($P<0.01$) and interactions between weight over time and stress ($P<0.05$) and time and diet ($P<0.001$). Data are expressed as mean $\pm$ SEM, N=10-15/group. HCD: high-carbohydrate diet; HFD: high-fat diet; CC: control receiving chow; HCC: control receiving chow and high-carbohydrate diet; HFC: control receiving chow and high-fat diet; IC: isolated receiving chow; HCI: isolated receiving chow and high-carbohydrate diet; HFI: isolated receiving chow and high-fat diet.

**Figure 2**. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on consumption of palatable diets during the period of isolation stress. **A.** Caloric consumption. Two-way ANOVA showed an effect of diet ($P<0.001$). HCD had a higher caloric consumption compared to other groups. **B**. Consumption of HCD and HFD. Repeated measures ANOVA showed an interaction between isolation stress and diet ($P=0.005$) and diet and time ($P<0.001$), effect of diet ($P<0.001$) and time ($P<0.001$). Data are expressed as mean $\pm$ SEM, N=4-10/group. HCD: high-carbohydrate diet; HFD: high-fat diet; HCC: control receiving chow and high-carbohydrate diet; HCI: isolated receiving chow and high-carbohydrate diet; HFC: control receiving chow and high-fat diet; HFI: isolated receiving chow and high-fat diet.

**Figure 3**. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on consumption of palatable diets until 60 days of age. **A**. Caloric consumption. Repeated measures ANOVA showed effect of diet ($P<0.001$) [higher in animals with HCD] and time ($P<0.001$). **B**. Consumption of palatable diets. Repeated measures ANOVA showed an effect of time ($P<0.001$), diet ($P<0.001$) and an interaction between time and diet ($P<0.05$). Data are expressed as mean $\pm$ SEM, N=2-4/group. CC: control receiving chow; HCC: control receiving chow and high-carbohydrate diet; HFC: control receiving chow and high-fat diet; IC: isolated receiving chow; HCI: isolated receiving chow and high-carbohydrate diet; HFI: isolated receiving chow and high-fat diet.

**Figure 4**. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on antioxidant enzyme activities, total thiol and free radicals (DCFH test) production in liver of adult rats. Data are expressed as mean $\pm$SEM. N= 5-7/group. **A**. SOD (expressed as U/mg protein), **B.** CAT (expressed as micromoles of $H_2O_2$ consumed/min/mg protein), **C**. GPx (expressed as nmol NADPH oxidized/min/mg protein), **D**. total Thiol (expressed as nmol TNB/mg protein) and **E**. DCFH (expressed as nmol of DCF formed/mg protein). Two-way ANOVA showed an interaction between stress and diet on SOD ($P<0.05$), and CAT ($P<0.01$) activities, and on DCFH ($P=0.01$); effect of stress on CAT activity ($P<0.01$); effect of diet on GPx activity ($P<0.001$), on total thiol ($P=0.01$) and on DCFH ($P<0.005$). HCD: high-carbohydrate diet; HFD: high-fat diet.

# Figure 1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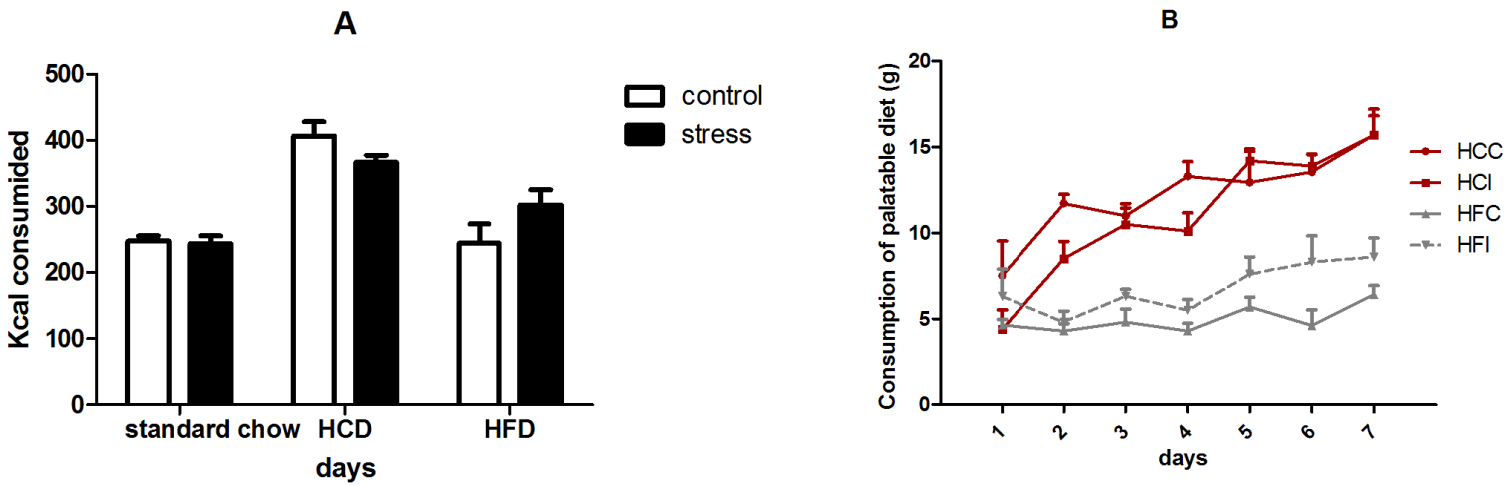
# Figure 2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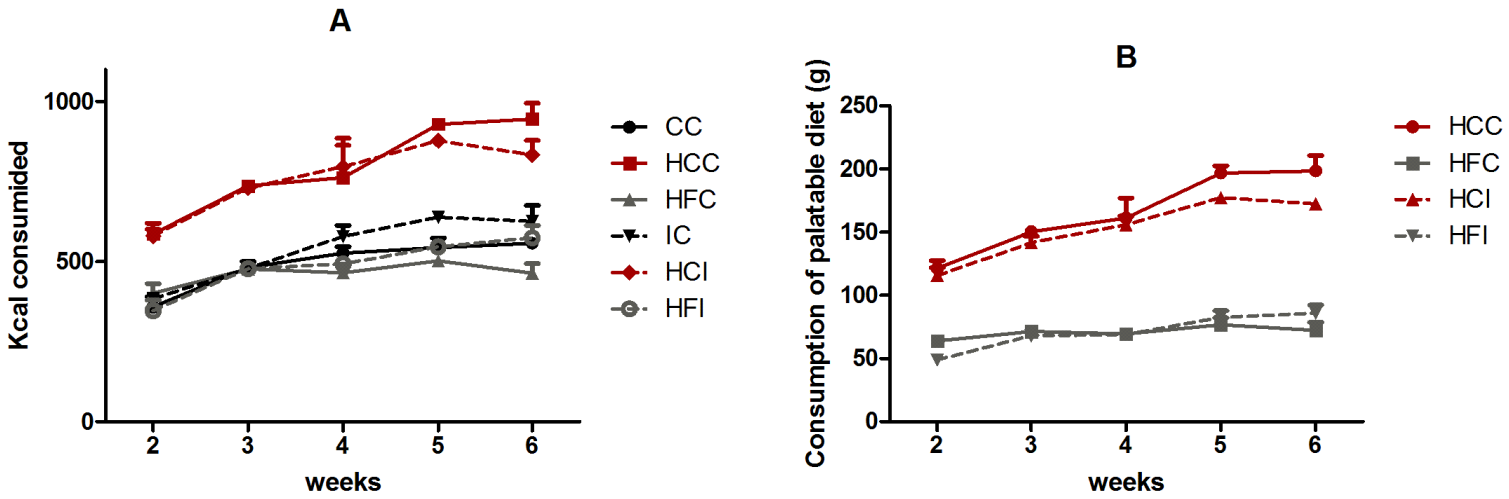
# Figure 3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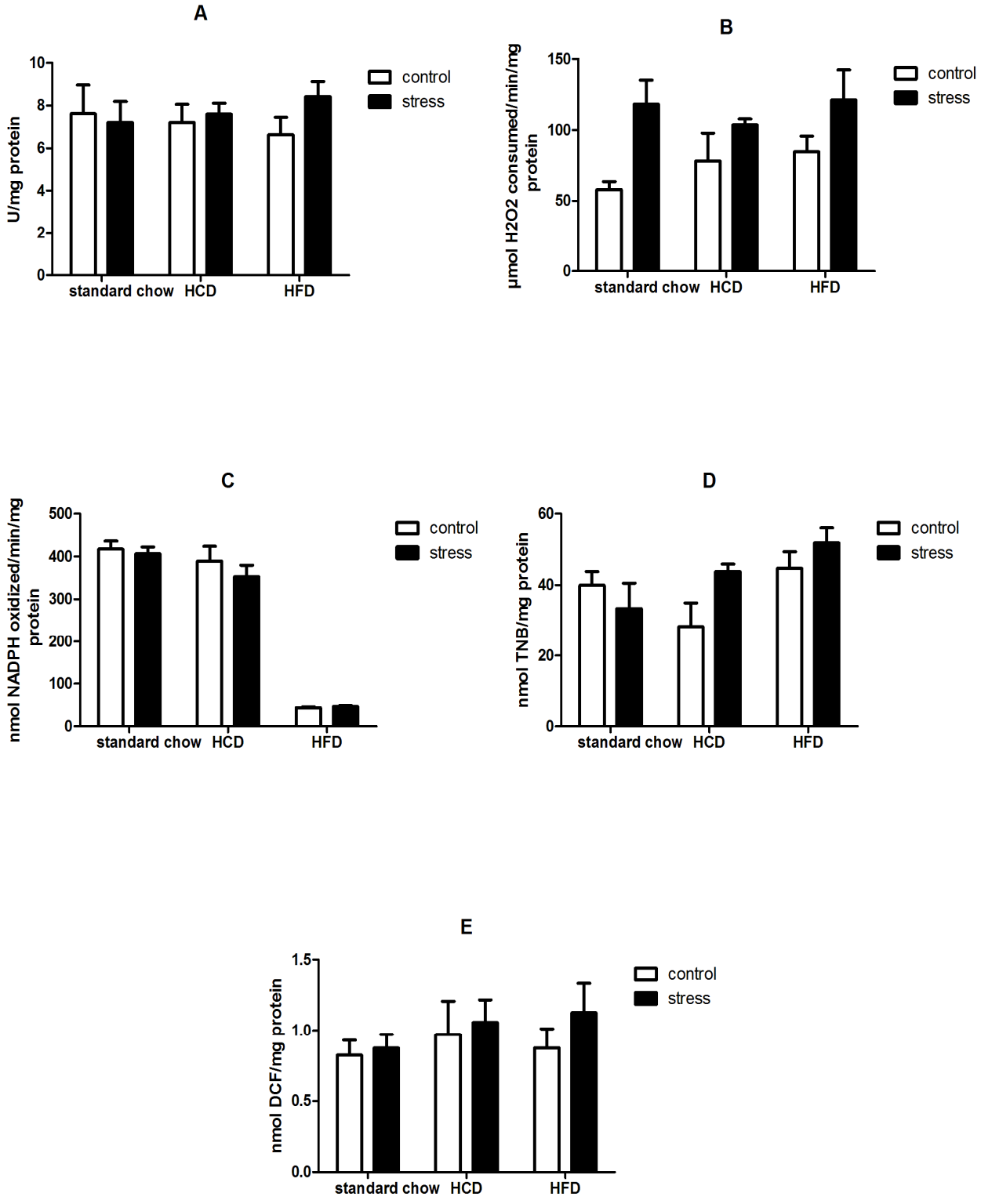
# Figure 4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## 3.2 Capítulo 2

