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EFEITOS METABÓLICOS DA EXPOSIÇÃO PERINATAL AO NONILFENOL EM CAMUNDONGOS

METABOLIC EFFECTS OF PERINATAL EXPOSURE TO NONYLPHENOL IN MICE

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências da Saúde, Universidade de Brasília, como requisito parcial à obtenção do título de Mestre em Ciências Farmacêuticas.

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Resumo

SOUSA, Ana Beatriz. Study of the metabolic effects of perinatal exposure to nonylphenol in mice. Brasília, 2025. Dissertação (Mestrado em Ciências Farmacêuticas) – Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília, 2025.

A obesidade é uma doença crônica caracterizada pelo acúmulo excessivo de tecido adiposo e representa um importante problema de saúde pública global, devido à sua associação com aumento da morbidade e mortalidade. Seu desenvolvimento é multifatorial, envolvendo suscetibilidade genética, e fatores ambientais, a exemplo de estilo de vida, como dietas ricas em gordura (DRGs), além de distúrbios hormonais e metabólicos. Nos últimos anos, tem havido crescente atenção aos compostos desreguladores endócrinos (DEs) como contribuintes ambientais para a obesidade, uma vez que eles podem interferir na sinalização hormonal, na homeostase energética e no desenvolvimento do tecido adiposo. Alguns DEs, conhecidos como obesogênicos, promovem a diferenciação de adipócitos e o acúmulo de gordura, especialmente quando a exposição ocorre durante janelas críticas do desenvolvimento. O nonilfenol (NF), um alquilfenol amplamente utilizado e xenoestrogênio derivado de surfactantes industriais, é um DE persistente e lipofílico capaz de interagir com receptores de estrogênio e desregular o metabolismo. Estudos experimentais sugerem que a exposição ao nonilfenol pode influenciar a adipogênese e os desfechos metabólicos, com efeitos aparentemente mais pronunciados após exposição precoce na vida. O objetivo deste estudo foi investigar o efeito da exposição ao NP durante períodos críticos do desenvolvimento sobre desfechos metabólicos em camundongos C57Bl/6 machos e fêmeas. Camundongos C57BL/6J machos e fêmeas foram utilizados como geração parental (F0) e sua prole como geração descendente (F1). As fêmeas da F0 foram expostas a uma concentração ambientalmente relevante de nonilfenol (0,25 mg/kg/dia) ou ao veículo (DMSO), através da água de beber, por uma semana antes do acasalamento e durante toda a gestação e lactação (com duração total de 7 semanas de exposição). A geração F1 foi dividida e alimentada com dieta controle (CD) ou DRG contendo 30% de gordura. Os animais da F1 foram submetidos à avaliação do peso corporal e do consumo alimentar, à análise do gasto energético por meio de um sistema de monitoramento metabólico, além dos testes de tolerância à glicose e à insulina, e à eutanásia para avaliação da função mitocondrial do tecido adiposo branco subcutâneo (TABs) por respirometria de alta resolução. A DRG aumentou o peso corporal em todos os grupos de machos. A exposição ao NF associada à DC promoveu aumento do peso corporal dos machos em comparação ao grupo veículo, além de reduzir o gasto energético. Esse efeito sobre o ganho de peso não foi observado nos machos expostos simultaneamente ao NF e à DRG. No entanto, os machos do grupo DRG+NF apresentaram redução do gasto energético. O metabolismo da glicose não foi alterado em nenhum dos grupos. A análise mitocondrial do TABs de machos revelou uma redução do consumo de oxigênio no grupo NF alimentado com DC e no grupo exposto ao veículo e alimentado com DRG, sobre o complexo I do sistema de transporte de elétrons. Além disso, um efeito da dieta sobre o vazamento de prótons referente à respiração dependente dos complexos I + II e sobre a capacidade máxima do sistema de transporte de elétrons foi observado. Apesar disso, não foram observadas interações significativas entre dieta e exposição ao NF. Não houve diferença entre os grupos de fêmeas em nenhuma das análises. Este estudo sugere que a exposição perinatalao NF altera a programação metabólica em descendentes machos, especialmente no contexto da dieta, ao afetar o gasto energético e a função mitocondrial no TABs. A ausência de efeitos semelhantes nas fêmeas destaca a relevância do dimorfismo sexual nas respostas metabólicas a DEs. Esses achados reforçam a necessidade de compreender melhor como a exposição precoce a DEs interage com a dieta e a função mitocondrial durante o desenvolvimento. Além disso, o uso de uma dieta moderadamente rica em gordura mostrou-se suficiente para induzir ganho de peso relevante, permitindo a identificação de interações entre dieta e exposição química sem provocar disfunções metabólicas graves.

Palavras-chave: Obesidade; Doenças metabólicas; Disruptores endócrinos.

Abstract

SOUSA, Ana Beatriz. Study Of The Metabolic Effects Of Perinatal Exposure To Nonylphenol In Mice. Brasília, 2025. Dissertação (Mestrado em Ciências Farmacêuticas) – Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília, 2025.

Obesity is a chronic disease characterized by excessive accumulation of adipose tissue and represents a major global public health problem due to its association with increased morbidity and mortality. Its development is multifactorial, involving genetic susceptibility and environmental factors, such as lifestyle, including high-fat diets (HFDs), as well as hormonal and metabolic disturbances. In recent years, increasing attention has been given to endocrine-disrupting chemicals (EDCs) as environmental contributors to obesity, as they can interfere with hormonal signaling, energy homeostasis, and adipose tissue development. Some EDCs, known as obesogens, promote adipocyte differentiation and fat accumulation, particularly when exposure occurs during critical windows of development. Nonylphenol (NP), a widely used alkylphenol and xenoestrogen derived from industrial surfactants, is a persistent and lipophilic EDC capable of interacting with estrogen receptors and disrupting metabolic processes. Experimental studies suggest that exposure to nonylphenol may influence adipogenesis and metabolic outcomes, with effects appearing to be more pronounced following early-life exposure. The aim of this study was to investigate the effects of NP exposure during critical periods of development on metabolic outcomes in male and female C57BL/6 mice. Male and female C57BL/6J mice were used as the parental generation (F0), and their offspring constituted the descendant generation (F1). F0 females were exposed to an environmentally relevant concentration of nonylphenol (0.25 mg/kg/day) or vehicle (DMSO) via drinking water for one week prior to mating and throughout gestation and lactation, resulting in a total exposure period of seven weeks. The F1 generation was divided and fed either a control diet (CD) or a high-fat diet (HFD) containing 30% fat. F1 animals were evaluated for body weight and food intake, energy expenditure using a metabolic monitoring system, and glucose and insulin tolerance tests. Animals were then euthanized for assessment of mitochondrial function in subcutaneous white adipose tissue (scWAT) using high-resolution respirometry. HFD increased body weight in all male groups. Exposure to NF in combination with the control diet increased body weight in males compared with the vehicle group and was also associated with a reduction in energy expenditure. This effect on weight gain was not observed in males exposed to both NF and DRG. However, males in the DRG+NF group exhibited reduced energy expenditure. Glucose metabolism was not altered in any of the groups. Mitochondrial analysis of male scWAT revealed a decrease in oxygen consumption linked to complex I of the electron transport chain in both the NF-exposed group fed the control diet and the vehicle-exposed group fed the DRG. Furthermore, an effect of diet on proton leak associated with complex I+II–supported respiration and on the maximal electron transport system capacity was observed. Nevertheless, no significant interactions between diet and NP exposure were observed. No differences were detected among female groups in any of the analyses. This study suggests that perinatal exposure to NP alters metabolic programming in male offspring, particularly in the dietary context, by affecting energy expenditure and mitochondrial function in scWAT. The absence of similar effects in females highlights the relevance of sexual dimorphism in metabolic responses to EDCs. These findings reinforce the need for a deeper understanding of how early-life exposure to EDCs interacts with diet and mitochondrial function during development. Furthermore, the use of a moderately high-fat diet was sufficient to induce relevant weight gain, allowing the identification of interactions between diet and chemical exposure without inducing severe metabolic dysfunction.

Keywords: Obesity; Metabolic diseases; Endocrine disruptors.

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List of Abbreviations

ΔΨm – mitochondrial membrane potential
AA – antimycin A
ADP – adenosine diphosphate
ATP – adenosine triphosphate
BAT – brown adipose tissue
BMI – body mass index
BDNF – brain-derived neurotrophic factor
BPA – bisphenol A
CI ETS – measure from complex I in electron transport chain system
CI+CII ETS – measure from complex I and complex II
CI+CII OXPHOS – ADP synthase respiration with oxidative phosphorylation
CCCP – carbonyl cyanide m-chlorophenylhydrazone
CD – control diet
CEUA – Animal Ethics Committee of the University of Brasília
CLAMS – comprehensive laboratory animal monitoring system
CO_2 – carbon dioxide
CONCEA – National Council for the Control of Animal Experimentation
DMSO – dimethyl sulfoxide
EDCs – Endocrine disrupting chemicals
EE – energy expenditure
ER – estrogen receptor
ERα – estrogen-receptor-α
ETS – electron transport system
F0 – parental generation
F1 – prole generation
GTT – glucose tolerance test
HRR – high-resolution respirometry
ITT – insulin tolerance test

FFA – free fatty acid

HFD – high fat diet

MRS – mitochondrial respiratory system

NAFLD – nonalcoholic fatty liver disease

NOAEL – no observed adverse effect level

NP – Nonylphenol

O_2 – oxygen

OXPHOS – Oxidative phosphorylation

PM – pyruvate and malate

PPARγ – peroxisome proliferator-activated receptor gamma

PPG1α – peroxisome proliferator-activated receptor gamma coactivator 1-alpha

RER – carbon dioxide production / oxygen consumption or VCO_2 / VO_2

ROT – rotenone

RXR – retinoid X receptor

S – succinate

SEM – standard error of mean

T2DM – type 2 diabetes mellitus

TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin

UCP1 – Uncoupling Protein 1

VCO_2/VO_2 – respiratory exchange ratio

WAT – white adipose tissue

Summary

1. Introduction

1.1 Obesity

Obesity is defined as a chronic disease characterized by accumulation of adipose tissue, which negatively impacts health and is associated with an increased risk of morbidity and mortality (PICHÉ; TCHERNOF; DESPRÉS, 2020; RUBINO *et al.*, 2025). It is considered a global health issue and an epidemic that can affect adults, children, and adolescents (NG *et al.*, 2025). The causes can be multifactorial and may be divided into primary and secondary ones. Primary causes include genetic ones (e.g. family history), monogenic disorders (leptin deficiency), and syndromes (Prader-Willi); and secondary origins include neurological (brain injury or tumor, hypothalamic obesity), endocrine (Cushing syndrome, GH deficiency), psychological (depression, eating disorders), and drug-induced (oral contraceptives, tricyclic antidepressants, glucocorticoids) causes. There are also contributing factors, including sleep disorders, lifestyle and environmental or socioeconomic issues (APOVIAN *et al.*, 2015; SAFAEI *et al.*, 2021). A hyperlipidic diet, rich in saturated fats and fats of animal origin, is already known for causing overweight and obesity, and it also contributes to metabolic disorders such as insulin resistance, hepatic lipid accumulation, cholesterol metabolism and disbalance in energy intake, in both humans and rodents (BLACK *et al.*, 2013; LIU, YOUHUA *et al.*, 2025; YANG *et al.*, 2012). Despite preclinical studies typically use high fat diet (HFD) containing 45% or more of total calories from fat, the percentage of fat commonly found in obesogenic human diets variate between 33% and 37% (ALFIERI; POMERLEAU; MICHAEL GRACE, 1997; AUSTIN; OGDEN; HILL, 2011; JEON et al., 2011).

