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The metabolic and anti-adiposity effects of oral copaiba-oil supplementation are influenced by sex and obesity

Os efeitos metabólicos e anti-adiposidade da suplementação oral com óleo de copaíba são influenciados pelo sexo e obesidade

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ABSTRACT

Copaiba oil (CO-oil) is commonly used as a topical healing and anti-inflammatory product in Brazil. However, recent data indicate that oral ingestion of CO-oil has anti-adiposity effects. In the present study, we evaluated the effects of oral CO-oil supplementation on body weight (BW), food intake (FI), adiposity and metabolism in male and female obese and non-obese rats. Monosodium glutamate (MSG; 4g/Kg) was administered during the first week after birth to induce hypothalamic obesity; control (CON; non-obese) rats received equimolar saline. After weaning (30 days of life), MSG and CON males and females were randomly subdivided into CO-oil supplemented (0.5mL/Kg; 3 times/week/8 weeks) and non-supplemented (NS) groups (n = 10-15 rats/group). BW, FI, feed efficiency (FE) and adiposity were registered, as well as fasting glucose (GLU), triglycerides (TGL) and total cholesterol (TC) values. Insulin resistance (IR) was assessed using the triglyceride-glucose index (TyG). Integrative principal component analysis (PCA) showed that chronic CO-oil supplementation alters FI and FE in MSG-obese and non-obese male rats, without modifying adiposity or metabolism. However, CO-oil supplementation of MSG-obese females reduced adiposity, TGL and improved IR, in relation to non-obese females. Thus, our data indicate that CO-oil oral supplementation influences males and females differently, having greater anti-adiposity effects and benefits on the metabolic state of obese female rats.

Keywords: oil-resin; natural compounds; antioxidant; obesity

RESUMO

O óleo de copaíba (CO-óleo) é comumente utilizado como produto tópico cicatrizante e antiinflamatório no Brasil. No entanto, dados recentes indicam que a ingestão oral de óleo de CO tem efeitos anti-adiposidade. No presente estudo avaliamos os efeitos da suplementação oral de óleo de CO no peso corporal (PC), ingestão alimentar (IA), adiposidade e metabolismo em ratos obesos e não obesos machos e fêmeas. Glutamato monossódico (MSG; 4g/Kg) foi administrado durante a primeira semana após o nascimento para induzir obesidade hipotalâmica; ratos controle (CON; não obesos) receberam solução salina equimolar. Após o desmame (30 dias de vida), machos e fêmeas MSG e CON foram subdivididos aleatoriamente em grupos suplementados com óleo de CO (0,5mL/Kg; 3 vezes/semana/8 semanas) e não suplementados (NS) (n = 10-15 ratos/grupo). Foram registrados PC, IA, eficiência alimentar (EA) e adiposidade, valores de glicemia de jejum (GLI), triglicerídeos (TGL) e colesterol total (CT). A resistência à insulina (RI) foi avaliada pelo índice triglicerídeo-glicose (TyG). A análise integrativa de componentes principais (PCA) mostrou que a suplementação crônica de óleo com CO altera IA e EA em ratos machos obesos e não obesos-MSG, sem modificar a adiposidade ou o metabolismo. No entanto, a suplementação com óleo de CO de ratas obesas-MSG reduziu a adiposidade, o TGL e melhorou a RI, em relação às ratas não obesas. Nossos dados indicam que a suplementação oral com óleo de CO influencia diferentemente machos e fêmeas, tendo maiores efeitos anti-adiposidade e benefícios no estado metabólico de ratas obesas.

Palavras-chave: óleo-resina; compostos naturais; antioxidante; obesidade.

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INTRODUCTION

Excessive white adipose tissue (WAT) deposition results in the disruption of metabolism, typically characterized by glucose intolerance, insulin resistance (IR), dyslipidemia and hypertension, conditions that define metabolic syndrome (MS). Obesity and MS elevate the risk of developing chronic diseases, in particular type 2 diabetes mellitus (T2DM) and cardiovascular disorders (Li *et al.*, 2022). For this reason, several surgical and pharmacological strategies have been used to mitigate the rapid progression of obesity. Healthy eating habits and the practice of regular exercise are well established methods to avoid excessive body weight gain; however, in some cases, this may not be sufficient to prevent fat mass accumulation and its deleterious effects to human health (Kao; Huang, 2021; Elgseer *et al.*, 2023). Thus, there is significant interest in the consumption of natural compounds that offer potential benefits for health and weight control in the global population (Shaik *et al.*, 2023; Naz *et al.*, 2023).