**Stress in the pre-pubertal period leads to long-term behavioral and biochemical alterations and are influenced by palatable diets in male adult rats**

Artigo a ser submetido para publicação na revista *Neurochemical Research*.

# STRESS IN THE PRE-PUBERTAL PERIOD LEADS TO LONG-TERM BEHAVIORAL AND BIOCHEMICAL ALTERATIONS AND ARE INFLUENCED BY PALATABLE DIETS IN MALE ADULT RATS

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**Abstract**

Social isolation during early development is one of the most potent stressors, and can cause changes in brain maturation processes, leading to behavioral and neurochemical changes that may persist into adulthood. In addition, exposure to palatable diets during development can cause changes in neural circuits with long-term repercussions. Since exposure to stress in the adult may induce anxious-like behavior and may increase oxidative stress, the aim of the present study was to investigate the long-term effects of isolation stress during the pre-pubertal period on exploratory and anxiety-like behavior, and on oxidative stress parameters and respiratory chain enzymes activities in the hippocampus of adult male rats. Additionally, since diets may affect the stress response, we also investigate the interactions of stress during this period with the chronic access to palatable diets, rich in sugar or fat. The results showed that isolation in the pre-pubertal period receiving or not high fat diet had an anxiolytic-like effect. The animals that were stressed and received high-carbohydrate diet, on the other hand, showed the opposite effects. Stress in the pre-pubertal period also leads a decreased activity of antioxidant enzymes, of complexes II and IV of the mitochondrial respiratory chain and decreased total thiol content. In turn, the high-fat diet when associated with stress reversed these effects. Taken together, these results show that the pre-pubertal period constitutes a critical window for stressful interventions during development, which lead to lower anxiety-like behavior and higher hippocampal oxidative stress susceptibility during adulthood, and these effects differ depending on the type of diet used.

*Key-words*: isolation stress; palatable diet; hippocampus; oxidative stress; anxiety; respiratory chain.

## 1. Introduction

The constant supply of palatable foods, rich in sugar and fat, associated with an increasingly sedentary lifestyle has led to obesity in a large parcel of the human population. In addition, it has been suggested that environmental factors, such as exposure to stress, are also related to obesity [1].

The stress response can potentially disrupt body homeostasis, known to cause changes in physiological and neurochemical factors in the organism [2]. During the development stage, when the plastic capacity is high, these changes become more important. Furthermore, the pre-pubertal period is known to be critical for the final maturation of the neural circuits controlling energy homeostasis and the stress responses [3] and exposure to stressors in this phase may influence neuroendocrine and behavioral responses in adulthood [4]. During this period, exposure to social isolation, considered a type of psychological stress in rats [5-6], can lead to behavioral, anatomical and neurochemical changes that may remain during adulthood, when these animals are compared to their socially-reared litter mates [6-7].

The stress response involves the neuroendocrine activation of sympatho-adrenomedullar system, release of catecholamines, and activation of hypothalamic–pituitary–adrenal (HPA) axis, culminating in the release of glucocorticoids (GCs) [8]. Glucocorticoid receptors (GR) mediate the negative feedback of GCs on the HPA axis following stress. In brain, these receptors are localized in some structures, including hippocampus, prefrontal cortex and hypothalamus, but the hippocampus has more GRs, thus it is the most stress-sensitive brain region [9-10]. Glucocorticoids have been associated with increased palatable food intake [11]. Increased stress has been associated with increased preference for consumption of high-caloric foods [12-14] and

a model of reward-based eating has been suggested as a mean to reduce the stress response [15]. Moreover, the type of palatable food consumed will regulate the HPA axis differentially: Diets high in calories and sugar reduce this axis response to stress [16], while diets rich in fat enhance stress-induced levels of glucocorticoids [17-18]. Additionally, stress may induce changes in emotional behavior, which are related to anxiety-like behavior [19]. Further, stress promotes consumption of palatable diets, rich in sugar and fat, supporting the hypothesis that palatable food may be used as compensation during periods of stress ("comfort foods") [20-21].

Moreover, stress exposure and elevated GCs levels may lead to an increased the generation of reactive oxygen species (ROS) [22-25]. The imbalance between high cellular levels of ROS in relation to cellular antioxidant defenses, namely oxidative stress [26], can lead to damage to cell structures like proteins, lipids, membranes and DNA [27-28], and may be involved in the pathogenesis of several brain diseases [29,25, 30-31]. Also, the adaptive response to stress involves important changes in mitochondrial functions, enabling them to adjust bioenergetics, thermogenesis, oxidative and/or apoptotic responses [32]. ROS can induce mitochondrial dysfunction, disruption of energy pathways [33], damage to neuronal precursors and impairments in neurogenesis [34]. In addition, some studies have demonstrated the association between the presence of ROS and excessive intake of fatty foods causing breaks in cellular DNA [35-39]. Thus, the consequences of stress exposure in animals with *ad libitum* access to palatable diets needed to be better understood.

Since the pre-pubertal period is critical for development, being important to the stress response and neurobehavioral changes, the aim of our study is to verify whether stress by social isolation during the pre-puberty period in animals with chronic access to palatable diets until adulthood may lead to cellular alterations in the hippocampus and

anxiety-like behavior. We used elevated plus maze as a test for anxiety-like behavior and evaluated oxidative stress parameters and respiratory chain enzymes activities in adulthood.

## 2. Material and Methods

### *2. 1. Experimental subjects*

All animal proceedings were performed in strict accordance to the recommendations of the Brazilian Society for Neurosciences (SBNeC) and Brazilian Law on the use of animals (Federal Law 11.794/2008), and were approved by the Institutional Ethical Committee. All efforts were made to minimize animal suffering, as well as to reduce the number of animals used.

Animals were housed in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust, and were maintained on a standard 12h dark/light cycle (lights on between 7:00h and 19:00h), temperature of 22 ± 2°C. On postnatal day (PND) 21, sixty-three Wistar rats were weaned. Only male pups were used from each litter, and these pups were divided into groups, in such a way that only one animal per litter was used in each group. Male pups were weighed at PND 21 and distributed into 3 groups, according to the diet that they received: (1) receiving standard lab chow (50% carbohydrate, 22% protein and 4% fat); (2) receiving both chow and a diet with a high content of simple carbohydrate [40] and (3) receiving chow and a high-fat diet (25% carbohydrate, 28% protein and 42% fat). Therefore, animals from these last two groups could choose the diet they consumed from the two diets available. Half of the animals on each diet were housed in groups of 4 per cage; the other half were stressed by isolation (one animal in a smaller home cage, 27x17x12 cm) [41], in such a way that six

groups were obtained; controls receiving chow (CC), controls receiving chow and high-carbohydrate diet (HCC), controls receiving chow and high-fat diet (HFC), isolated animals receiving chow (IC), isolated animals receiving chow and high-carbohydrate diet (HCI), and isolated animals receiving chow and high-fat diet (HFI). The isolation stress occurred between postnatal days 21-28. On PND 28, isolated animals were returned to regular home cages (65 x 25 x 15 cm) in groups of four. During 40 days, beginning on PND 21, amounts of palatable diets and standard lab chow were offered *ad libitum.* At postnatal day 60, two behavioral tests were performed consecutively: Plus Maze and Open Field. After one week, the animals were killed by decapitation and the brain was removed, dissected (hippocampus) and frozen at -80°C for further biochemical evaluations.