In humans, clinically, the diagnosis of overweight and obesity is made by assessing an individual's weight and height to calculate the body mass index (BMI), defined as weight in kilograms divided by height in squared meters (kg/m²). For adults, children, and adolescents, BMI classifications for obesity differ based on age and sex (WORLD HELTH ORGANIZATION, 2025). For adults, a BMI of 25 to 29.9 kg/m^2 is defined as overweight and a BMI of 30 kg/m^2 or higher is defined as obesity. A global study estimated that in 2021, 1 billion men and 1,11 billion women aged twenty-five had overweight or obesity. In this scenario, China had the highest number of adults with overweight/obesity (402 million), followed by India (180 million) and the USA (172 million) (NG *et al.*, 2025). In Brazil 55.4% of the population were overweight and 19,8% are obese (ABESO, [N. d.]). For 2050, the estimative is that over 3,8 billion adults will be affected (more than half the global adult population) (NG *et al.*, 2025). For children and adolescents, this data is an emerging worldwide health concern, since 1 of 5 children or adolescents globally experienced excess weight (ZHANG, XINYUE *et al.*, 2024).

Adipocyte dysfunction, marked by a progressive loss of essential adipose tissue functions and a reduced capacity to safely store excess lipids, creates a permissive environment for metabolic disorders, with type 2 diabetes (T2D) being the most prevalent (HAGBERG; SPALDING, 2024; RUZE *et al.*, 2023). T2D is a disease characterized by a nonautoimmune heterogeneously progressive loss of adequate islet β cell insulin secretion frequently in the presence of insulin resistance and metabolic syndrome (LU, XI *et al.*, 2024).

Hyperglycemia might arise from reduced insulin sensitivity due to a decline in functional β-cell mass, with obesity playing a key role in its onset and progression. This process is driven by enhanced genetic and epigenetic susceptibility, microenvironmental factors that impair insulin signaling, β-cell dysfunction, and disruption of the microbiome–gut–brain axis (RUZE *et al.*, 2023).

Another consequence of adipocyte dysfunction is the Metabolic dysfunction–Associated Steatotic Liver Disease (MASLD), in which the set of insulin resistance and lipolysis result in elevated circulating FFAs and leptin along with reduced adiponectin. Leptin is a hormone secreted by adipose tissue that regulates hunger, metabolism, and body weight. In obesity, high leptin levels lead to leptin resistance. Adiponectin, on the other hand, is a type of adipokine that is associated with insulin sensitivity, fat oxidation and reduced inflammation. Together, these changes contribute to fat accumulation in the liver (steatosis). Without appropriate obesity management, ongoing lipotoxicity, glucotoxicity, and cellular stress promote MASLD progression, potentially leading to cirrhosis and impaired hepatic regeneration (POLYZOS et al., 2019). In obesity, the alteration and disbalance in hormones, proinflammatory cytokines and immune cells are linked to many others metabolism disorders, like cardiovascular diseases and hypertension caused by vascular alterations in structure and function (SERAVALLE et al., 2017). Also, obesity has tumoral-promoting effects both locally—through adipose tissue inflammation and changes in the surrounding microenvironment—and systemically, via circulating metabolic and inflammatory factors linked to that inflammation (IYENGAR *et al.*, 2016). The consequences of obesity are severe and progress every year. Therefore, understanding the mechanisms linked to its causes is an important way for intervein, control, and prevent this epidemic.

1.2 Physiopathology of obesity

Human adipose tissue is increasingly recognized as a complex and dynamic organ involved in energy homeostasis, immune response, and endocrine function. It comprises not just fat-storing adipocytes, but also various immune, endothelial, and progenitor cells, that interact in intricated ways (CYPESS, 2022). Adipocytes are classified at least into three distinct type: white adipocytes, which are adapted explicitly for fat storage, and na expansive fat vacuole with perilipin border and very few mitochondria, they form the white adipose tissue (WAT). Brown adipocytes specialized for non-shivering thermogenesis and characterized by vascularization, and abundant mitochondria, which confer their distinctive brown colo, they form the brown adipose tissue (BAT); and the last, beige adipocytes, which develop within white adipose tissue depots and exhibit increased mitochondrial content, uncoupling Protein 1 (UCP1) expression, and non-shivering thermogenic capacity (LUSTIG *et al.*, 2022).

Adipose tissue can expand through either adipocyte enlargement (hypertrophy) or the recruitment of new adipocytes (hyperplasia). A hyperplastic response helps to prevent excessive cell enlargement and protects against the development of dysfunctional adipose tissue. However, adipocyte hypertrophy is a hallmark of metabolic risk in obesity, which is linked to elevated inflammation (SMITH et al., 2016). Inflamed adipocytes release proinflammatory cytokines and adipokines locally and systemically, impairing the normal function of adipose tissue and distant organs (HOTAMISLIGIL, 2017). Dysfunctional adipocytes can also increase levels of free fatty acids (FFA), which typically occur during fasting, exercise, or stress, when the body needs to mobilize stored energy. However, in obesity, the excess of FFA in WAT can induce its abnormal expansion, which results in hypoxia and remodeling-induced senescence. This process triggers a chronic state of inflammation mediating not only metabolic dysfunctions but also the adaptive reactions necessary to maintain physiological conditions in WAT (KAWAI et al., 2020).

Obesity is also commonly associated with elevated circulating levels of leptin, a condition known as hyperleptinemia. Leptin is a hormone predominantly produced by WAT, and its secretion is regulated by factors such as food intake, total body fat, and various other hormones (OBRADOVIC *et al.*, 2021). In individuals with excess adipose tissue, leptin levels increase, which can lead to a reduced responsiveness of the hypothalamus to leptin signals, and this diminished sensitivity results in a state known as leptin resistance, leading to an increase in appetite and energy expenditure (SETH *et al.*, 2020).

1.3 Endocrin disrupting chemicals

Endocrine disrupting chemicals (EDCs) are exogenous chemicals, or mixture of chemicals, which can interfere in any aspect of hormone action, on a time and potency dependent manner (THOMAS ZOELLER *et al.*, 2012). A study of chemical pollution analyzed 22 chemical inventories from 19 countries and found over 350,000 chemicals and mixtures of chemicals registered for production and use. Three times as many as previously estimated, over 50,000 chemicals were claimed as confidential and 70,000 were ambiguously described (WANG *et al.*, 2020). Nevertheless, the European Union lists only 131 substances legally identified as endocrine disruptors (ED LISTS, 2024).

The primary source of EDCs lies in many everyday products, including plastic and plasticizers, metal food cans, detergents, flame retardants, food, household and industrial chemicals, children's products, cosmetics, pesticides, and water pollution (GORE *et al.*, 2024; YILMAZ *et al.*, 2019). The most detected classes in the general population are bisphenol A (BPA) and phthalates. BPA is predominantly used in food packaging, toys, and plastics, while phthalates are used as liquid plasticizers found in a wide range of products including plastics, coatings, cosmetics, and medical tubing (GORE et al., 2015).

The key characteristics of EDCs were established based on the observation that hormone regulation and action share common biological features, regardless of the wide variety of hormonal effects that occur throughout the life cycle. Because of these shared mechanisms, chemicals that disrupt endocrine function also tend to act through a set of common biological processes. EDCs generally exert their effects through mechanisms that include: interacting with or activating hormone receptors; antagonizing hormone receptors; altering signal transduction in hormone-responsive cells; inducing epigenetic modifications in hormone-producing or hormone-responsive cells; altering hormone synthesis; affecting hormone transport across cell membranes; changing hormone distribution or circulating hormone levels; modifying hormone metabolism or clearance; and altering the development, survival, or function of hormone-producing or hormone-responsive cells (LA MERRILL et al., 2019).

Although definitive causal relationships between exposure to EDCs and specific disease outcomes have not yet been fully established, substantial evidence underscores the importance of recognizing the widespread presence of EDCs in the environment. Given the broad range of phenotypic effects associated with EDC exposure, it is unlikely that these compounds operate through a single, uniform mechanism. Indeed, EDCs often produce non-monotonic dose-response relationships, suggesting that the diverse biological effects observed at varying exposure levels may result from distinct underlying mechanisms of action.

1.4 Obesogens

Obesogens are chemicals that have been shown to promote the differentiation of adipocytes in vivo. This means they can increase WAT by directly influencing stem cell commitment, promoting their differentiation into mature adipocytes, and altering their number, size, and triglyceride content. Examples are the bisphenols, that can promote obesity via epigenetic mechanisms; phthalates that can stimulate adipogenesis via the PPARγ pathway; and parabens that can increased both weight and weekly food intake, as well as down-regulate of the leptin receptor (HEINDEL *et al.*, 2022). The mechanisms through which the known obesogens exert their effects remain poorly understood. However, some studies have identified the peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptor (RXR) pathways as key regulators of adipogenesis, and several obesogens act through the activation of this heterodimeric complex, highlighting its central role in the development of adipose tissue. Also, it is established that some obesogenic substances can regulate the thermogenic fat and gut microbiome (MOHAJER *et al.*, 2021)

1.5 Early Life Effects of EDC Exposure

As a consequence of their biochemical properties, EDC's are capable of crossing the placental barrier and reaching the developing embryo or fetus. This exposure can lead to abnormal genetic and epigenetic regulation, with sex-specific modifications, thereby contributing to pregnancy and placental disorders. Exposure during these critical developmental periods may alter metabolic programming through epigenetic mechanisms, increasing the risk of disease later in life (ROLFO *et al.*, 2020). Evidence from both animal models and human epidemiological studies indicates that early-life exposure to these substances is associated with the development of obesity and metabolic disorders in adulthood (HAJJAR *et al.*, 2024; JUAREZ *et al.*, 2023).