The Brazilian Amazon rainforest has a large diversity of plants with different medicinal activities, including the genus Copaifera, which is comprised of the species C. multijuga, C. reticulata, C. Officinalis and C. Langsdorffii, the most frequently encountered species in Brazil. Copaifera is a tree that can reach a height of 20 m, and its trunk yields a yellow brown oil resin that can be used for medical purposes. Copaíba-oil (CO-oil) is considered to be one of the most important Brazilian Amazon rainforest products for the economy (Frazão *et al.*, 2023).

Applications for the use of CO-oil as a diuretic, laxative, anti-tetanic, tumoral inhibitor and anti-inflammatory agent have previously been studied. However, due to its toxic oral effects, its known principal effects refer to it topical use (Pieri *et al.*, 2010; Garcia; Yamaguchi, 2012). Furthermore, the use of oral and topical routes for CO-oil administration have shown different effects on health outcomes. CO-oil consists of several compounds, including sesquiterpene hydrocarbons (β-caryophyllene, α-copaene, β-elemene, α-humulene, and germacrene D) and diterpene acids (copalic acid, kaurenoic acid, alepterolic acid, and polyalthic acid). Diterpene acids are commonly found in copaifera oleoresins and have shown remarkable biological activities, such as antibacterial, anti-inflammatory, antiproliferative and wound-healing effects (Trindade; Silva; Setzer, 2018). Interestingly, recent studies indicate that oral CO-oil administration could exert anti-adiposity effects (Telles *et al.*, 2022; Paula et al, 2024).

Obesity is frequently associated with a pro-inflammatory process that contributes to IR, dyslipidemia, and hyperglycemia (Kosmas *et al.*, 2023). Kaurenoic acid, a component of CO-oil, has been reported to decrease lipogenic effects in hepatic steatosis (Kim; Younossi, 2008), while oral administration of CO-oil to obese male rats resulted in a reduction in BW gain, adiposity, IR, plasma interleukin 6 (IL6) levels, and improved oxidative stress in WAT (Horácio *et al.*, 2017). However, the effects of CO-oil supplementation were different in non-obese male animals (Paula *et al.*, 2023). Hyperinsulinemia and IR play a central role in the pathophysiology of obesity, which is intimately associated with the disruption of the principal intracellular pathway that is stimulated by the insulin receptor in peripheral tissues (pI3K/Akt/mTOR). Importantly, an in vitro study has shown that this pathway could be modulated by copaiba essential oil (Urasaki *et al.*, 2020).

The administration of high doses of monosodium glutamate (MSG) during the neonatal period, in rats, results in hypothalamic lesions and obesity development during adulthood (Hernandez *et al.*, 2018). The principal hypothalamic nuclei to be damaged by MSG treatment is the arcuate nucleus (ARC), which participates in the control of metabolism, mediated by autonomic nervous system (ANS) activity, hormonal axis secretion modulation and energetic homeostasis (Hernandez Bautista; Mahmoud; Guerrero, 2018). Thus, as a consequence of ARC lesions, MSG-treated rats present massive visceral WAT deposition (Jais, Bruning, 2022), hyperinsulinemia, IR, dyslipidemia, glucose intolerance and cardiovascular dysfunctions (von Diemen; Trindade; Trindade, 2006; Dolnikoff *et al.*, 2011), thereby reproducing the principal characteristics of MS observed in obese humans. Moreover, due to the rupture of the hypothalamus-hypophyses axis (HPA), MSG-obese rats present a significant reduction in growth hormone (GH) and hypercortisolism, in addition to decreased sexual hormone production (Betran *et al.*, 1992;

NARDELLI *et al.*, 2011; HIRATA *et al.*, 2003; DE PAULA *et al.*, 2021). MSG-induced ARC lesions cause an imbalance of the ANS, resulting in lower metabolic energy expenditure without hyperphagia (OLNEY, 1969; ROMAN-RAMOS *et al.*, 2011).

Obesity exerts different metabolic consequences in male and female rodents (RUDYK *et al.*, 2018), and this response is also observed in the MSG-induced obesity model (de Paula *et al.*, 2021). The anti-inflammatory effects of intragastric CO-oil administration have been reported to be similar in both sexes (DALENOGARE *et al.*, 2019). However, it remains unclear whether sexual dimorphism modulates the anti- adiposity and beneficial metabolic effects of CO-oil supplementation. As such, we herein evaluated the effects of CO-oil supplementation on BW, FI, adiposity and metabolism in male and female MSG-obese and non-obese rats.