*2.2 Diets*

The nutritional compositions of each diet used are displayed in Table 1. The high-carbohydrate diet (HCD) used in this study was enriched in simple carbohydrates, and made with condensed milk, sucrose, vitamins and a salt mix, powder standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet is similar to that of a standard lab chow (including 22% protein and 4-6% fat), however most carbohydrates in the palatable diet were sucrose [40]; in contrast, the standard lab chow contained carbohydrates obtained mainly from starch.

The high fat diet (HFD) used in the study was enriched with fat (42%) from lard and soy oil. In addition, this diet contained vitamins and a salt mixture, purified soy protein, methionine, lysine and starch [adapted from 42].

*2.3 Behavioral tests*

Before each behavioral task, rats were placed in the test room (temperature 21±2°C) for one hour to allow habituation with the environment and researcher. All tasks were performed between 1:00 and 6:00 p.m. The behavior was recorded and analyzed using the ANY-Maze video-tracking system (Stoelting, CO). Between each trial, apparatuses were cleaned with ethanol 70%.

*2.3.1 Elevated plus-maze test* (EPM)

The elevated plus maze test was conducted after 60 days of treatment, using a standard plus maze apparatus kept 50 cm above the floor, consisting of four arms arranged in the shape of a cross (arms measured 50 x 15 cm). The four arms were joined at the center by a 10 cm square platform. Two of the arms, opposite to each other, had no walls (open arms); the two other arms (closed arms) had 49 cm high walls. The experiment was conducted in a room illuminated by red light. The light intensity at the center of the apparatus was 30 lx. This test is considered sensitive to the anxiety state of the animal, based on the principle that exposure to an elevated and open arm leads to an approach conflict that is stronger than that evoked by exposure to an enclosed arm maze [43]. Animals were placed individually on the center of the maze, on the junction between open and closed arms, facing one of the open arms, and performance was scored during 5 min. A rat was considered to have entered one arm of the maze when all four feet were within the arm. Conventional parameters of anxiety-like behavior were monitored, i.e., % entries into open arms (calculated entries in the open arms divided by the sum of the entries in open and closed arms multiplied by 100), % time in open arms (calculated time in the open arms divided by total time in the arms multiplied by 100) and number and time of head dips.

*2.3.2 Exposure to the Open Field*

Exposure to the open field was used to evaluate motor activity and habituation to a new environment. A 50-cm high, 50×50-cm open field was used. The floor was subdivided with white lines into 16 equal 12.5- by 12.5-cm squares, and the animals were gently placed facing the left corner and allowed to explore the arena for 5 min. The performance was observed and evaluated: distance traveled (m) and number of rearings. Animals were subjected to two sessions of this task, with an interval of 24h between sessions.

*2.4 Assessment of oxidative stress parameters*

The animals were killed by decapitation. Their hippocampus was quickly dissected out and stored at $-70^{o}$ C until analysis, when the structures were homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged at 1000 x g for 10 min at $4^{o}$ C and the supernatant was used.

*2.4.1 Superoxide Dismutase Activity*

Superoxide dismutase activity was determined using a RANSOD kit (Randox Labs., USA), which is based on the procedure described by Delmas-Beauvieux, et al. [44]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed spectrophotometrically at 492 nm at $37^{o}$ C. The inhibition of the production of the chromogen is proportional to the activity of SOD present in the sample; one unit of SOD causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

*2.4.2 Glutathione Peroxidase Activity*

Glutathione peroxidase activity was determined according to Wendel [45], with modifications. The reaction was carried out at 37$^{o}$C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione, and 0.4 U glutathione reductase. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

*2.4.3 Catalase Activity*

Catalase activity assessment is based upon the spectrophotometric establishment of the rate of $H_2O_2$ degradation at 240 nm at 25$^{o}$C [46]. CAT activity was calculated in micromoles of $H_2O_2$ consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 $M^{-1}$ $cm^{-1}$.

*2.4.4 Evaluation of free radical production by the chemical oxidation of dichlorodihydrofluorescein (DCFH)*

The samples were incubated with 2',7'-dichlorodihydrofluorescein diacetate (100 μM) at 37°C for 30 minutes. DCFH is released by cellular esterases and oxidized by reactive oxygen/nitrogen species. The formation of the fluorescent derivative dichlorofluorescein (DCF) was monitored by excitation and emission wavelengths of 488 and 525nm, respectively, using a spectrum photometer. The formation of oxidized reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmol of DCF formed per mg of protein [47].

*2.4.5 Determination of total thiol content*

This assay is based on the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by thiol groups, which become oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm [48].

*2.5 Respiratory chain activity determination*

Brain structures were homogenized with a teflon–glass homogenizer (1:20, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base), pH 7.4, for determination of respiratory chain complexes activities. The homogenates were centrifuged at 1000g for 10 min at $4^{o}$ C and the supernatants were immediately kept at -70°C until used for enzyme activity determination. Mitochondrial energy metabolism was evaluated using enzymatic analysis of the electron transport chain (ETC) activities. The activities of the ETC complexes I–III, II and IV were determined in homogenates according to standard methods previously described in the literature [49-51]. The activity of complex I–III (complex I + CoQ + III) was assessed by measuring the increase in absorbance due to cytochrome c reduction at 550 nm according to the method described by Schapira, Mann et al. [51]. The reaction mixture contained 5 to 10 µg protein and 20 mM potassium phosphate buffer with 2 mM KCN, 10 mM EDTA and 50 mM cytochrome c, pH 8.0. The reaction was initiated adding 25 mM NADH and was monitored at 25ºC for 3 min before addition of 10 mM rotenone, after which the activity was measured for an additional 3 min. Complex I–III activity was the rotenone sensitive NADH: cytochrome c reductase activity. The activity of complex II (succinate: DCIP oxiredutase) was determined according to Fischer et al [49], following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm. After a pre-incubation with 30 to 60 µg protein for 20 min, the reaction was carried out at 30ºC in a

medium consisting of 40 mM potassium phosphate buffer containing 16.0 mM sodium succinate and 8 mM DCIP, pH 7.4. After that, 4 mM sodium azide, 7 mM rotenone and 40 mM DCIP were added to the medium and monitored for 5 min. Cytochrome c oxidase (COX, complex IV) activity was determined according to Rustin et al [50], following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm. The reaction was initiated adding 0.175mg reduced cytochrome c in a medium containing 10 mM potassium phosphate buffer, 0.6 mM n-dodecyl-β-D-maltoside, pH 7.0 and 1.5 to 3 µg protein. The activity of complex IV was measured at 25ºC for 10 min. The activity of respiratory chain complexes were calculated and expressed as nmol per min per mg protein.

*2.6 Protein Assay*

The protein concentration was determined in the samples using the method described by Lowry et al. [52], with bovine serum albumin as the standard.

*2.7 Statistical Analysis*

Data are expressed as mean ± SE of the mean, and analyzed using two-way ANOVA, with isolation stress and diet as factors. ANOVA tests were followed by the Tukey multiple range test, when indicated. For comparison between the two sessions of the open field task, a repeated measures ANOVA was used. All analyses were performed using SPSS software and a $P \leq 0.05$ was considered significant.

**3. Results**

*3.1 Elevated plus-maze test* (EPM)

Results from elevated plus maze are shown in *Fig. 1*. No difference was found in the number of entries in open and closed arms (two-way ANOVA, $P>0.05$, data not shown). In relation to percentage of entries in open arms, there was no difference between the groups ($P>0.05$, *Fig. 1a*). An interaction between stress and diet was found in percentage of time in open arms [$F(2,53)=4.15$, $P<0.05$]: isolation stress increased % of time in open arms in groups receiving standard chow and HFD but decreased in HCD (*Fig. 1b*). The animals receiving the HFD increased the number [$F(2,55)=4.71$, $P=0.01$, followed by Tukey post-hoc] and time of head dips (HD) [$F(2,55)=4.45$, $P<0.05$, followed by Tukey post-hoc] compared to HCD. Moreover, stress decreased both the number [$F(2,55)=3.17$, $P<0.05$] and time of HD [$F(2,55)=4.99$, $P=0.01$] in the group receiving the HCD, but increased in others groups (*Fig. 1c and 1d*).

*3.2 Open Field*

Results from the open field are shown in *Table 2*. When comparing rearings in the two sessions of the open field tasks between groups, there was a difference between sessions in all groups [$F(1, 56)=42.48$, $P < 0.01$], since all groups showed a reduction in the number of rearings in the second session. There were no interactions and no other effects (from stress or diet factors). The same results were observed for distance traveled [$F(1, 56)=30.75$, $P < 0.01$], since all groups showed a lower distance traveled in the second session, and no interactions and no other effects (from stress or diet factors) were observed.

*3.3 Antioxidant enzyme activities, total thiol content and free radical production*

Oxidative stress parameters were analyzed to verify whether there was an oxidative imbalance in the hippocampus after exposure to isolation stress during the

pre-pubertal period, when palatable diets were chronically offered (*Fig. 2*). When evaluating SOD activity, an interaction between stress and diet was shown [two-way ANOVA, $F(2,21)=6.23$, $P<0.01$]: isolation stress led to increased SOD activity in the group receiving the HFD while decreasing this activity in the other groups (*Fig. 2a*). There was also a marginally significant main effect of stress [$F(1,21)=3.86$, $P=0.06$]. Similar results occurred in relation to catalase activity, where interaction between stress and diet was found [$F(2,21)=12.52$, $P<0.001$]: isolation stress increased CAT activity in group receiving the HFD, and decreased this activity in the other groups (*Fig. 2b*). Exposure to stress decreased GPx activity [$F(1,21)=6.89$, $P<0.05$] and there was an effect of diet [$F(2,21)=6.03$, $P<0.01$; no significant difference was observed in post-hoc test] (*Fig. 2c*). For total thiol content, an interaction between stress and diet was observed [$F(2,27)=6.28$, $P<0.01$]: stress increased total thiol content in the group receiving HFD, decreasing this content in the other groups (*Fig. 2d*). No significant difference was found in relation to free radicals production, as evaluated by the DCFH test ($P>0.05$; *Fig. 2e*).