Adipocyte number is largely set by the end of childhood, and any early-life increase in this number tends to be permanent. Therefore, the timing of exposure to obesogenic agents is critical, since disruptions occurring during early developmental stages may persist in adulthood and are unlikely to be reversed (SPALDING *et al.*, 2008). A previous study has shown that prenatal exposure to BPA alters the methylation patterns of genomic sites associated with obesity, suggesting that early-life exposure to this metabolic disruptor may play a causal role in the development of obesity, including in humans (VAN ESTERIK *et al.*, 2014). Also, a recent cohort study revealed that prenatal exposure to EDCs, assessed by their urinary levels during pregnancy, was linked to an increased risk of obesity in children. The study identified that specific deoxyribonucleic acid (DNA) methylation patterns partially mediated this association (LV *et al.*, 2024).

Additionally, exposure to environmental contaminants with metabolic-disrupting activity may result in transgenerational effects, characterized by the manifestation of metabolic disease phenotypes not only in the directly exposed generation but also in subsequent, unexposed generations (EGUSQUIZA; BLUMBERG, 2020). Tributyltin, an organotin compound widely used in industrial applications, has been identified as a contaminant in plastics and detected in household dust samples. Studies have shown that exposure of female mice to tributyltin during pregnancy and lactation led to increased adiposity in the third generation, which had no direct contact with the compound, thereby demonstrating a transgenerational metabolic impact in phenotype (CHAMORRO-GARCIA *et al.*, 2017). Although the mechanisms underlying transgenerational inheritance are not yet fully understood, growing evidence suggests that epigenetic modifications induced by endocrine and metabolic disruptors in the germline may play a central role and be passed on to future generations (EGUSQUIZA; BLUMBERG, 2020).

This supports the growing body of evidence linking environmental exposures during early life to increased susceptibility to chronic diseases in adulthood, highlighting the importance of considering epigenetic mechanisms in the study of EDCs.

1.6 Nonylphenol

Nonylphenol (NP) is a synthetic organic chemical that belongs to a group of substances called alkylphenols, characterized by a phenol ring bonded to a nine-carbon nonyl group (SOARES *et al.*, 2008). In the environment, NP comes from the degradation of nonylphenol ethoxylates, and is widely used in many industrial applications, including the production of detergents, surfactant, emulsifiers, surface modifiers, flotation dispersants, paints, and plasticizers (ZHAO *et al.*, 2024) It is a highly lipophilic compound, and this feature contributes to its persistence in the environment and makes it resistant to degradation (AHEL; GIGER, 1993). Recognized as a xenoestrogen, NP can interact with the estrogen receptor (ER) and mimic the actions of natural estrogens, thereby interfering with normal hormonal signaling. This disruption can affect various physiological processes, including reproductive function, metabolism, and brain activity. Furthermore, NP exposure can also lead to hormonal imbalances, immune system disturbances, and an increased risk of tumor development across a wide range of species, including humans (HUANG et al., 2019). The NOAEL (no observed adverse effect level) is 650 ppm NP in the diet (approximally 50 mg/kg/day) (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION, 2026). The environmental presence of nonylphenols and their isomers is a matter of considerable concern. This is supported by extensive, detailed, and well-documented evidence regarding the toxicity of these compounds across various biological models (DE LA PARRA-GUERRA; ACEVEDO-BARRIOS, 2023; TATO *et al.*, 2018). Due to its chemical properties and widespread industrial use, NP has emerged as a significant environmental contaminant and a potential risk to human health. Consequently, further research is essential to better understand its behavior and mitigate its adverse effects.

A previous study conducted by our research group exposed adult mice directly to environmentally relevant concentrations of NP while administering a high-fat diet. Although in cell culture NP showed an adipogenic effect, the results in adult mice model indicated no significant changes in body weight or in the mRNA expression of genes associated with adipogenesis (RIBEIRO *et al.*, 2023). However, it is important highlight the period of exposure in this study, since in early development, but not necessarely in adulthood, animals are more sensitive to environmental changes. Another study exposing pregnant mice to an environmental concentration of NP showed an adipogenic effect in offspring mice (HAO *et al.*, 2012). Nonetheless, the metabolic consequences of NP exposure during intrauterine life and lactation remain insufficiently explored and warrant further investigation.

1.7 Sexual Dimorphism

Sex-specific differences in obesity are well established, particularly regarding its incidence and associated health outcomes. Males and females exhibit distinct patterns of fat deposition, mobilization, and utilization, as well as differing physiological consequences resulting from both excessive and insufficient adipose tissue (HEINDEL *et al.*, 2016). Females generally possess more total body fat than males. Moreover, pre-menopausal females tend to accumulate more subcutaneous WAT, primarily in peripheral and lower body areas, whereas males typically develop a higher abundance of visceral WAT in the upper body regions (BLOOR; SYMONDS, 2014). The susceptibility to obesity varies between sexes, primarily due to the influence of sex hormones. For instance, female rats exhibit reduced weight gain compared to males when exposed to metabolic challenges such as a high-fat diet, however, this sex-based difference is abolished following ovariectomy, highlighting the regulatory role of ovarian hormones in metabolic responses (STUBBINS *et al.*, 2012).

Estrogens are involved in the regulation of many complex physiological processes, including processes regulating energy homeostasis on the brain or through activation of estrogen receptors (ER) on adipocytes. This hormone helps to protect against increased body fat and obesity by reducing appetite and promoting greater energy expenditure. The primary form of estrogen, estradiol, contributes to appetite suppression by strengthening the effects of appetite-reducing signals, such as cholecystokinin, apolipoprotein A-IV, leptin, and brain-derived neurotrophic factor (BDNF), while weakening the effects of appetite-stimulating signals. In contrast, testosterone shows a suppressive effect on lipoprotein lipase activity in femoral subcutaneous fat in men, that is associated with an increas central fat expansion, insulin resistance and systemic inflammation (PALMER; CLEGG, 2015).

Nevertheless, sexual dimorphism is a well-recognized factor in metabolic research, despite the influence of sex has been often overlooked in experimental studies. The majority of investigations have been conducted using single-sex animal models, predominantly male. Especially regarding EDC's studies, the role of sex hormones is particularly significant, as they are essential regulators of cellular function and metabolic processes. It has been demonstrated that numerous EDCs interfere with sex hormone signaling in distinct and consequential ways, underscoring the importance of considering sex-specific effects in such research. This lack of sex-specific analysis highlights the critical need to incorporate sexual dimorphism into the study of obesity and metabolic health, as it may significantly influence physiological responses and disease outcomes.

2. Objective

To investigate the effect of exposure to nonylphenol during critical periods of development on metabolic outcomes in C57Bl/6 male and female mice.

2.1 Specific objectives

To investigate the effects of NP exposure in C57BL/6 mice during intrauterine life and lactation, as well as its interaction with diet in adulthood, on the following parameters:

- Body weight regulation, energy intake, and metabolic efficiency;
- Glucose homeostasis and insulin responsiveness;
- Whole-body energy expenditure;
- Oxidative metabolism in white adipose tissue.

3. Metodology

3.1 Animal Model

Five-week-old C57BL/6J mice (12 male and 24 female) were obtained from the State University of Campinas and comprised the parental generation (F0). All experimental procedures were reviewed and approved by the Animal Ethics Committee of the University of Brasília (CEUA–UnB), in accordance with ethical principles and the guidelines of the National Council for the Control of Animal Experimentation (CONCEA), the certificate can be found in Annex I. The mice were kept in ventilated cages, four to five animals per cage, temperature of 25ºC, fixed light-dark cycles of 12 hours each and free access to diet (AIN-93 G food provided in pellet form) and drinking water. At 14 weeks of age the females were exposed for seven weeks to 0.25 mg/kg/day of NP (Sigma-Aldrich, 46018) in drinking water, which was based on environmental concentrations (LU, 2021; Mao et al., 2012), or dimethyl sulfoxide (DMSO) in concentration of 1mL/L (0,1%), which was used as vehicle. The animals were submitted to mating at 15 weeks of age. One male was individualized with two females for two days, period during which the exposure was interrupted. The females were separated from males when the copulation plug was visible, and the exposure was resumed. The exposure was maintained during gestational period and for three weeks after the birth of the prole generation (F1). Thereafter, the F1 was weaned and the F0 was euthanized by isoflurane anesthesia. To avoid bias related to litter effect, offspring with four animals were not analyzed (VUGUIN, 2007). At seventh weeks of age, the F1 generation was randomly divided into eight groups according to perinatal exposure, diet during adulthood, and sex, as described in Table 1.

Table 1. Relationship between sex, maternal exposure, diet, and number of experimental animals per group.

Group	Sex	Maternal exposure	Diet	No of animals
CD + vehicle	Male	Vehicle	CD	6
CD + vehicle	Female	Vehicle	CD	12
HFD + vehicle	Male	Vehicle	HFD	6
HFD + vehicle	Female	Vehicle	HFD	12
CD + NP	Male	NP	CD	12
CD + NP	Female	NP	CD	8
HFD + NP	Male	NP	HFD	12
HFD + NP	Female	NP	HFD	9

CD: control diet (16% kcal from fat); HFD: high-fat diet (30% kcal from fat); NP: nonylphenol.

The diet was initiate in the 7th week of life of the F1 animals, the composition of HFD is described in Table 2, and the composition of CD is described in Table 3.

Table 2. Composition of a high-fat diet.

Ingredient	Amount (%)	Amount (g/mL)	Energy from fat (%)
Corn starch	35.35	4.27735	0
Casein	20.0	2.42	0.46
Dextrinized starch	10.0	1.21	0
Sucrose	10.0	1.21	0
Soybean oil	4.0	0.484	8.31
Lard	10.6	1.2826	22.02
Microcrystalline cellulose	5.0	0.0605	0
Mineral mix AIN-93G	3.5	0.4235	0
Vitamin mix AIN-93G	1.0	0.121	0
L-cystine	0.3	0.0363	0
Choline bitartrate	0.25	0.03025	0
Butylated hydroxytoluene (BHT)	0.005	0.000605	0.01
Blue dye	0.005	0.000605	0
Total of energy from fat			**30,8**

The percentage of energy derived from fat was calculated based on the energy density of macronutrients (9 kcal/g for lipids and 4 kcal/g for proteins and carbohydrates) (FAO/WHO, 2003).