MATERIALS AND METHODS

Ethical aspects:

Male (n=50) and female (n=50) Wistar rats were subdivided into subgroups, based on obesity induction and CO-oil supplementation. During the lactation and growth period, rats received food and water ad libitum, and were maintained under controlled conditions with constant temperature (12 h light/cycle; 21 ± 2ºC), following the guidelines of the National Council for Control of Animal Experiments and based on the international standards for animal care and maintenance (PERCIE *et al.*, 2020). The Ethics Committee on Animal Use (CEUA) of the Western Parana State University (UNIOESTE) approved the experimental protocols on November 13, 2020, prior to the start of experimentation.

Hypothalamic obesity induction:

Pregnant female Wistar rats (n = 30), acquired from the university's central vivarium, were kept in individual cages, with free access to water and rodent chow in a satellite vivarium with controlled luminosity and temperature, as described above. After birth, the offspring size was adjusted to 6 – 8 pups per dam to assure adequate nutritional lactation, while maintaining a similar distribution of males (m; n=3 or 4) and females (f; n=3 or 4) animals.

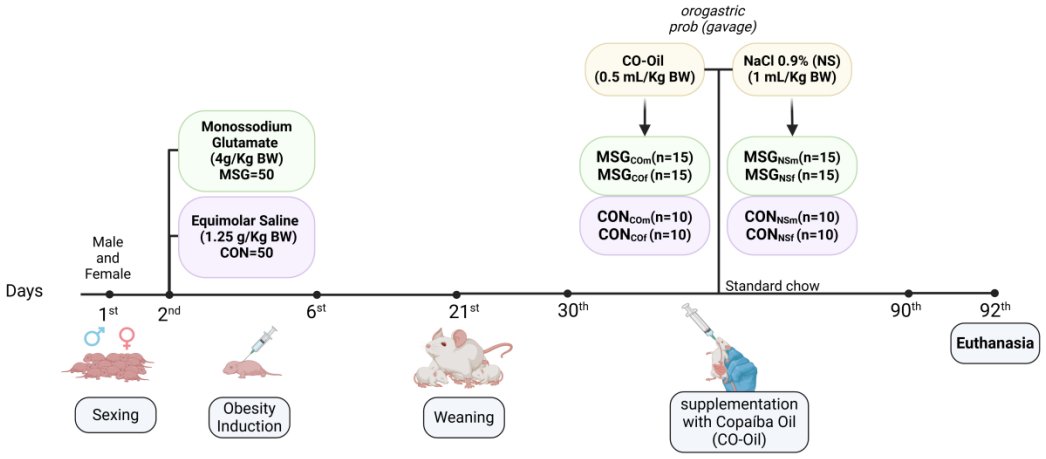
MSG was administered daily from the 2nd to the 6th days of life, both to male (n = 25) and female (n = 25) pups at a dose of 4g/Kg of BW, according to Olney´s (1969) original protocol with adaptations (BALBO *et al.*, 2007; GUARESCHI *et al.*, 2019). The control (CON) or non-obese male (m; n = 25) and female (f; n = 25) rats received, during the same time and period, an equivalent saline solution dose (1.25g/kg of BW). The MSG and CON groups were weaned on the 21st day of life, and then regrouped into 3-4 rats (in cages).

Chronic supplementation of CO-oil:

At one week after weaning, the CON and MSG male and female animals were subdivided, according to the chronic supplementation protocol. We adopted a chronic oral supplementation protocol, in which Co-oil was administered over a long period of time to guarantee sustained effects throughout life. Moreover, oral supplementation involves incorporating one nutrient or compound into a regular diet, permitting the normal process of digestion and absorption at the intestinal level. This is important, since many health benefits of Co-oil have been evaluated through topic application (FRAZÃO *et al.*, 2023).

Part of the CON animals (n=10 in each group; m and f) and of the MSG animals (n = 15 in each group; m and f) received CO-oil supplementation (AMAZONOIL, BRAZIL) at a dose of 0.5mL/Kg of BW, 3 times/week, for 8 weeks (administered via orogastric probe). The CO-oil dose used in this study was adapted from previous reports that demonstrated safe concentrations for CO-oil use in rats, ensuring non-toxicity (SACHETTI *et al.*, 2009). The composition of the CO-oil used in this study can be accessed via the link, https://amazonoil.com.br/produtos-da-floresta/oleo-essencial-de-copaiba/. The non-supplemented (NS) groups (CON AND MSG, MALE AND FEMALES) received saline solution (NaCl 0.9%), using the same via, dose and frequency. Therefore, 8 experimental groups were formed, according to MSG-obesity induction, CO-oil supplementation and gender, as shown in Figure 1. All animals received free water and rodent chow during the protocol and were maintained under controlled luminosity and temperature conditions, as previously mentioned.