### *3.4 Respiratory chain activity determination*

Mitochondrial energy metabolism in the hippocampus was evaluated using enzymatic analysis of electron transport chain (ETC) activities (*Fig. 3*). The group receiving the HFD had decreased complex I + III activity compared to standard chow group [two-way ANOVA, $F(2,22)= 3.75$, $P<0.05$, followed by Tukey post-hoc] (*Fig. 3a*). Isolation stress decreased complex II activity [$F(1,22)=8.54$, $P<0.01$]; however, in the group receiving the HFD, stress increased complex II activity [$F(2,22)=5.19$, $P=0.01$] (*Fig. 3b*). Similar results occurs for complex IV activity, when stress decreased activity [$F(1,23)=10.10$, $P<0.01$], but this effect did not occur in animals stressed

receiving HFD, when isolation stress cause an increase [F(2,23)=5.56, P=0.01] (*Fig. 3c*).

## 4. Discussion

The major findings in the present study demonstrated that stress by isolation early in life, in adult male rats, induced decreased activity of antioxidant enzymes, total thiol content, and respiratory chain complexes II and IV activities. Moreover these animals showed an anxiolytic effect. On the other hand, access to different diets altered long-term effects of isolation stress in the pre-pubertal period. Stress associated with high fat diet showed opposite effects on the biochemical parameters evaluated: there was increased SOD and CAT activities, total thiol content, and complexes II and IV activities. Regarding behavior evaluation, however, the animals that received a high carbohydrate diet showed anxiogenic effects in the elevated plus maze task.

Long-term behavioral changes were observed in the animals subjected to isolation stress during the prepubertal period and these changes were dependent on the diet the animals had. The stressed animals with access to standard chow and high-fat diet remained higher % time in open arms on the EPM, indicating an anxiolytic-like effect, since the elevated plus maze is based on the natural aversion of rodents for open spaces [53-54]. Furthermore, these animals showed increased number and duration of head-dips compared to group stressed receiving high carbohydrate diet. Head-dips are the frequency with which the animal lowered its head over the sides of the open arm toward the floor and are considered "risk assessment" behavior. These results indicate that these rats had reduced reluctance to leave relatively safe areas of the maze and increased exploration in the potentially dangerous open arms, a behavioral pattern that

strengthens the conclusion of an anxiolytic-like action. In the open field, used to assess locomotion capacity, there was no difference between the groups. All groups of animals showed a reduction in the exploratory behavior from the first to the second section, indicating habituation to the new environment and adequate memory for this task [55]. A previous study from our laboratory [56], assessing social isolation in the pre-pubertal period in young rats (28 days of age), verified that stress had an anxiogenic-like effect in these animals when evaluated before puberty. This effect, however, does not remains into adulthood, according to the present results. These behavioral responses in adulthood can be compared with other early interventions, such as neonatal handling, which also leads to anxiolytic-like behavior in adulthood [57-59]. Therefore, interventions in distinct periods early in life may induce altered anxiety in adulthood. Some authors have suggested that the disruption of social development induced by isolation can impair social fear learning [60-61]; thus, the anxiolytic effect observed here can be due to a decreased fear when exposed to the new environment, when animals isolated during the pre-pubertal period are compared to animals not subjected to isolation. Anyway, memory of habituation to the open field was adequate in all groups, suggesting that if fear learning is compromised, other types of memory do not appear to be so. It must be considered that some other studies show distinct results, having observed an increase in anxiety-like behavior in socially isolated animals [62-64], and higher locomotor activity on exposure to a novel environment [65-66, 62]. This difference when compared to our results may be due to the fact that these other studies used more prolonged time of social isolation, when the animals were subjected to isolation from the pre-pubertal period until adulthood, while in our study a short period of isolation was used, just in the pre-pubertal period.

The stressed animals that received the high-carbohydrate diet exhibited a different behavior, compared to the animals receiving other diets. These animals had lower % time in open arms and less time and number of head-dips in EPM test. Thus, the association of stress and HCD appears to reverse stress the long-term effect of isolation stress during the pre-pubertal period on anxiety-like behavior. It would be expected that a diet high in sugar can act as a "comfort food" [16], reducing the stress response; this could be the reason why this diet was able to reverse stress effects. It should be pointed out, however, that in adult rats stressed by restraint, a diet rich in simple sugars was able to reverse stress-induced anxious-like behavior [37].

Some studies also show a correlation between oxidative stress and anxiety-like behavior [67-69]. However, in this study, the results do not corroborate this relationship. It is known that the brain is especially vulnerable to free radicals-induced damage because of its high oxygen consumption, abundant lipid content and a relative paucity of antioxidant enzymes [70-72]. Several studies have reported that stress results in the imbalance of the antioxidant status, which at long term, leads to increased oxidative stress [73-75]. From these considerations, we evaluated oxidative stress parameters, such as antioxidant enzymes activities (SOD, GPx and CAT), free radical production (DCFH oxidation test) and total thiol content. The data showed that stress by isolation during the pre-pubertal period decreased the activities of antioxidants enzymes and total thiol content in the adulthood. In addition, it decreased the activity of respiratory chain complexes II and IV. A previous study from our laboratory showed that soon after exposure to stress (at 28 days of age) [76] there was a decrease in the activity of SOD, but no change in the activities of others antioxidant enzymes, as well as an increased free radicals production and DNA damage in hippocampus. The present study shows that the effects on free radicals production are stabilized over time, but the

antioxidant enzymes activities are decreased, as well as total thiol content, suggesting that the hippocampus of these animals remains susceptible to oxidative imbalance during the adult life. In relation to the decreased content of total thiol in these animals, this could represent damage to proteins, or this reduction may reflect a lower content of glutathione, which is a substrate for the antioxidant enzyme glutathione peroxidase, and this fact could be related to decrease GPx found in these isolates animals. It is important to consider that the decrease found in complex II and IV activities in adult isolated rats may facilitate the escape of electrons through these complexes. As the activity of antioxidant enzymes in these animals are decreased, they would be more susceptible to oxidative damage in situations that could lead to increased free radicals production.

Interestingly, when stress was associated with a high-fat diet, the observed effects were opposite; there was increased activity of the antioxidant enzymes: SOD and CAT, increased content of total thiol and also an increase in the activity of respiratory chain complexes II and IV. These results are intriguing, since studies show that a high-fat diet seems to exacerbate stress responses by increasing the activity of the HPA axis [77,18]. Therefore, in this case, if isolation stress was associated with a high-fat diet, it was expected an exacerbation of the effects of stress. However, another study showed that the high-fat diet decreased HPA axis activity [78]. This finding would be consistent with our results, since the HFD appeared to reverse the effects of stress. Another possibility for explaining this finding could be related to a decrease in the glucocorticoid receptors (GR) in the hippocampus. The hippocampus is a brain structure sensitive to the stress response, since it has higher density of glucocorticoid receptors [9-10]. Some studies found that exposure to early-life stress during development was associated with reductions in hippocampal GR and these changes were normalized with

access to HFD [79-80]. Thus, the high-fat diet would improve responses to stress in the hippocampus; this possibility deserves further studies.

In conclusion, our findings showed that isolation stress during the pre-pubertal period leads long-lasting behavioral alterations (reducing anxious behavior), and neurochemical changes in the hippocampus of male rats, causing decreased antioxidant enzymes, decreased total thiol content and decreased activity of respiratory chain complexes, making these animals more susceptible to oxidative insults. On the other hand, access to different diets may alter the outcomes induced by this early experience, since the high simple carbohydrate diet when associated with stress led to opposite effects on behavior, and the high-fat diet reversed stress effects on the neurochemical parameters evaluated, possibly by acting in the HPA axis activity or its inhibition. The mechanisms, by which these changes occur, however, need to be further studied. This study also points to the importance of environmental factors during different stages of development when studying behavioral and neurochemical disturbances. Understanding how interventions during the development and the type of diet used may affect brain and behavior, will help to elucidate the pathophysiological mechanisms related with these alterations.

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**Table 1**. Nutritional composition/100 g of the food used in the studies performed. HCD: high carbohydrate diet; HFD: high-fat diet.

| Food | Energy (kcal) | Total protein (g) | Total carbohydrate (g) | Total fat (g) |
|---|---|---|---|---|
| Standard Chow[a] | 324 | 22 | 55 | 4.5 |
| HCD[b] | 450 | 25 | 65 | 10 |
| HFD[c] | 588 | 28 | 25 | 42 |

**a** Nuvilab®

**b** Souza CG, et. al. Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like beharior. Life Sci, 2007, 81:198-203.

**c** Adapted Ziegler DR, et. al. A ketogenic diet increases protein phosphorylation in brain slices of rats. J Nutr 2002, 132:483-487.