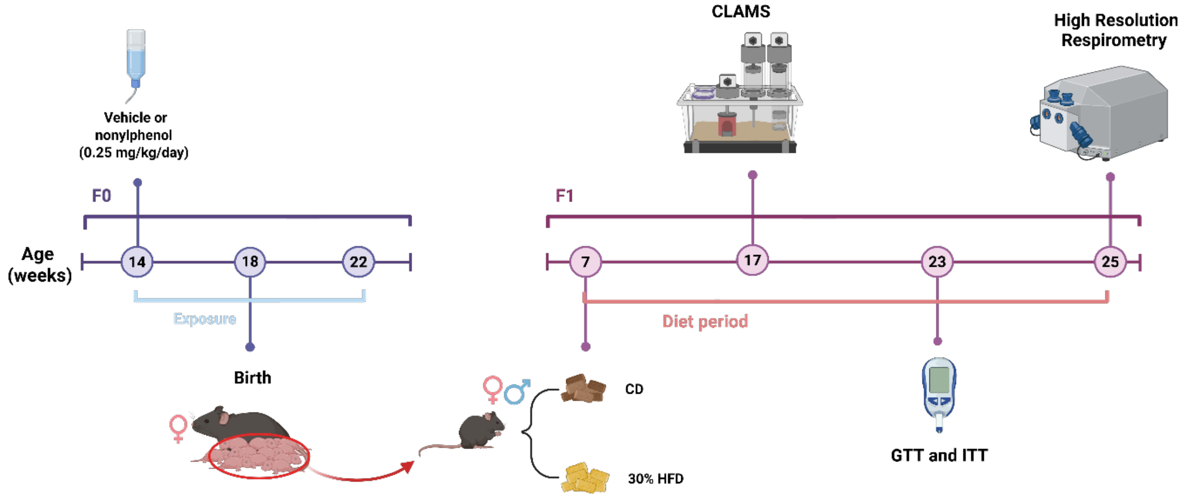
Table 3. Composition of the control diet (AIN-93G).

Ingredient	Amount (%)	Amount (g/mL)	Energy from fat (%)
Corn starch	39.75	4.80975	0
Casein	20.0	2.42	0.46
Dextrinized starch	13.2	1.5972	0
Sucrose	10.0	1.21	0
Soybean oil	7.0	0.847	15.95
Microcrystalline cellulose	5.0	0.605	0
Mineral mix AIN-93G	3.5	0.4235	0
Vitamin mix AIN-93G	1.0	0.121	0
L-cystine	0.3	0.0363	0
Choline bitartrate	0.25	0.03025	0
Butylated hydroxytoluene (BHT)	0.0014	0.000169	0.003
Total of energy from fat			**16,413**

The percentage of energy derived from fat was calculated based on the energy density of macronutrients (9 kcal/g for lipids and 4 kcal/g for proteins and carbohydrates) (FAO/WHO, 2003).

Animals were submitted to body weight and diet consumption measurement, assessment of energy expenditure using comprehensive laboratory animal monitoring system (CLAMS, Columbus®), glucose and insulin tolerance test (GTT and ITT) and high-resolution respirometry. At the age of 28 weeks, mice were euthanized with isofluorane anesthesia followed by decapitation, and inguinal WAT was collected for assessment of mitochondrial activity by high-resolution respirometry (Figure 1).

Figure 1. Schematic representation of the experimental design



F0 represents pregnant mice that were exposed to NP 0.25 mg/kg/day or vehicle DMSO in drinking water. The F1 represents the offspring that were divided into 8 groups (CD+Vehicle, HFD+Vehicle, CD+NP, HFD+NP for male and for female). At From 17 to 20 weeks of age F1 was submitted to CLAMS analysis, at 23 weeks of age, to GTT and ITT tests, and at 25 weeks of age, to euthanasia and concurrently for oximetry. Made by Biorender.

3.2 Body Weight and Diet Consumption

The animals and the diet were weighed weekly. One week before the beginning of CLAMS analysis, there was excessive manipulation of animals. Therefore, to avoid bias, weight and food intake data until 16 weeks of age were analyzed.

3.3 Lee Index

The Lee Index, introduced by Lee in 1928, is calculated by dividing the cubic root of body weight (in grams) by the naso-anal length (in centimeters) (BERNARDIS; PATTERSON, 1968). On the day of euthanasia, animals were weighed and after anesthesia, a picture from the animal with a ruler was obtained. ImageJ software was used to calculate the length measures.

3.4 Energy expenditure

Energy expenditure (EE) was calculated using the modified Weir equation, by determining the product of the caloric equivalent of oxygen (3.815 + 1.232 × RER) and oxygen consumption (VO_2), where RER represents the respiratory exchange ratio (carbon dioxide production / oxygen consumption or VCO_2/VO_2). The animals were submitted to CLAMS between 18 and 20 weeks of age. They were placed in individualized cages with free access to water and food for 96 h. The first 24 h analysis was discarded, since they comprised the acclimatation period. HEAT (energy expenditure (EE) (kcal/h/kg)) measures were used. Six animals per group (N=6) were used in EE assessment, except for the HFD+Vehicle group, where N=5.

3.5 Glucose Tolerance Test

Mice were fasted for 6 h during the morning, and blood glucose levels were assessed before (0 min) and after (15, 30, 60 and 120 min) the administration of glucose intraperitoneally (2 g/kg of body weight, in 0.9% saline) ALQUIER; POITOUT, 2017). Blood was collected from the tail vein and measured using a handheld glucometer (Accuchek Performa, Roche, Germany).

3.6 Insulin Tolerance Test

Six mice per group were fasted for 6 h during the morning, and blood glucose levels were assessed before (0 min) and after (15, 30, 60 and 90 min) the administration of regular insulin intraperitoneally (0.05 UI g/kg of body weight, human insulin Novolin® R) (ALQUIER; POITOUT, 2017). Blood was collected from the tail vein and measured using a handheld glucometer (Accuchek Performa, Roche, Germany). Some animals were unable to complete the test because several mice developed severe hypoglycemia during the procedure, requiring glucose administration. Sample sizes for the experimental groups remained as follows: for males, CD+Vehicle (n=4), HFD+Vehicle (n=6), CD+NP (n=4), and HFD+NP (n=6); for females, CD+Vehicle (n=6), HFD+Vehicle (n=4), CD+NP (n=5), and HFD+NP (n=6).

3.7 High-resolution respirometry

Oxygen consumption was assessed using high-resolution respirometry (HRR) with an Oroboros 2k Oxygraph (Oroboros Instruments, Innsbruck, Austria) at 37°C. The oxygraph system is a closed chamber that measures changes in oxygen (O_2) concentration. Any variation in O_2 levels is attributed to the samples, which consume oxygen and utilize the substrates or drugs added during the experiment, enabling the assessment of specific mitochondrial states.

Subcutaneous white adipose tissue (scWAT) was collected and cut into small pieces (1-2 mm). The tissue was submerged in 2.5 mL of BIOPS K^+ (CaK_2 EGTA 2.77mM, K_2 EGTA 7.23mM, Calcium 0.1uM, Imidazole 20mM, Taurine 20mM, K-MES 50mM, DTT 0.5mM, $MgCl_2$ 6.56mM; pH=7.1) and 0.1% BSA fatty free, and stirred at 100 RPM for 20 min. Thereafter, the tissue was dried and submerged in another solution of 2.5 mL of BIOPS + 0.05 mg/mL of Saponin, and stirred at 100 RPM for 20 min. Following this, the tissue was dried, weighted and added to a 2mL chamber containing MIR 05 ($MgCl_2.6H_2O$ 3mM, Lactobionic acid 60mM, Taurine 20mm, EGTA 0.5mM, KH_2PO_4 10mM, HEPES 20mM, D-sacarose 110 mM, BSA Fatty Free 1g/L; pH=7.1) and 40 µL of 0.1% BSA fatty free. Initially, pyruvate and malate (PM) 5 mM and 2.5mM, respectively, were added, followed by succinate (S) 10mM and adenosine diphosphate (ADP) 5 mM. Subsequently, oligomycin (OMY) 0.3 mg/mL was added to inhibit ATP synthase. Next, ~8 µM uncoupler protonophorecarbonyl cyanide m-chlorophenylhydrazone (CCCP) was added by titration, followed by rotenone (ROT) 0.75 µM and sequentially antimycin A (AA) 1 µM. The combination of PM substrates fuels complex I (CI), and S directly fuels complex II (CII) from electron transport system (ETS). Therefore, the combination of PM+S measures the activity of both complexes (CI+CII ETS). The addition of ADP represents the measures of CI and CII activity coupled with oxidative phosphorylation, when the proton gradient is harnessed to synthesize ATP (CI+CII OXPHOS). Conversely, OMY measures the CI and CII respiration without the function of ATP synthase. The addition of CCCP represents the maximum oxygen consumption rate, achieved by shuttling protons across the inner membrane, effectively uncoupling the ETS. The ROT addition can inhibit CI, blocking the transfer of electrons from NADH, and decreasing the oxygen consumption depending on that complex. Finally, AA can inhibit complex III (CIII) and reduce oxygen consumption to baseline levels.

3.8 Statistical analysis

Outlier Grubb's test was performed, and outlier animals were removed from all the analyses. The Shapiro-Wilk normality and lognormality test was also performed, and the results indicated a normal distribution. Data were presented as mean ± standard error of mean (SEM), and analyses were performed using GraphPad Prism 10.4.1. Two-way ANOVA was performed for all data, followed by post-hoc Turkeys' analysis. Statistical significance was considered as $p < 0.05$.