Figure 1- Experimental design.



Legend: BW: body weight; CON: control; MSG: monosodium glutamate; $CON_{NSm,}$ non-supplemented control male; CON_{COm}, CO-oil supplemented control male; $CON_{NSf,}$ non-supplemented control female; $CON_{COf,}$ CO-oil supplemented control female; $MSG_{NSm,}$ non-supplemented MSG male; $MSG_{COm,}$ CO-oil supplemented MSG male; $MSG_{NSf,}$ non-supplemented MSG female; $MSG_{COf,}$ CO-oil supplemented MSG female.

Body weight, growth, and food consumption:

All the experimental groups had free access to rodent chow (BIOBASE, SC, BRAZIL) and drinking water from 30 to 90 days of life, each received an individual tail mark and were weighed three times/week. The following calculation was used for registering food intake (FI): food offered minus the leftover chow in the last 48 hours, divided by number of animals in the cages. The median chow consumption/rat was then divided by the individual BW of the rat, to obtain values in g/g of BW. Food consumption and BW ponderal evolution curves were obtained and respective area under curves (AUCs) were generated. Additionally, the feed efficiency (FE) was calculated using the formula: delta (Δ) of BW(g)/Σ of food consumption (g) * 100, as proposed by Freitas *et al.* (2012).

Euthanasia, biometric and adiposity parameters

At 90 days of life, and at 48h after the final CO-oil administration, animals (after 12h of fasting) were individually weighed and measured. Subsequently, they were briefly desensitized in a CO2 camera and decapitated immediately. The final BW (g) and nasal--anal length (NAL; cm) were used to obtain the Lee Index (LI) value, using the formula LI = 3√ BW (g) / NAL (cm) x 1000). The LI is a well-established indicator of a rodent's obesity, as previously established by Bernardis & Patterson (1968). After euthanizing, animals' blood was immediately collected in heparinized tubes for posterior biochemistry parameters dosage. Subsequently, the abdominal cavity of each animal was opened and WAT from the visceral (perigonadal) and subcutaneous (inguinal) depots were excised, cleaned, and weighed, and results expressed in g/100g BW.

Plasma biochemical parameters and insulin resistance:

For the measurement of plasma biochemical parameters, the truncal blood was immediately collected in heparinized tubes for plasma separation. Blood was centrifuged (3000 rpm/min; 15 min) and the plasma obtained was used for measuring glucose (GLU), triglycerides (TGL) and total cholesterol (TC), using colorimetric enzymatic kits (LAB TEST, BR; GPO-TRINDER, BR, RESPECTIVELY), according to the manufacturer's instructions. The IR was determined by the Triglyceride-glucose index (TYG INDEX), using fasting values of plasma glucose (mg/dL) and TGL (mg/dL), according to previous descriptions (SIMENTAL-MENDIA; RODRIGUEZ-MORAN; GUERRERO-ROMERO, 2008).

Statistical analysis and Principal Component Analysis (PCA)

Data were submitted to normality and the homoscedasticity Shapiro-Wilk test and are presented as means and standard errors of the mean (SEM). Variance analysis (two-way ANOVA) was performed to evaluate the F value of CO-oil supplementation, sex and interaction in the CON and MSG subgroups. When F was significant ($p<0.05$), the Tukey post-hoc test was applied. For statistical analysis and graphical elements, the Graph Pad Prism software, version 8.0 for Macintosh (GRAPHPAD SOFTWARE) was used.

The biometric and metabolic variables registered in this study were submitted to PCA, considering two fixed factors (obesity and CO-oil supplementation). For this, the Kaiser-Meyer-Olkin (KMO >0.5) test was applied initially to evaluate data adequation for PCA. Next, using "FactoMineR" (R PROGRAM), relevant variables were selected ("Weight", "Naso.anal.length", "Lee.Index", "Food.intake", "Food.efficiency", "Weight.gain", "Perigonadal Fat", "Inguinal Fat", "Glycemia", "TC", "TGL" and "TyG"). Thus, auto-values and auto-vectors were calculated, and the variables with greater influences were determined and disposed of in dimension (Dim). Auto-values and auto-vectors were used to generate a "ggplot2" diagram, in which CONNS; CONCO; MSGNS and MSGCO were disposed according to sex. The "ExpDes.pt" was used to evaluate significant difference with Analysis of Variance Double Factor and the Tukey-HSD post-test (R CORE TEAM, 2023).