**Table 2**. Effect of isolation stress during the prepubertal period and chronic access to palatable diets on behavior in the Open field of adult rats.

| | Control | | | Stress | | |
|---|---|---|---|---|---|---|
| | Standard Chow | HCD | HFD | Standard Chow | HCD | HFD |
| *Session 1* | | | | | | |
| Distance traveled | 15.37+5.86 | 16.50+5.87 | 16.14+4.98 | 13.83+6.65 | 15.01+5.16 | 15.59+3.39 |
| Rearings | 33.73+10.60 | 35.10+10.26 | 35.58+6.56 | 29.00+12.53 | 34.60+9.25 | 34.50+11.82 |
| *Session 2* | | | | | | |
| Distance traveled | 33.73+10.60 | 35.10+10.26 | 35.58+6.56 | 29.00+12.53 | 34.60+9.25 | 34.50+11.82 |
| Rearings | 21.40+12.78 | 20.80+14.68 | 21.67+13.64 | 20.20+14.01 | 25.20+10.46 | 27.00+14.73 |

Data are expressed as mean ± S.E.M. for each parameter. N=10–12/group. Repeated measures ANOVA showed a difference between sessions in all groups for rearings ($P < 0.01$) and distance traveled ($P < 0.01$).

**Legends to Figures**

**Fig. 1** Effect of isolation stress during the pre-pubertal period with chronic access to palatable diets on behavior in the Plus Maze of adult rats. Data are expressed as means±S.E.M. N= 8-12/group. **a** % entries into open arms, **b** % time in open arms, **c** number of head dips, **d** time of head dips. Two-way ANOVA showed an interaction between stress and diet on % time in open arms ($P<0.05$), number ($P<0.05$) and time ($P=0.01$) of head dips and also effect of diet on number ($P=0.01$) and time ($P<0.05$) of head dips. HCD: high-carbohydrate diet; HFD: high-fat diet.

* Significantly different from HCD (Tukey post-hoc, $P<0.05$)

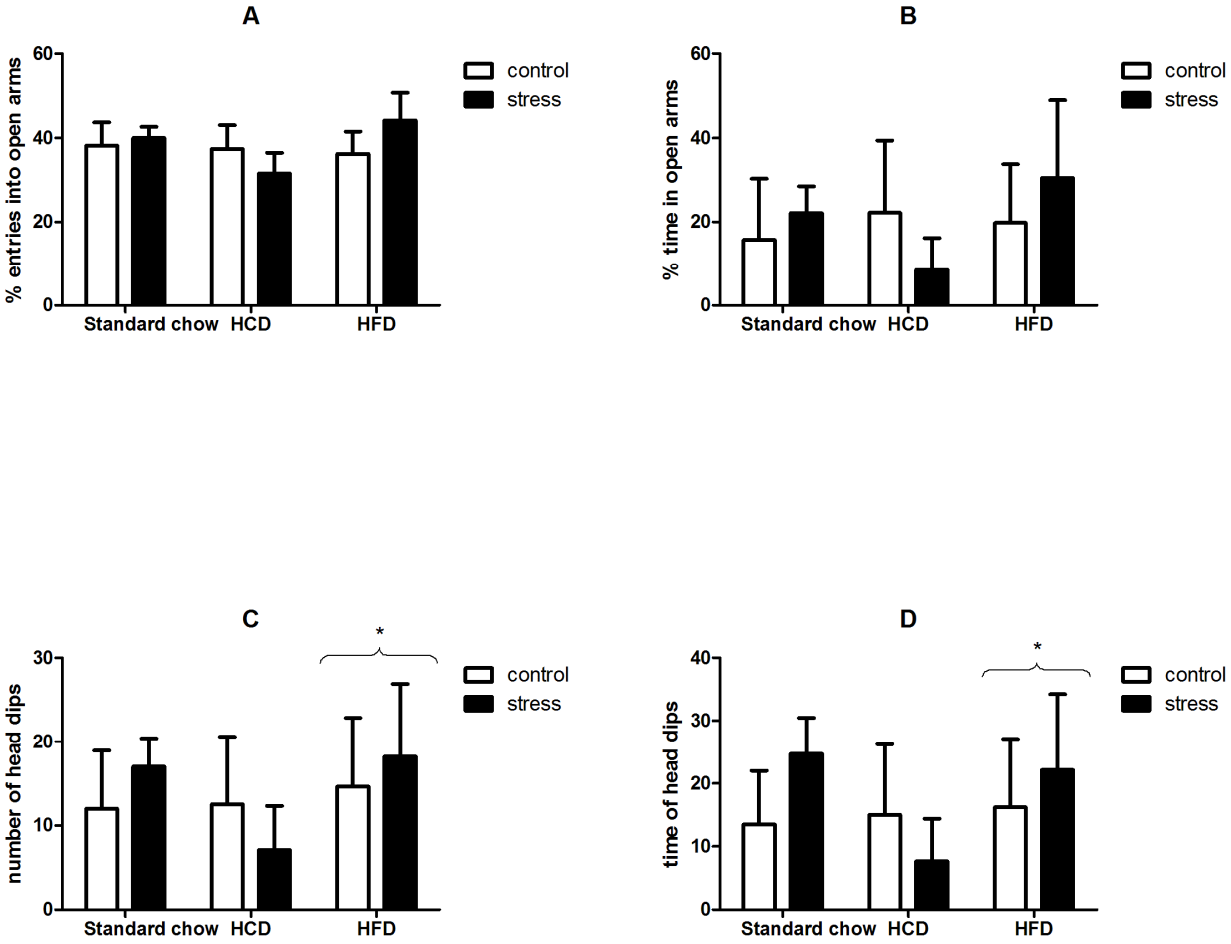
**Fig. 2** Effect of isolation stress during the pre-pubertal period with chronic access to palatable diets on antioxidant enzyme activities, total thiol and free radicals (DCFH test) production in hippocampus of adult rats. Data are expressed as mean $\pm$SEM. N= 5-7/group. **a** SOD (expressed as U/mg protein), **b** CAT (expressed as micromoles of $H_2O_2$ consumed/min/mg protein), **c** GPx (expressed as nmol NADPH oxidized/min/mg protein), **d** total Thiol (expressed as nmol TNB/mg protein) and **e** DCFH (expressed as nmol of DCF formed/mg protein). Two-way ANOVA showed an interaction between stress and diet on SOD ($P<0.01$), CAT ($P<0.001$) activities, and on total thiol ($P<0.01$); effect of stress ($P<0.05$) and effect of diet ($P<0.01$) on GPx activity; and no effect on DCFH ($P>0.05$). . HCD: high-carbohydrate diet; HFD: high-fat diet.

**Fig. 3** Effect of isolation stress during the pre-pubertal period with chronic access to palatable diets on respiratory chain enzymes activities in hippocampus of adult rats. Data are expressed as mean ± SEM N = 4–5/group. **a** complex I–III activity (nmol

cytocromo c reduced/min/mg protein), **b** complex II activity (nmol DCIP reduced/min/mg protein) and **c** Complex IV activity (nmol cytocromo c oxidized/min/mg protein). Two-way ANOVA showed an effect of diet on complex I-III activity ($P<0.05$); effect of stress on complex IV ($P<0.01$) and complex II ($P<0.01$); an interaction between stress and diet on complex IV ($P=0.01$) and complex II ($P=0.01$). HCD: high-carbohydrate diet; HFD: high-fat diet.

* Significantly different from standard chow (Tukey post-hoc, $P<0.05$)