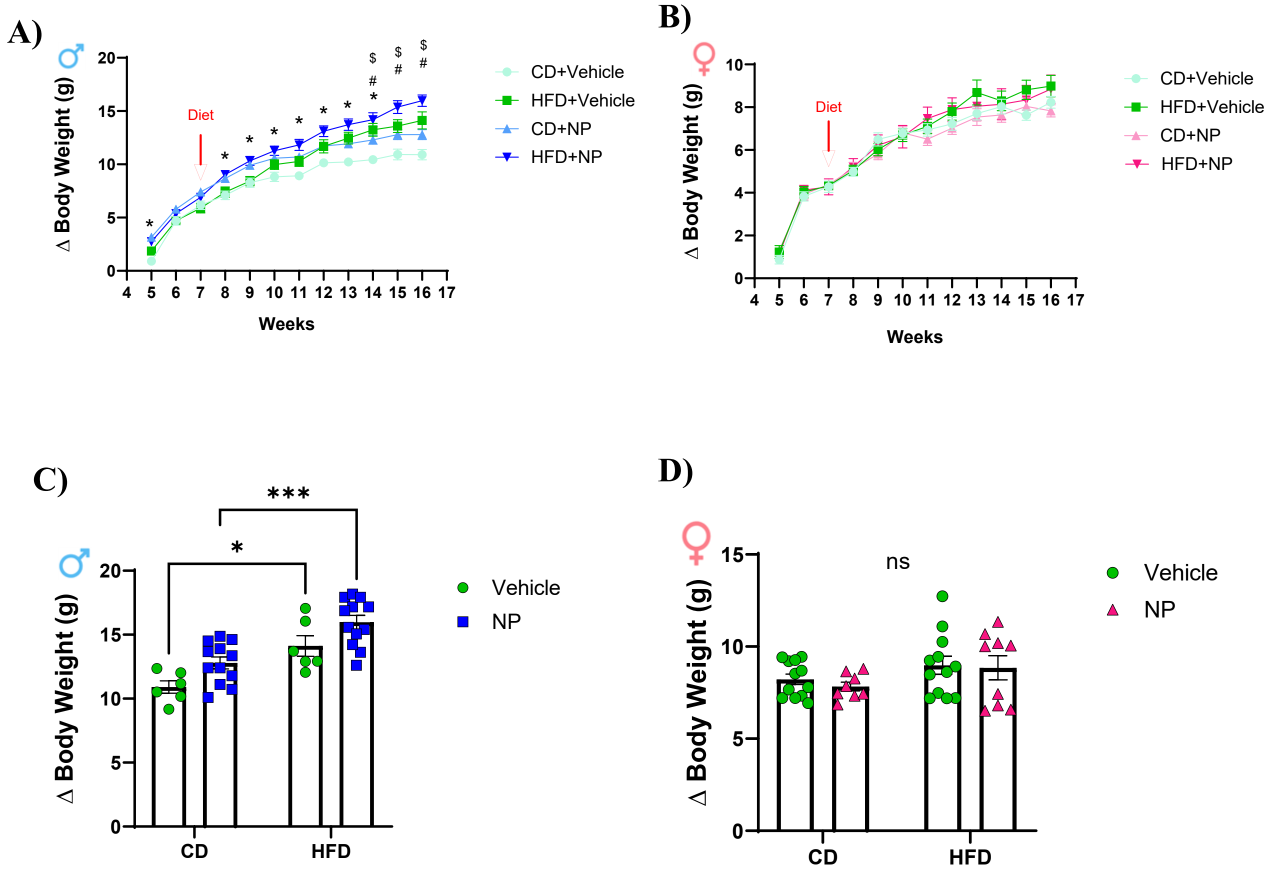
4. Results

4.1 Perinatal exposure to nonylphenol affects weight gain in adulthood in a sexually dimorphic fashion

For males, there was a significant interaction between treatment and diet in animals' weight (P<0.001). CD+NP group had increased weight gain when compared with CD+Vehicle group (P<0.05), indicating that NP can increase weight gain, independently from the diet. At the final weeks of evaluation, there was an increase of body weight in the HFD+Vehicle and HFD+NP groups when compared with CD+Vehicle (P<0.05) and CD+NP (P<0.05) groups, respectively, implying that the 30% HFD could significantly increase body weight (Figure 2A). The Δ body weight between the last and the first week of weighting revealed HFD-fed mice in both exposures (vehicle and NP) had increased body weight: CD+Vehicle x HFD+Vehicle (P<0.05) and CD+NP x HFD+NP (P<0.001). However, there were no differences between vehicle and NP exposed animals within the same diet, either under control diet or HFD conditions (Figure 2C).

There were no significative differences between any weight data from female mice groups (Figure 2B, D).

Figure 2. Nonylphenol increases weight gain from male fed with CD, but not with HFD.

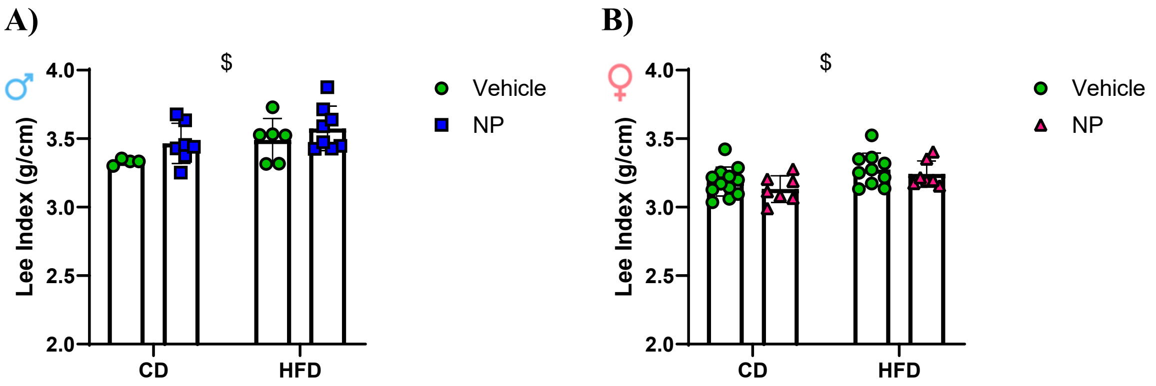


(A) Line graphic of delta body weight gain in male mice until 16 weeks of age. (B) Line graphic of delta body weight in female mice until 16 weeks of age. (C) Grouped bar graph showing the cumulative change in body weight in male mice up to 16 weeks of age. (D) Grouped bar graph showing the cumulative change in body weight in female mice up to 16 weeks of age. Data presented as mean ± SEM and analyzed by two-away ANOVA followed by post-hoc Tukey test. N = 7 males, 12 females (CD+Vehicle), N = 6 males, 12 females (HFD+Vehicle), N = 12 males, 8 females (CD+NP), N = 12 males, 9 females (HFD+NP). *p<0.05, ***p<0.001 between CD+Vehicle and CD+NP; $^{\#}$p<0.05 between CD+Vehicle and HFD+Vehicle; $^{\$}$p<0.05 between CD+NP and HFD+NP. ns = not significant. Outlier animals were removed.

4.2 Perinatal exposure to nonylphenol does not affect the Lee index during adulthood

The ANOVA performed for Lee Index showed a significant effect of diet in both males ($P<0.05$) and females ($P<0.05$). However, the post-hoc analysis demonstrated no significative differences between groups in either males or females (Figure 3A, B).

Figure 3. Nonylphenol did not alter Lee Index.

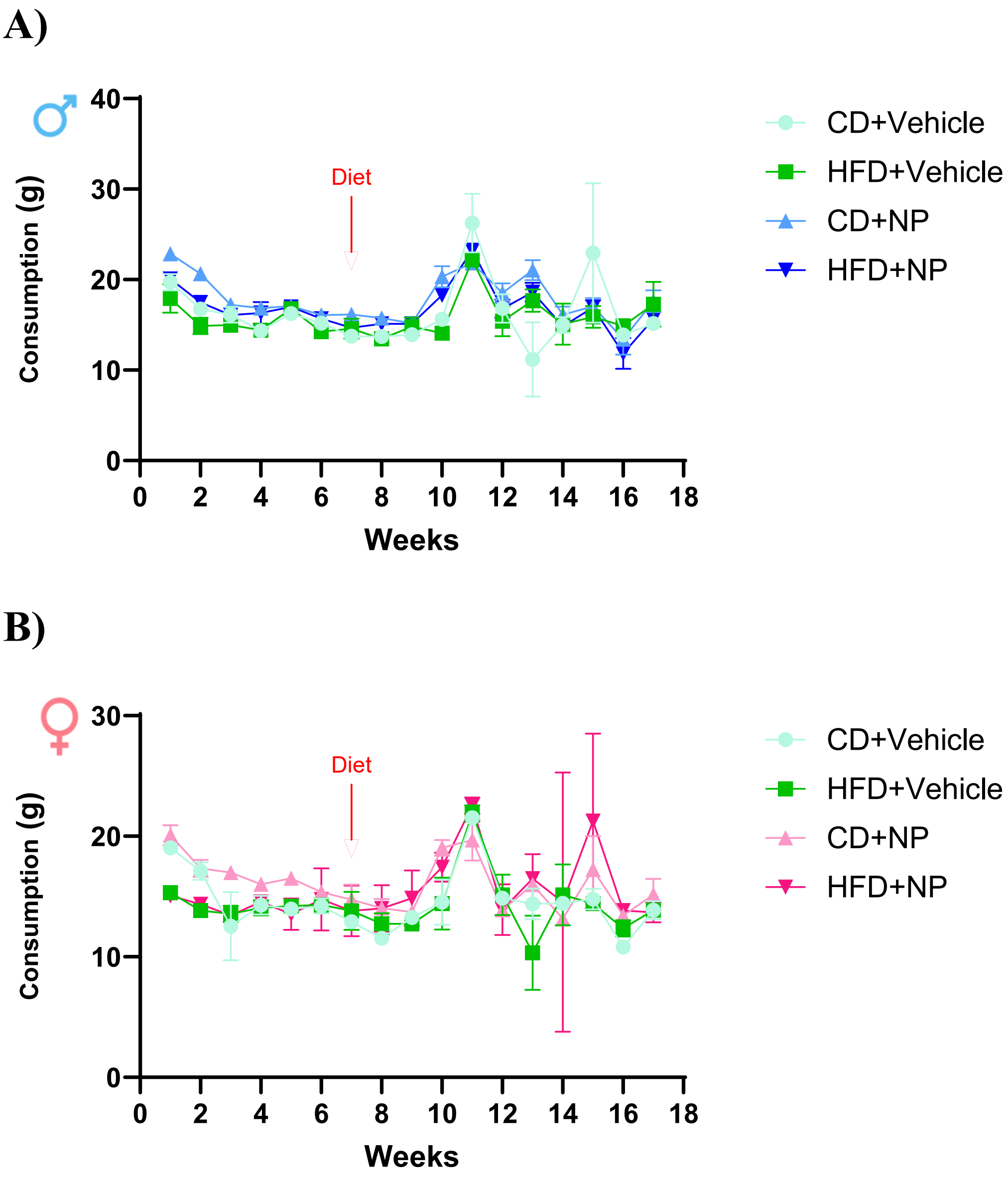


(A) Lee Index from male mice. (B) Lee Index from female mice. Data presented as mean ± SEM and analyzed by two-away ANOVA followed by post hoc Tukey test. N = 5 males, 12 females (CD+Vehicle), N = 6 males, 10 females (HFD+Vehicle), N = 7 males, 7 females (CD+NP), N = 8 males, 7 females (HFD+NP). $^{\$}p<0.05$ in ANOVA, for diet. Outlier animals were removed.

4.3 Perinatal exposure to nonylphenol does not affect food consumption in adulthood

The ANOVA test performed for food consumption showed no differences between groups for either male or female group (Figure 4A, B).

Figure 4. Nonylphenol did not alter diet consumption.

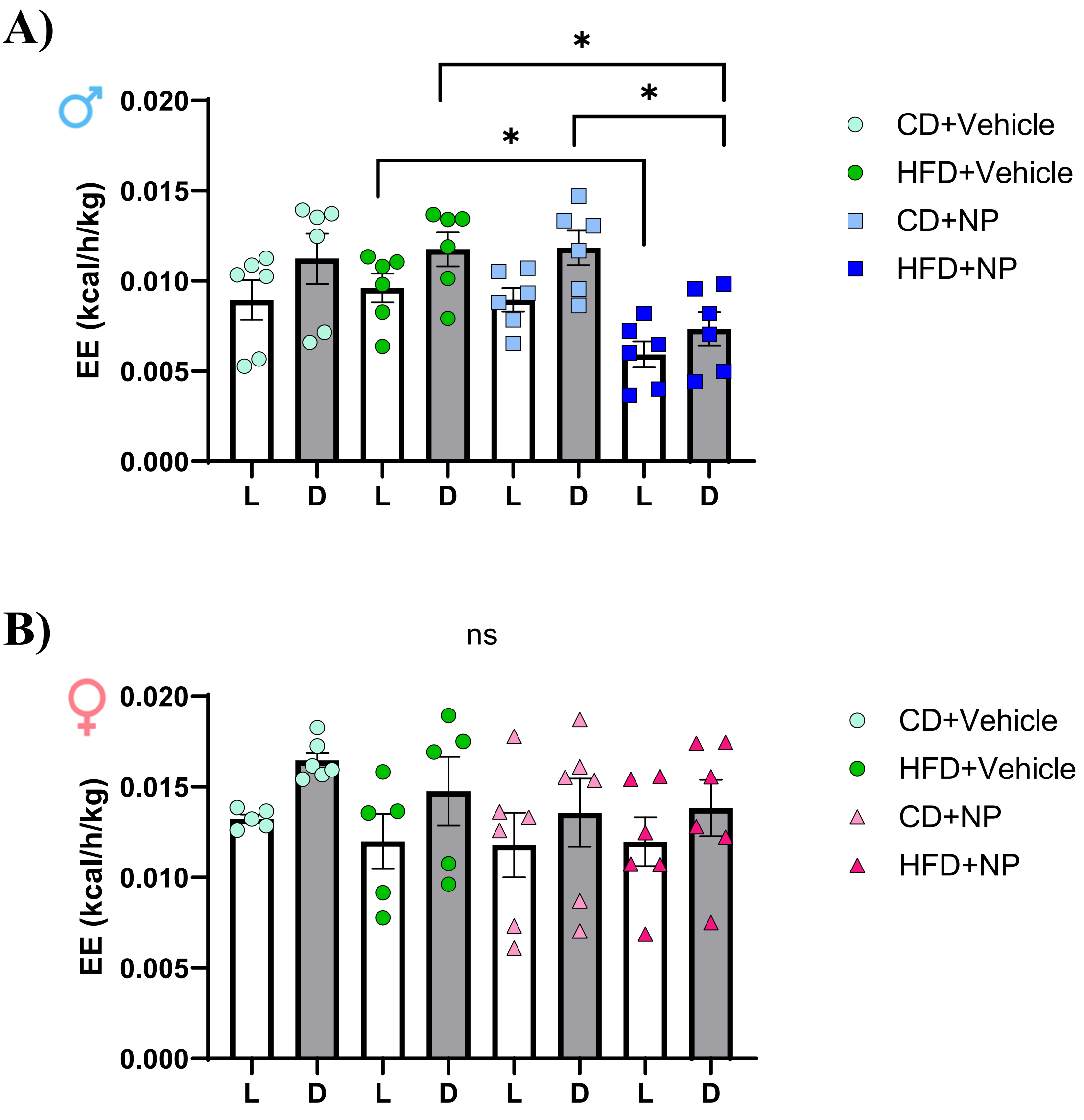


(A) Line graphic of diet consumption from male mice until 18 weeks of age. (B) Line graphic of diet consumption from female mice until 18 weeks of age. Data presented as mean ± SEM and analyzed by two-away ANOVA followed by post hoc Turkey test. ns = not significant. N = 7 males, 12 females (CD+Vehicle), N = 6 males, 12 females (HFD+Vehicle), N = 12 males, 8 females (CD+NP), N = 12 males, 9 females (HFD+NP). Outlier animals were removed.

4.4 Perinatal exposure to nonylphenol affects energy expenditure in adulthood in a sexually dimorphic and diet-dependent fashion

A decrease in EE for male mice from HFD+NP group was observed when compared with HFD+Vehicle, in both light and dark cycle (P<0.05), and with CD+NP (P<0.05), in dark cycle, demonstrating that NP along with the 30% diet can decrease the EE (Figure 5A). There were no differences between female groups in EE (Figure 5B).

Figure 5. Nonylphenol can decrease EE in male mice.

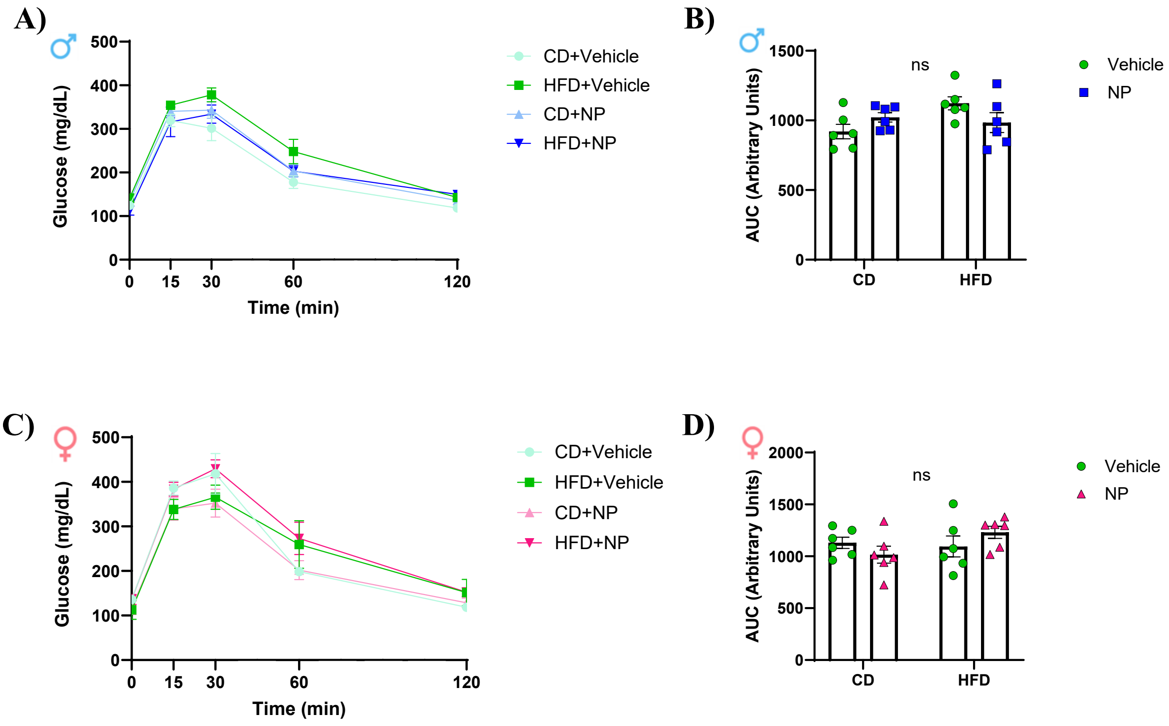


(A) HEAT measure in light and night cycles of male mice. (B) HEAT measure in light and night cycles of female mice. Data presented as mean ± SEM and analyzed by two-away ANOVA followed by post hoc Turkey test. *p < 0,05; ns = not significant. N = 5 for female HFD+Vehicle and N = 6 for all other groups. Outlier animals were removed. L (light) and D (dark).

4.5 Perinatal exposure no nonylphenol does not affect glucose tolerance in adulthood

Neither NP exposure nor 30% diet could interfere in glucose tolerance in both male and female offspring mice (Figure 6A, B, C, D).

Figure 6. Nonylphenol did not alter glucose tolerance.

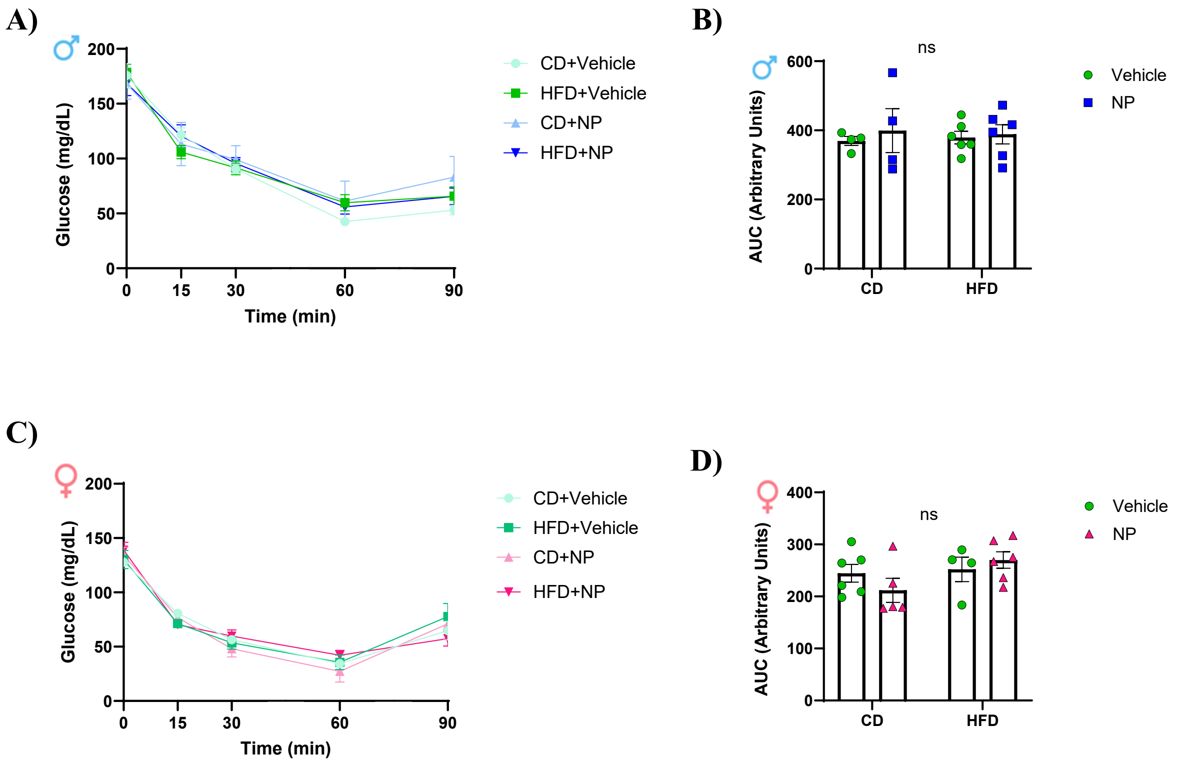


(A) Line graphic of GTT measures from male mice in different timepoints. (B) AUC of GTT from male mice. (C) Line graphic of GTT measures from female mice in different timepoints. (D) AUC of GTT from female mice. Data presented as mean ± SEM and analyzed by two-away ANOVA followed by post hoc Turkey test. ns = not significant. N = 6 for all groups.