# Fig. 1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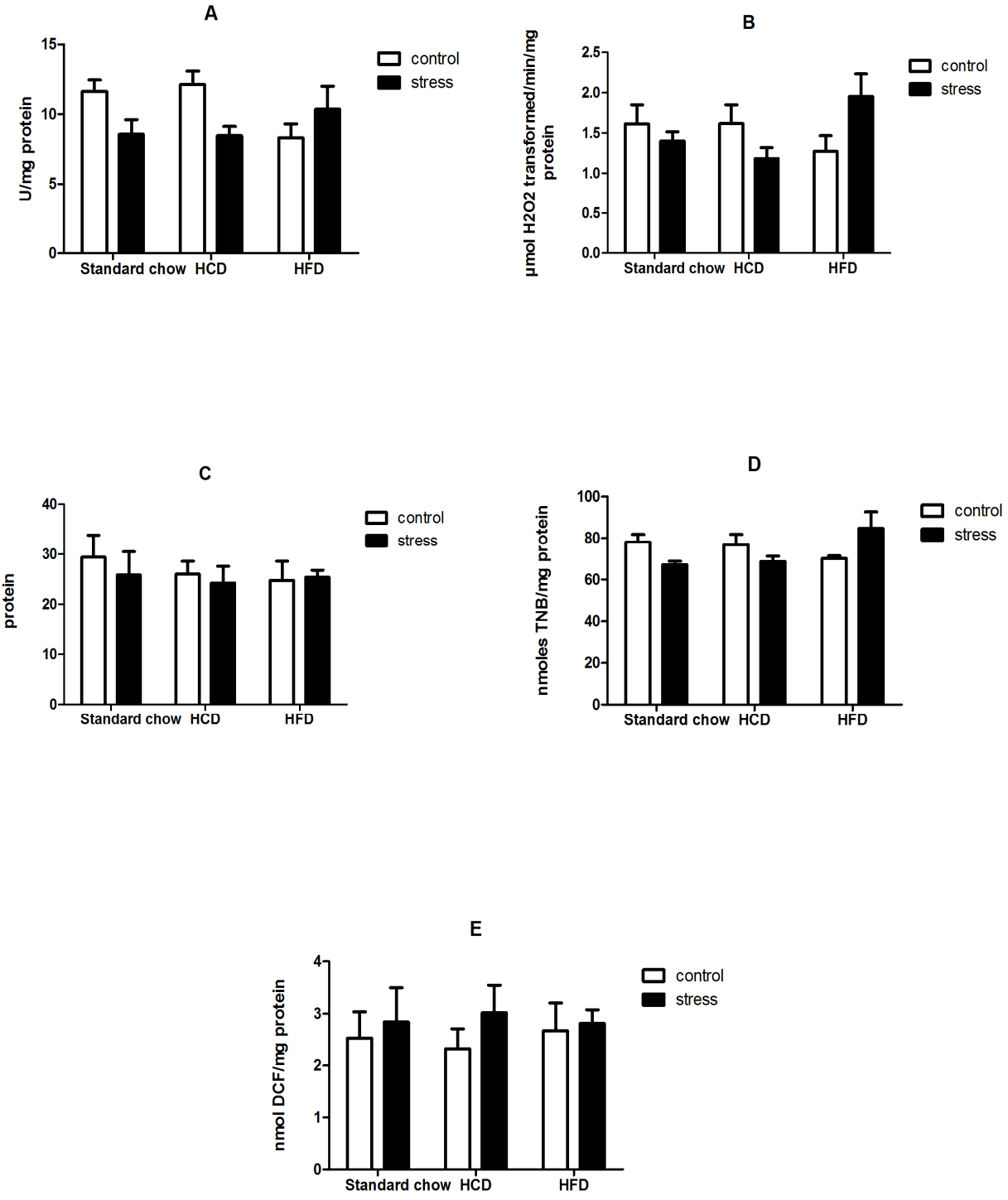
# Fig. 2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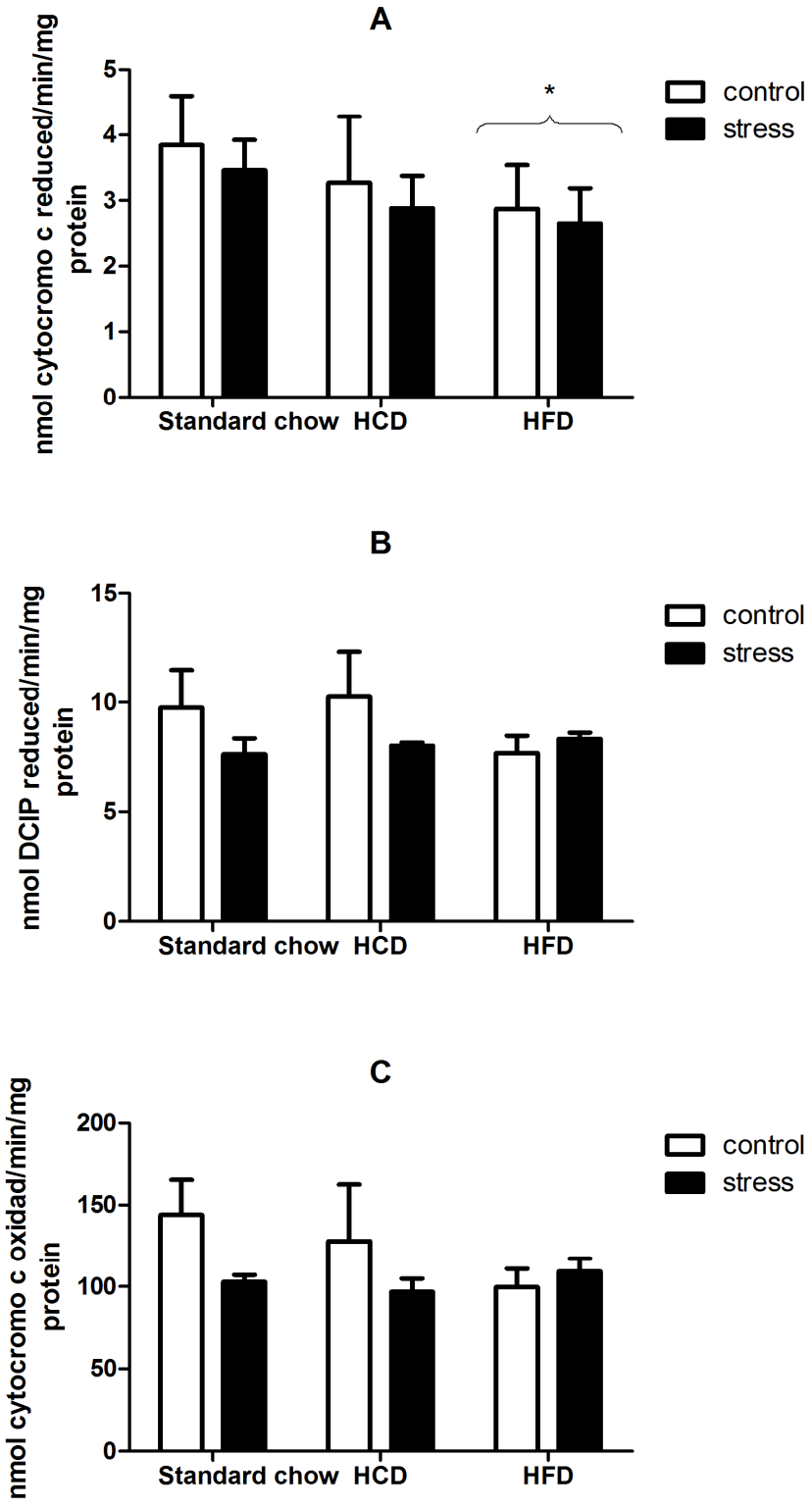
# Fig. 3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# 4. DISCUSSÃO

No presente trabalho, observamos que os animais que receberam uma dieta rica em carboidratos apresentaram maior ganho de peso e consumo calórico comparado aos outros grupos, sendo esse aumento devido a um maior consumo de dieta palatável e não de ração padrão, já que eles tinham a opção de escolha na caixa moradia. Por outro lado, o grupo que recebeu a dieta hiperlipídica apresentou maior acúmulo de gordura abdominal, sendo que os animais que receberam essa dieta e foram estressados apresentaram uma potenciação desse efeito. Além disso, o estresse por isolamento induziu um comportamento do tipo ansiolítico. Entretanto, o grupo estressado que teve acesso à dieta rica em carboidratos mostrou comportamento do tipo ansiogênico no Labirinto em Cruz Elevado.

Analisando os efeitos metabólicos, a associação do estresse com a dieta hiperlipídica aumentou os níveis de LDL-colesterol, enquanto que a exposição ao estresse diminuiu esse parâmetro nos demais grupos. Além disso, os animais que receberam a dieta rica em gordura apresentaram aumento da glicemia e da atividade da colinesterase plasmática. O acesso às duas dietas hiperpalatáveis foi associado com um aumento nos níveis de leptina, enquanto que o isolamento social mostrou uma diminuição dos níveis desse hormônio. Em relação aos resultados relacionados ao desequilíbrio oxidativo, observou-se que, no fígado, houve um aumento de produção de espécies reativas com o isolamento social, especialmente nos grupos que receberam as dietas hiperpalatáveis. Além disso, o estresse aumentou a atividade da SOD no grupo que recebeu dieta hiperlipídica e aumentou a atividade da CAT em todos os grupos. A atividade da GPx diminuiu com as dietas hiperpalatáveis, principalmente no grupo com acesso a dieta hiperlipídica. Observamos também parâmetros de estresse oxidativo em uma estrutura cerebral, o hipocampo, que é uma estrutura bastante relacionada com as respostas ao estresse. Nessa estrutura, o isolamento social apresentou diminuição da

atividade das enzimas antioxidantes, do conteúdo de tióis totais e da atividade dos complexos II e IV da cadeia respiratória. É interessante observar que a associação do estresse com a dieta rica em gordura foi capaz de reverter os efeitos observados do estresse nesta estrutura, aumentando a atividade das enzimas antioxidantes CAT e SOD, o conteúdo de tióis totais e a atividade dos complexos II e IV.

Conforme descrito no capítulo I, os animais com acesso a dieta hiperlipídica na semana do isolamento social (21-28 dias de idade) ganharam menos peso comparado aos outros grupos. O consumo calórico, porém, foi similar aos controles, sugerindo uma baixa eficiência calórica nesses animais. Isso não ocorreu nos animais estressados que receberam essa dieta, os quais apresentaram um aumento do consumo e o ganho de peso foi similar aos controles. Já os animais que receberam dieta rica em carboidratos aumentaram o consumo calórico, mas não apresentaram ganho de peso equivalente. O estresse aumentou o consumo das dietas hiperpalatáveis, que está de acordo com a hipótese que alimentos palatáveis podem ser usados como uma compensação em período de estresse (Dallman et al., 2005).

Após o isolamento, os animais retornaram para junto de seus congêneres e permaneceram assim até a idade adulta. Considerando esse período após isolamento até idade adulta, os animais com acesso a dieta rica em carboidratos apresentaram um maior ganho de peso, o qual está relacionado com um aumento do consumo de dieta. Os grupos que sofreram isolamento e receberam dietas palatáveis aumentaram o ganho de peso. Além disso, o acesso às duas dietas palatáveis levou a um aumento do acúmulo de gordura abdominal nesses animais, que é um importante fator de risco para síndrome metabólica (Lottenberg et al., 2012; Spolidoro et al., 2012). Esse aumento de gordura abdominal é refletido pelo maior consumo no grupo que recebeu dieta rica em carboidratos. Já o grupo que recebeu dieta hiperlipídica não mostrou aumento de ganho

de peso comparado aos controles, o que sugere menor massa magra. Além disso, a exposição ao estresse associada à dieta hiperlipídica aumentou o acúmulo de gordura abdominal, porém teve efeito oposto quando associada à dieta rica em carboidratos. Uma explicação para isso está no fato que a dieta hiperlipídica aumenta os níveis de glicocorticoides induzidos pelo estresse (Tannenbaum et al., 1997; Kamara et al., 1998), e assim pode aumentar o acúmulo de gordura abdominal, enquanto que uma dieta rica em açúcar reduz as respostas do eixo HHA ao estresse (Pecoraro et al., 2004).

A leptina, um hormônio que tem uma importante correlação positiva com o tecido adiposo, sinaliza estoques de energia e ingestão de alimentos (Benoit et al., 2004). Para correlacionar com os resultados da gordura abdominal, foi realizada a dosagem desse hormônio e observamos que as duas dietas hiperpalatáveis aumentaram os níveis de leptina no plasma, que pode ser correlacionado com o aumento da gordura abdominal encontrada nesses animais. Além disso, o aumento nos níveis de leptina pode agir no hipotálamo diminuindo o apetite (Casanueva & Dieguez, 1999). A partir disso, um fato interessante foi o aumento do consumo destes animais que tiveram acesso à dieta rica em carboidratos. Esse fato pode ser explicado devido a uma baixa sensibilidade à leptina no hipotálamo desses animais (Jequier, 2002; Zhang & Scarpace, 2006), já que a leptina não foi capaz de reduzir o consumo desta dieta. Além disso, o estresse causou uma diminuição nos níveis de leptina nos animais que receberam dietas hiperpalatáveis. Dados da literatura mostram que o estresse está relacionado a um aumento nos níveis de leptina, inclusive em pacientes com terapia com glicocorticoides (Rieth et al., 2009; Fardet et al., 2012). Porém, o resultado encontrado foi diferente dos achados da literatura, podendo ser pelo fato de que foi utilizado um estressor sub-agudo em uma fase crítica do desenvolvimento.

Os animais estressados tiveram um pequeno aumento na glândula adrenal. Mostrando que o isolamento social aplicado no período pré-pubere teve efeitos a longo-prazo, já que essa medida foi realizada na idade adulta. Esse resultado demonstra que o cérebro pode ser bastante suscetível a influências ambientais durante períodos de intensa maturação cerebral (Grossman et al., 2003; Meaney & Szyf, 2005; Friederici, 2006). Além disso, o estresse por isolamento social nesse período pode ser associado a alterações no eixo HHA (Gogtay et al., 2004; Weiss et al., 2004) que podem persistir até a idade adulta.

Nas avaliações do perfil lipídico desses animais, os animais com acesso à dieta hiperlipídica associado ao estresse por isolamento, apresentaram maiores níveis de LDL-colesterol. Outros parâmetros analisados não apresentaram diferença significativa. O estresse por isolamento social é uma intervenção aversiva que aumenta a atividade do eixo HHA (Douglas et al., 2004; McCormick & Mathews, 2007). Sabe-se que os glicocorticoides podem aumentar a circulação de ácidos graxos livres através de um aumento da ingestão de gorduras (Peckett et al., 2011), e isso está de acordo com o resultado obtido, já que os animais que receberam dieta hiperlipídica aumentam o consumo durante o período de isolamento. Porém, esse efeito não persistiu até a idade adulta, demonstrando que a programação da ingestão deste tipo de dieta não é modificada a longo prazo, enquanto que a dieta rica em carboidratos continua a ser mais consumida por algumas semanas após o estresse. Além disso, os animais estressados tiveram uma diminuição nos níveis de LDL-colesterol (exceto o grupo que recebeu dieta hiperlipídica), quando comparado ao grupo controle. Uma possível explicação para isso seria pela síntese de hormônios esteroidais, que ocorrem tanto na adrenal como nos ovários e testículos e empregam LDL-colesterol principalmente obtido da circulação (Kanat et al., 2007; Hoekstra et al., 2010). Assim, seria possível que a

diminuição nos níveis de LDL-colesterol, que ocorre como resultado do estresse seria uma consequência do redirecionamento desses lipídeos para a síntese de hormônios esteroidais, ou seja, o estresse induziria a esteroidogênese, e que estaria de acordo também com o resultado do pequeno aumento da adrenal nesses animais.

A dieta hiperlipídica também apresentou aumento nos níveis de glicose plasmática. Sabe-se que a resistência à insulina pode ser induzida por diferentes fatores ambientais, incluindo hábitos alimentares (Riccardi et al., 2004). Uma dieta rica em gordura pode contribuir para isso, podendo levar a uma deficiência na ligação da insulina e/ou dos transportadores de glicose, por mudanças na composição dos ácidos graxos da membrana induzida por modificação do tipo de gordura ingerida na dieta (Lichtenstein & Schwab, 2000). Além disso, foi observado um aumento da atividade da colinesterase plasmática nos animais com acesso a dieta rica em gordura. Embora não se saiba exatamente a função dessa enzima, o aumento de sua atividade vem sendo associado com hiperlipidemias, diabetes e obesidade (Randell et al., 2005). Contudo, os animais que receberam uma dieta rica em gordura apresentaram aumento nos níveis de glicose plasmática e aumento da atividade da colinesterase plasmática, que possivelmente são fatores de risco para síndrome metabólica e doenças cardiovasculares.

Alterações bioquímicas sugerindo um desequilíbrio oxidativo foram observadas no fígado dos animais submetidos ao estresse por isolamento, sendo mais proeminente nos animais que receberam dieta rica em gordura. O aumento das enzimas antioxidantes (SOD e CAT) pelo estresse e a diminuição da atividade da GPx pelas dietas hiperpalatáveis assim como um aumento da produção de espécies reativas pelo estresse mostram que o isolamento social no período pré-pubere induziu um aumento na produção de espécies reativas no fígado a longo-prazo, concomitantemente com o

aumento da atividade das enzimas antioxidantes. Poderíamos pensar que este aumento das enzimas antioxidantes pode ser resultado da resposta à produção de espécies reativas, como forma de proteger as células contra danos oxidativos. Além disso, este aumento parece ser maior no grupo com acesso a dieta hiperlipídica. É interessante que essa dieta também induziu uma drástica redução na atividade da GPx. Estudos mostram que a dieta hiperlipídica está relacionada ao estresse oxidativo (Milagro et al., 2006; Noeman et al., 2011), causando a formação de intermediários tóxicos que podem diminuir a atividade de enzimas antioxidantes (Thampi et al., 1991; Lee et al., 2009). Uma possível explicação para a atividade diminuída da GPx pode ser pelo rápido consumo e esgotamento dessa enzima, devido a reações com as espécies reativas geradas. Entretanto, essa diminuição da atividade da GPx pode levar a um desequilíbrio oxidativo, tornando o fígado vulnerável a uma produção aumentada de peróxido de hidrogênio produzido pela SOD nos animais estressados com acesso a dieta hiperlipídica, já que a atividade da SOD está aumentada nesses animais. Esses dados encontrados são relevantes, considerando que o estresse oxidativo pode levar a várias formas de lesão hepática crônica (Ha et al., 2010).

Os resultados apresentados no capítulo I sugerem que o acesso crônico a dietas hiperpalatáveis, especialmente a rica em gordura, levou a mudanças em parâmetros relacionados a fatores de risco para doenças cardiovasculares e síndrome metabólica (como aumento de peso corporal, gordura abdominal, níveis de leptina, glicemia e atividade colinesterase). Nossos resultados também sugerem que esta condição torna-se pior com a exposição a um estressor durante o período pré-pubere. Além disso, a exposição ao estresse levou a um desequilíbrio oxidativo no fígado que foi mais pronunciado nos animais com acesso a dieta hiperlipídica.

Visto que a associação das dietas hiperpalatáveis com o estresse no período pré-pubere foi capaz de causar alterações periféricas no metabolismo desses animais, procuramos avaliar se estas alterações também ocorreriam no SNC, mais especificamente, no hipocampo. Além disso, analisamos se o comportamento do tipo ansioso e atividade exploratória poderiam estar correlacionados com algum desses efeitos.

Para isso realizamos, na idade adulta, as tarefas do Campo Aberto e Labirinto em Cruz Elevado, conforme descrito no capítulo II. Foi observado que os animais que foram isolados no período pré-pubere com acesso a ração ou dieta rica em gordura permaneceram maior % tempo nos braços abertos no Labirinto em Cruz Elevado. Adicionalmente, esses animais apresentaram um maior número e tempo de mergulhos da cabeça (*head-dips*, considerado um comportamento de risco), quando comparado ao grupo estressado que recebeu dieta rica em carboidratos, indicando um efeito do tipo ansiolítico nesses animais. No Campo aberto, utilizado para avaliar a capacidade locomotora, não houve diferenças entre os grupos. Todos os grupos mostraram uma redução do comportamento exploratório em relação à primeira sessão para a segunda, indicando uma habituação ao aparato e memória adequada para esta tarefa. Um estudo prévio realizado no nosso laboratório (Marcolin Mde et al., 2012), onde o isolamento social aplicado no período pré-pubere em ratos jovens (28 dias), mostrou que o estresse teve um efeito do tipo ansiogênico quando avaliado antes da puberdade. Esse efeito, entretanto, não persistiu até a idade adulta, de acordo com os resultados encontrados. Essas respostas comportamentais na idade adulta podem ser comparadas com outros tipos de intervenções, como por exemplo, a manipulação neonatal, que na idade adulta também leva a comportamento do tipo ansiolítico (McIntosh et al., 1999; Silveira et al., 2005; Boufleur et al., 2012b). Desse modo, intervenções em períodos distintos do

desenvolvimento podem induzir alterações na ansiedade na idade adulta. Alguns autores sugerem que uma interrupção no desenvolvimento social induzido pelo isolamento social, pode prejudicar o aprendizado do medo (Voikar et al., 2005; Yusufishaq & Rosenkranz, 2013). Assim, o efeito ansiolítico observado pode ser devido a uma diminuição do comportamento de medo quando os animais são expostos a um ambiente novo, em comparação aos animais que não passaram pelo isolamento. Além disso, a memória de habituação ao Campo aberto foi adequada em todos os grupos, sugerindo que se houve comprometimento do aprendizado do medo, outros tipos de memória não parecem ser afetados. Importante salientar que outros estudos demonstram distintos resultados, mostrando um aumento no comportamento do tipo ansioso em ratos isolados socialmente (Karim & Arslan, 2000; Green et al., 2012; Ros-Simo & Valverde, 2012) e maior atividade locomotora quando expostos a um ambiente novo (Hall et al., 1997; Smith et al., 1997; Karim & Arslan, 2000). Essas diferenças em relação aos nossos resultados podem ser pelo fato desses outros estudos usarem um período de isolamento mais prolongado, ou seja, o estresse aplicado é de forma crônica. Neste trabalho o estresse aplicado é de curto prazo e realizado antes do processo de maturação sexual, o que pode explicar essas diferenças.