4.6 Perinatal exposure no nonylphenol does not affect insulin sensibility.

Neither NP exposure nor 30% diet could interfere in insulin sensibility in both male and female offspring mice. (Figure 7A, B, C, D). Due to severe hypoglycemia in some animals, the following groups had animals removed: CD+Vehicle male, CD+NP male, CD+NP female and HFD+Vehicle female.

Figure 7. Nonylphenol did not alter insulin sensibility.

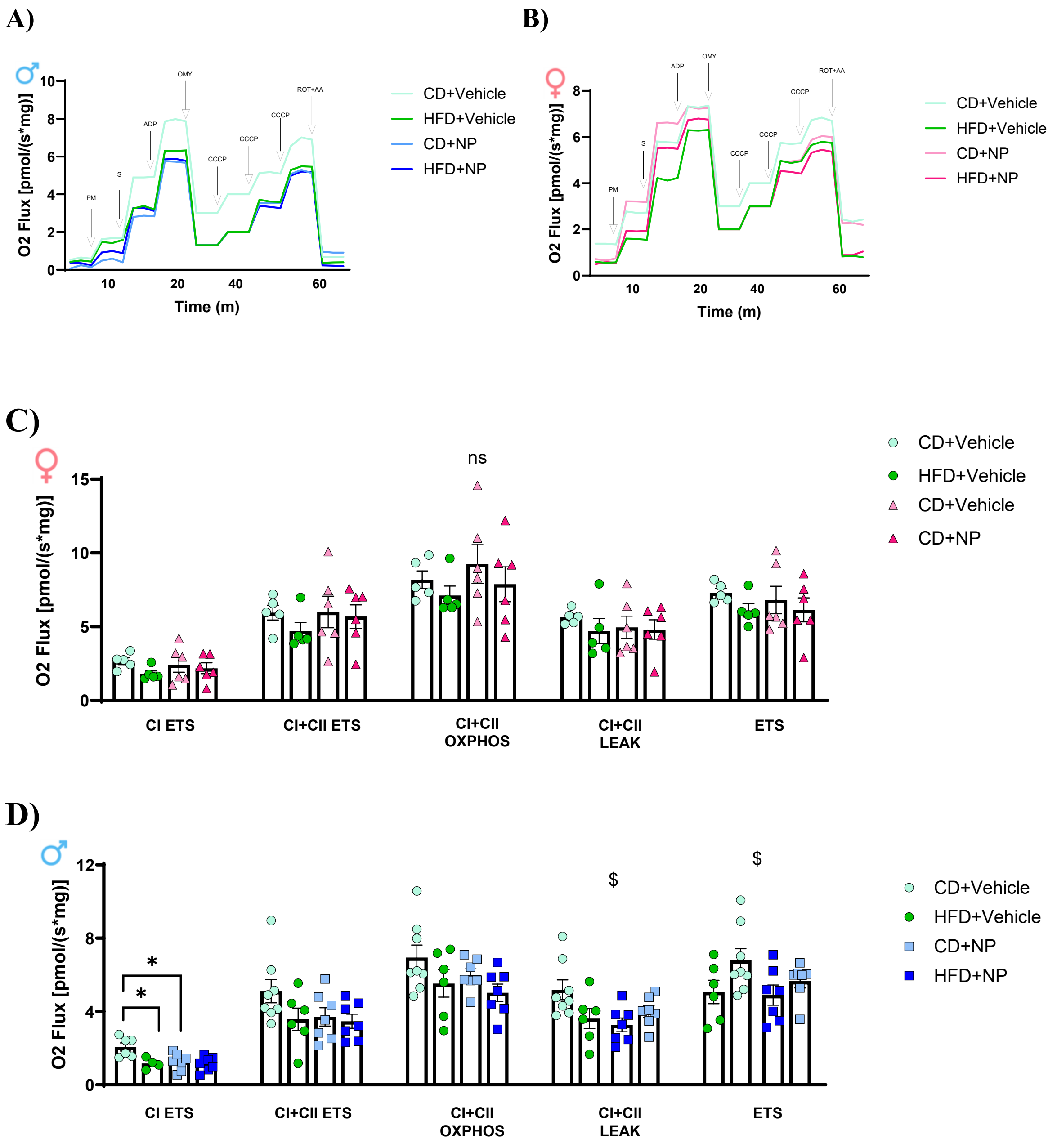


(A) Line graphic of ITT measures from male mice in different timepoints. (B) AUC of ITT from male mice. (C) Line graphic of ITT measures from female mice in different timepoints. (D) AUC of ITT from female mice. Data presented as mean ± SEM and analyzed by two-away ANOVA followed by post hoc Turkey test. ns = not significant. N = 4 males, 6 females (CD+Vehicle), N = 4 males, 5 females (CD+NP), N = 6 males, 4 females (HFD+Vehicle), N = 6 males, 6 females (HFD+NP).

4.7 Perinatal exposure to nonylphenol affects mitochondrial complex I electron transport system in inguinal white adipose tissue

In male offspring, a decrease in mitochondrial respiration was observed inCD+NP (P<0.05), and HFD+Vehicle (P<0.05) groups, when compared with CD+Vehicle. An effect of diet in CI+CII LEAK (P<0.05) and ETS (P<0.05) was also observed. Nonetheless, the post hoc test in CI+CII LEAK and ETS did not show differences between groups (Figure 8C). In female offspring, no significant effects were observed (Figure 8D).

Figure 8. Nonylphenol causes an effect in male scWAT mitochondrial complex I.



(A) Representative graph of HRR analysis of scWAT from all male groups. (B) Representative graph of high-resolution respirometry analysis of scWAT from all female groups. (C) High-resolution respirometry analysis of scWAT from male mice. (D) High-resolution respirometry analysis of scWAT from female mice. Data presented as mean ± SEM and analyzed by two-away ANOVA followed by post hoc Turkey test. ns = not significant. N = 6 male, 5 females (CD+Vehicle), N = 4 male, 5 females (HFD+Vehicle), N = 7 male, 6 females (CD+NP), N = 7 male, 6 females (HFD+NP). Outlier animals were removed. $^{\$}$p<0.05 in ANOVA for diet, $^{\#}$p<0.05 in ANOVA for treatment.

5. Discussion

The adipogenic potential of NP has already been reported. NP and its derivatives act as obesogens, promoting adipocyte differentiation, lipid accumulation, and the development of obesity through the activation of molecular pathways related to adipogenesis, especially the PPARγ receptor (HAO *et al.*, 2012; KASSOTIS *et al.*, 2022; ZHANG, QI *et al.*, 2022). Nevertheless, there still is a lack of studies showing this potential in offsprings. The gestational phase is a critical moment for fetal development, therefore, the exposure to EDCs at this moment can lead to many consequences, such as gestational diabetes mellitus, spontaneous abortions, and many others epigenetic changes. Importantly, increased EDC exposure has been associated with dose-dependent impairments in fetal growth, characterized by proportional reductions in offspring birth weight and body length (PUCHE-JUAREZ *et al.*, 2023). Compared to adults, fetuses exhibit reduced or absent expression and activity of several cytochrome P450 enzymes responsible for the metabolism of environmental chemicals and pharmaceuticals. Furthermore, numerous time-dependent and highly coordinated developmental processes occur during early life, and their disruption may increase the susceptibility to diseases during childhood (BRAUN, 2017).

In this study, a deregulation effect of NP, an EDC, was observed in male mice exposed during critical periods of development. The NP was capable of increasing weight gain in mice fed with CD, despite this effect not being observed in mice fed with HFD. This data suggests that a HFD may mask the NP effect. The weight gain induced by a HFD is already known, however, this effect might be so pronounced that additional changes caused by an EDC might become difficult to be statistically biologically detected (ALONSO-MAGDALENA *et al.*, 2019). On the other hand, in female mice, an increase in body weight was not observed. This might be explained by male and female mice different responses to HFD. Males typically develop rapid hyperphagia, pronounced weight gain, and severe metabolic disturbances, whereas females show relative resistance to these effects, a protection that can be linked to estrogen-mediated suppression of food intake (HUANG, KUEI PIN *et al.*, 2020). These findings suggest an obesogenic potential of NP in male offspring (Figure 2).

When interpreting weight gain, it is important to consider that obesity in humans is clinically defined by a body mass index (BMI) ≥ 30 kg/m^2, which reflects excessive weight gain. This excessive weight gain largely arises from an imbalance between caloric intake and energy expenditure, with excessive dietary energy consumption serving as the main driver of its development. Epidemiological evidence indicates that typical Western diets provide approximately 30–40% of total energy from fat (LAI; CHANDRASEKERA; BARNARD, 2014). In experimental research, however, rodent models often employ HFDs containing up to 60% of calories from fat, which can induce rapid and severe metabolic disturbances, what does not accurately reflect the gradual onset of obesity and metabolic syndrome observed in humans (STOTT; MARINO, 2020). Diets with extremely high content of fat can provoke excessive food intake, accelerated weight gain, and metabolic overload responses that rarely occur in human populations (SKALSKI *et al.*, 2024). In contrast, moderate HFDs, containing 30–45% of total energy from fat, resemble more closely human dietary patterns and promote a slower and progressive development of insulin resistance and adiposity (STOTT; MARINO, 2020). This approach allows for more physiologically relevant modeling of chronic metabolic disorders. Furthermore, it enables the study of complex interactions with other factors, such as genetics, aging, and environmental endocrine disruptors, without eliciting confounding non-physiological stress responses. Therefore, moderate-fat diets are generally considered more appropriate for translational studies aiming at modeling human obesity and its related metabolic dysfunctions.

Besides the difference between NP-treated and untreated mice, we also observed a difference between male mice feed a 30% and a 16% fat diet (Figure 2C), proving that a moderate increase of fat in diet can also result in weight gain in animals, inducing more physiologically relevant outcomes. In addition, the lack of change in dietary intake (Figure 4) may indicate that neither NP nor 30% HFD affect the feeding behavior of the mice, which means that the obesogenic effect was not induced by increased consumption.

Despite increasing body weight, neither NP nor the 30% diet were able to induce obesity in mice using the Lee index (Figure 3). However, it is important to emphasize that the Lee index is an indirect measure of body fat, and, therefore, can be considered inaccurate (WEBB, 1980).

Some studies suggested that NP and other EDCs exposure can lead to diabetes development in some animal models, through mechanisms dependent on insulin resistance and disfunction of β cells (LI *et al.*, 2017; LIND; LIND, 2018; MARTÍNEZ-PINNA *et al.*, 2023). However, our study showed no difference between vehicle and NP exposure groups in GTT and ITT. Even with the diet intervention, the offspring mice exposed to NP did not present glucose or insulin intolerance. One possible explanation could be that neither the 30% diet nor NP at ambient concentrations can decrease glucose tolerance and insulin sensitivity.