Os animais estressados que receberam uma dieta rica em carboidratos, no entanto, exibiram um comportamento oposto quando comparados aos animais que receberam outras dietas. Eles apresentaram menor % tempo nos braços abertos e menor número e tempo de mergulhos no teste do Labirinto em Cruz Elevado. Assim, a associação do estresse e dieta rica em carboidratos parece reverter os efeitos do isolamento social no comportamento do tipo ansioso. Seria esperado, nesse caso, que a dieta rica em glicídios pudesse agir como um alimento “confortante” (“comfort foods”) (Pecoraro et al., 2004), diminuindo as resposta ao estresse, e esta poderia ser a razão

pela qual esse tipo de dieta foi capaz de reverter os efeitos do estresse. Entretanto, é importante salientar que, em ratos adultos estressados por contenção, uma dieta rica em glicídios simples foi capaz de reverter o comportamento do tipo ansioso induzido pelo estresse (Krolow et al., 2010).

Para analisar os efeitos do isolamento social e da exposição crônica a dietas hiperpalatáveis no SNC, avaliamos alguns parâmetros relacionados ao estresse oxidativo. Além disso, buscamos também relacionar com os achados da ansiedade, visto que alguns estudos mostram uma correlação entre estresse oxidativo e comportamento do tipo ansioso (de Oliveira et al., 2007; Masood et al., 2008; Salim et al., 2010). Porém, neste estudo, os resultados não confirmam esta relação. Os dados mostraram que o estresse diminuiu a atividade das enzimas antioxidantes, do conteúdo de tióis totais e a atividade dos complexos II e IV da cadeia respiratória. Um estudo prévio mostrou que logo após o isolamento social (28 dias) houve uma diminuição da atividade da SOD, mas sem mudanças nas outras enzimas antioxidantes, assim como um aumento na produção de espécies reativas e no dano ao DNA no hipocampo de ratos machos (Krolow et al., 2013). Os achados deste estudo mostram que os efeitos na produção de espécies reativas foram estabilizados com o tempo, porém as atividades das enzimas antioxidantes foram diminuídas, assim como o conteúdo de tióis totais, sugerindo que o hipocampo desses animais permanece suscetível ao desequilíbrio oxidativo na idade adulta. A diminuição do conteúdo de tióis totais nesses animais pode representar um dano a proteínas, ou pode refletir menor conteúdo de glutationa, que é um substrato para a enzima antioxidante GPx, e isto pode ser uma explicação para a diminuição da GPx encontrada nos animais estressados. É também importante considerar que a diminuição da atividade dos complexos II e IV nos ratos isolados na idade adulta pode facilitar o escape de elétrons através desses complexos. Como a atividade das enzimas

antioxidantes nesses animais está diminuída, pode-se esperar que esses animais estejam mais suscetíveis a danos oxidativos em situações que podem levar a um aumento na produção de espécies reativas.

Quando o estresse foi associado à dieta hiperlipídica, os efeitos observados foram opostos: aumento da atividade das enzimas antioxidantes SOD e CAT, aumento do conteúdo de tióis totais e aumento da atividade dos complexos II e IV da cadeia respiratória. Esses resultados são controversos, já que estudos mostram que a dieta hiperlipídica parece exacerbar as respostas ao estresse pelo aumento da atividade do eixo HHA (Tannenbaum et al., 1997; Legendre & Harris, 2006). Nesse caso, o esperado seria que o isolamento social associado com a dieta hiperlipídica aumentasse os efeitos do estresse e não o contrário, como observado nesses animais. Isso mostra a importância de se conhecer os possíveis mecanismos pelos quais a dieta rica em gordura pode estar agindo nessas mudanças. No entanto, outro estudo mostrou que a dieta hiperlipídica diminuiu a atividade do eixo HHA (Auvinen et al., 2012), e esse achado estaria de acordo com nosso resultado, onde a dieta rica em gordura parece reverter os efeitos do estresse. Outra explicação poderia ser por uma diminuição nos receptores de glicocorticoides (RG) no hipocampo. Sabe-se que o hipocampo é uma estrutura bastante sensível ao estresse e que possui grande quantidade de RG (Sapolsky, 2003; McEwen, 2008). Alguns estudos, porém, demonstram que a exposição ao estresse durante o desenvolvimento foi associada com redução de RG no hipocampo e que essas mudanças foram normalizadas com o acesso a dieta rica em gordura (Maniam & Morris, 2010a; Maniam & Morris, 2010b). Assim, a dieta hiperlipídica poderia estar agindo diminuindo as respostas ao estresse no hipocampo.

Desta forma, os resultados apresentados no capítulo II mostram que o isolamento social no período pré-pubere levou a mudanças comportamentais (reduzindo

comportamento do tipo ansioso) e neuroquímicas a longo-prazo. Pelas alterações observadas, pode-se supor que esses animais podem estar mais suscetíveis a um dano oxidativo no hipocampo. Por outro lado, o acesso a diferentes tipos de dietas podem alterar os efeitos associados ao isolamento social, visto que a dieta rica em carboidratos simples, quando associada ao estresse, levou a efeitos opostos no comportamento, e, além disso, a dieta rica em gordura associada ao estresse foi capaz de reverter os efeitos do isolamento, possivelmente por essa dieta estar agindo na atividade do eixo HHA ou sua inibição. Os mecanismos pelos quais isso ocorre, no entanto, necessitam ser melhores entendidos.

Analisando os resultados obtidos nos capítulos I e II, percebemos que o estresse no período pré-pubere induz mudanças comportamentais, metabólicas e neuroquímicas que podem ser observadas até a idade adulta. Também se observa que os efeitos da dieta rica em gordura diferem quanto ao local em que agem, ou seja, de forma periférica a dieta levou a mudanças em parâmetros relacionados a doenças cardiovasculares e síndrome metabólica, além de no fígado estar bem relacionada ao desequilíbrio oxidativo. Já no cérebro, mais precisamente no hipocampo, essa dieta age de maneira oposta, ou seja, ela reverte os efeitos de desequilibro oxidativo relacionado ao estresse, além de ter um efeito do tipo ansiolítico nos testes comportamentais. A nossa hipótese é que diferentes dietas modificam a regulação do eixo HHA. Possivelmente, a dieta rica em gordura estaria agindo no eixo aumentando a sua atividade. Importante considerar aqui que esse aumento pode estar relacionado a uma diminuição no número de RG no hipocampo, o que causaria uma redução no processo de retroalimentação negativa do eixo. Uma maior liberação de glicocorticoides levaria ao aumento do acúmulo de gordura abdominal encontrada nos animais estressados com acesso a dieta hiperlipídica. Assim, os efeitos do estresse são visualizados perifericamente nesses animais, porém

quando analisou-se em uma estrutura cerebral, observamos que a resposta do estresse é diminuída em animais recebendo dieta hiperlipídica, talvez pelo menor número de RG nesta estrutura cerebral. Além disso, observa-se que a dieta rica em carboidratos simples também modificou os efeitos observados pelo estresse no comportamento do tipo ansioso, mostrando que o acesso a diferentes dietas pode alterar os efeitos induzidos pelo estresse por isolamento no período pré-pubere em animais na idade adulta.

# 5. CONCLUSÕES

- O estresse no período pré-pubere e o acesso crônico a dietas hiperpalatáveis induziram diferentes efeitos comportamentais e bioquímicos que foram observados na idade adulta.
- Os animais com acesso a dieta rica em carboidratos tiveram maior ganho de peso e maior consumo calórico comparado ao controle, enquanto que o grupo que recebeu dieta hiperlipídica apresentou maior acúmulo de gordura abdominal, que é aumentado associado ao estresse.
- O estresse sozinho ou associado à dieta hiperlipídica tem efeito do tipo ansiolítico no Labirinto em Cruz Elevado, enquanto que os animais com acesso a dieta rica em carboidratos apresentaram efeitos opostos, ou seja, apresentaram efeito do tipo ansiogênio no mesmo aparato.
- O acesso crônico a dietas hiperpalatáveis, especialmente a rica em gordura, levou a mudanças em parâmetros relacionados a fatores de risco para doenças cardiovasculares e síndrome metabólica (como aumento de peso corporal, gordura abdominal, níveis de leptina, glicemia e atividade da colinesterase plasmática). Esta condição torna-se pior com a exposição a um estressor durante o período pré-pubere.
- A exposição ao estresse levou a um desequilíbrio oxidativo no fígado, que foi mais pronunciado nos animais com acesso a dieta hiperlipídica. No hipocampo, o estresse tornou os animais mais suscetíveis a um dano oxidativo. A dieta rica em gordura associada ao estresse, no entanto, foi capaz de reverter esses efeitos.

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