The calorimetry analyzes O_2 and carbon dioxide (CO_2) exchange dynamics, specifically the differences in O_2 depletion and CO_2 accumulation. The system calculates key metabolic parameters, including VO_2, CO_2 production (VCO_2), and the VCO_2/VO_2. This method delivers highly precise and repeatable measurements of whole-body EE, enabling reliable assessment of metabolic activity (BAYLOR COLLEGE OF MEDICINE, [N.d.]). EDCs are already known to alter the balance between energy intake and EE, reducing EE and promoting fat storage, especially during critical developmental windows (BALISE et al., 2019; NADAL et al., 2017). In the current research, the association of NP and HFD decreased the EE in both light and dark cycles of male mice. Despite males in HFD+Vehicle group presented weight gain compared to CD+Vehicle male group, we did not observe a decrease in their EE in HEAT analysis. However, the association of NP with HFD led to a decrease in EE. Also, a decrease in EE induced by HFD in NP-exposed males was observed, but not in vehicle-exposed groups, reinforcing that the association of NP with diet can cause a disbalance in energy intake in male offsprings. In female mice, the EE was not significantly different between groups (Fig 4 B), suggesting that NP could not affect the EE in female offspring mice. BAT is the principal driver of wholebody EE, since its mitochondria uncouple oxidative phosphorylation via, releasing heat instead of ATP (BOMBASSARO *et al.*, 2025; PEREZ *et al.*, 2022). A study with BPA combined with a HFD impaired BAT function through multiple mechanisms, including inflammation, thermogenic dysfunction, microvascular damage, and mitochondrial injury, leading to marked thermogenic dysfunction and lower EE in mice (ZHANG, HUIHONG *et al.*, 2025). These results could indicate that EDC exposure diminishes EE by disrupting BAT mitochondrial uncoupling, vascularization, and hormonal signaling, creating a mechanistic link between environmental chemicals and obesity--related energy imbalance. However, further studies are necessary to elucidate these findings. Unlike males, female mice did not suffer a decrease in EE, which can be explained through estrogen--mediated actions on BAT. Estradiol binds to estrogen receptor-α (ERα) in adipocytes, maintaining mitochondrial biogenesis and UCP1 expression, which sustains BAT uncoupling even when EDCs suppress sympathetic signaling (LAPID et al., 2014). In the RER analysis, no differences were detected between the groups (data not shown).

HRR provides an indirect calorimetric approach to assess metabolic heat changes by measuring oxygen consumption. Additionally, when integrated with assessments of mitochondrial membrane potential ($\Delta\Psi m$) generated by protons pumps (complex I, III and IV), respirometry offers deeper insights into bioenergetic efficiency, linking electron transport system activity to ATP synthesis and proton motive force dynamics (DOERRIER *et al.*, 2018).

Since oxidative phosphorylation (OXPHOS) represents a central process in cellular bioenergetics, extensive investigations have been conducted to elucidate the mechanisms of energy transduction within the mitochondrial electron transport system and to define mitochondrial respiratory regulation under physiological and pathological conditions (GNAIGER, 2020). In obesity context, it is important to investigate the role of cells such as white adipocytes. Although white adipocytes have fewer mitochondria, these organelles play critical roles in lipid handling and endocrine signaling. Mitochondria are essential for adipogenesis, lipid metabolism, endocrine signaling and redox homeostasis; consequently, mitochondrial dysfunction in WAT is recognized as a hallmark of obesity (ZHU; AN; SCHERER, 2022). Some *in vitro* studies in mouse cells lines and human and mouse adipocytes exposed to EDCs showed an impairment in $\Delta\Psi m$ and in oxygen consumption rate, accompanied by a decrease in the levels of mitochondrial respiration related genes (CHEHADE et al., 2022; HOWELL; YOUNG, 2024; LIU et al., 2021; WEN et al., 2024). In addition, in mice, the gestational exposure to the EDC benzene decreased energy homeostasis in both sexes, possibly through hypothalamic regulation (KOSHKO *et al.*, 2021).

Our study showed a significant decrease in CI ETS in CD+NP, when compared with CD+Vehicle, suggesting that NP exposure can interfere with the oxygen consumption of mitochondrial complex I. Complex I is the first enzyme in the mitochondrial respiratory system (MRS), initiating ETS by transferring electrons from NADH to drive ATP production. Proper oxidation of upstream metabolites, such as pyruvate and malate, is essential to sustain this mechanism. A study using tolylfluanid in isolated mitochondria from BAT demonstrated that this compound could reduce pyruvate uptake by directly inhibiting the MRS (CHEN *et al.*, 2018). This reinforces the idea that EDCs may affect MRS primarily by inhibiting the production of molecules necessary for the initial stages of the system.

HFD is already known to cause mitochondrial disfunction and decrease mitochondrial respiration in WAT (XIA *et al.*, 2024). In this work, we saw a decrease in CI ETS in HFD+Vehicle group, when compared with CD+Vehicle, indicating that HFD can interfere with the oxygen consumption of mitochondrial complex I. Furthermore, HFD+NP did not present a decrease when compared to HFD+Vehicle. This finding is consistent with the results of the weight analyses, which likewise indicated that the association of a HFD with NP might have masked the effects of the contaminant itself. Also, CI+CII LEAK, which represents respiration independent of ATP synthase, and ETS, that reflects the maximum capacity of the phosphorylation system, were both affected by diet in male mice. These results suggest a potential mechanism through which HFD decreases the oxygen flux. In a study where mice were fed with HFD for 24 weeks, maximal oxygen consumption (ETS) was markedly reduced compared with chow-fed controls, indicating a diminished maximal respiratory capacity (or ETS reserve). This impairment is associated with: downregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1a) and other mitochondrial biogenesis genes, resulting in fewer functional respiratory complexes; and accumulation of lipidic intermediates that inhibit electron flux through complexes I and II, thereby limiting the maximal electron transport that can be stimulated by CCCP (SCHÖTTL *et al.*, 2015). In addition to impairing uncoupled respiration, HFD also suppressed respiration independent of ATP synthase (CI+CII LEAK). Eight weeks after HFD, basal leak respiration of scWAT normalized to adipocyte number was significantly reduced, indicating compromised intrinsic proton conductance of the inner mitochondrial membrane. This reduction is associated with increased mitochondrial ROS production and changes in membrane lipid composition, which are likely to decrease membrane permeability and proton leak (POLITIS-BARBER *et al.*, 2020). These findings suggest that a HFD diminishes both the maximal uncoupled respiration (via loss of ETS capacity and mitochondrial content), and the respiration independent of ATP synthase (through membrane remodeling and oxidative damage), thereby impairing the overall oxidative flexibility of WAT. Nonetheless, the current results are not sufficient to fully elucidate this mechanism, and further studies are needed.

Finally, it is of notice that sexual dimorphism was clearly perceived in this study, since female mice groups did not present the same differences observed among male mice groups. The NP exposure did not affect female mice metabolism, and the absence of metabolic alterations in female offspring following gestational exposure to NP may be explained by sex-specific biological mechanisms. Estrogen itself exerts protective roles in energy metabolism by promoting mitochondrial biogenesis, oxidative phosphorylation efficiency and fatty acid oxidation through the activation of PGC-1α and other mitochondrial transcriptional regulators. These effects might counteract the mitochondrial oxidative stress and bioenergetic impairments typically induced by EDC (BAUZÁ-THORBRÜGGE *et al.*, 2019; BEIKOGHLI KALKHORAN; KARARIGAS, 2022; CAPLLONCH-AMER *et al.*, 2014). These mechanisms may underlie the observed sexual dimorphism in response to NP exposure, with estrogenic signaling conferring relative metabolic resilience to female offspring. A greater susceptibility of males compared to females to EDC has also been reported. In a study using the persistent environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), male mice exhibited more pronounced metabolic impairments than females, including a substantial loss of metabolic sensitivity following exposure (HOYECK et al., 2020). These findings underscore clear sex-specific differences in the metabolic response to EDC exposure. However, sex-specific responses to EDC exposure vary according to the nature of the compound, the administered dose, the developmental stage or age of the animals, and potentially through interactions with dietary composition (LUIZA DOS SANTOS RODRIGUES VAZ *et al.*, 2026). A substantial proportion of studies investigating the effects of EDC has been conducted exclusively in male mice, despite clear evidence that females are also susceptible to EDC-induced alterations (PALANZA *et al.*, 2021). Therefore, the inclusion of female mice in experimental designs is essential, as sex-dependent differences in hormonal regulation, metabolism, and tissue responsiveness can significantly influence the biological effects of EDC exposure. Accounting for these differences not only improves the translational relevance of animal models but also enables a more comprehensive understanding of the mechanisms of action of EDCs, thereby reducing bias and strengthening the interpretation of toxicological and metabolic outcomes.

6. Conclusion

Collectively, the findings from the present study highlight that gestational NP exposure can alter metabolic programming in male offspring, particularly under specific dietary conditions, by modulating energy expenditure and mitochondrial function in WAT. The absence of comparable effects in females underscores the importance of considering sexual dimorphism in metabolic toxicology. Further research is warranted to elucidate the precise molecular mechanisms underlying these observations, particularly the interaction between EDC exposure, diet, and mitochondrial bioenergetics during early developmental windows. Also, the study further reinforces that even moderate-fat diets (30% energy from fat) are sufficient to induce physiologically relevant weight gain in animal models, closely resembling human dietary patterns. Importantly, this approach allowed the detection of interactions between diet composition and chemical exposure without eliciting extreme metabolic disturbances.

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ANNEX I

Universidade de Brasília
Comissão de Ética no Uso Animal

Brasília, 6 de dezembro de 2023.

CERTIFICADO

Certificamos que o projeto intitulado **“Exposição a desreguladores endócrinos em períodos críticos do desenvolvimento e desfechos metabólicos e cognitivos na vida adulta”**, SEI nº 23106.040370/2023-00, sob responsabilidade do(a) pesquisador(a) Angélica Amorim Amato, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal – CONCEA. Esse projeto foi avaliado e aprovado pela Comissão de Ética no Uso Animal (CEUA) da Universidade de Brasília na 196ª reunião ordinária, em 30/06/2023 para fins de PESQUISA e para utilização de *Mus musculus* (camundongo, C57Bl/6, 60 machos e 72 fêmeas – total 132) provenientes do(a) Biotério Central da Universidade estadual de Campinas (Unicamp).

O presente certificado é válido pelo período de 06/12/2023 a 10/08/2027.

Dr. Bruno Stéfano Lima Dallago
Coordenador da CEUA – UnB

*Este documento se restringe à avaliação ética do projeto supracitado e não substitui outras licenças e permissões que porventura se façam necessárias.