RESULTS AND DISCUSSION

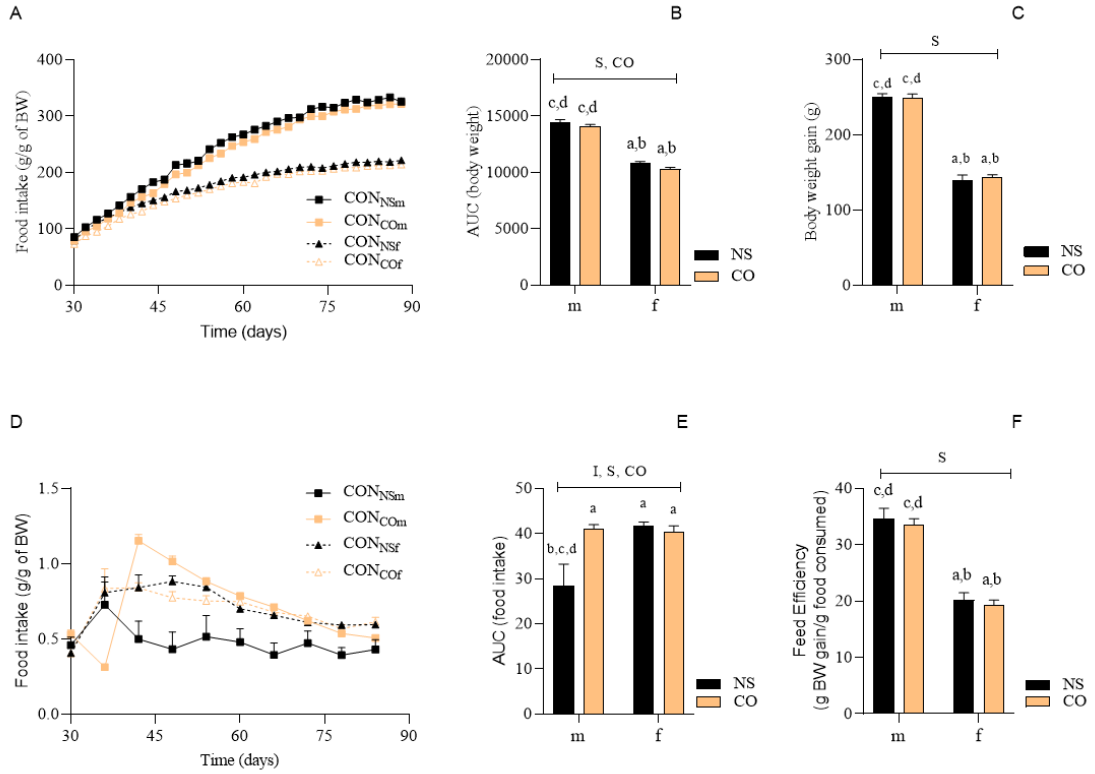
We herein evaluated the effects of chronic CO-oil resin supplementation on metabolism, food intake and adiposity in obese and non-obese rats, also evaluating the influence of sex. Commercially available CO-oil resin is frequently obtained from C. reticulata, C. multijuga or C. langsdorffii, which are the most common species found in South America (FRAZÃO *et al.*, 2023). Diterpenes (non-volatile) and sesquiterpenes (volatile) are the principal components of CO-oil resin, with the latter constituted of at least 90% oleoresin. The sesquiterpene constituents include α-humulene, β-caryophyllene, caryophyllene oxide, α-cadinol, Δ-cadinene, β-elemene, β-bisabolene, α-cubebene, trans-α-bergamotene, α-selinene, and β-selinene (LEE *et al.*, 2023), while the diterpenes are often made up of kaurane, clerodane, or labdane-type skeletons (LEE *et al.*, 2023). The anti-inflammatory, anti-microbial and anti-obesity effects of CO-oil resin have been reported over the years (LEE *et al.*, 2023). We used commercially available CO-oil resin, whose general composition aligns with that of the above mentioned studies.

Effects of CO-oil supplementation on body weight, food intake, adiposity and metabolic state in non-obese male and female animals

In the non-obese state, males that were supplemented with CO-oil presented reduced BW gain, despite an increased FI, resulting in lower FE. Similar data were obtained from diabetic mice supplemented with CO-oil (CARVALHO *et al.*, 2018). In our study, reduced FE was also observed in male and female MSG rats supplemented with CO-oil. The reduction in FE did not appear to be associated with any toxic effects of CO-oil ingestion, since the dose used in the present study was based on other studies that reported nontoxic effects at this dose (SACHETTI *et al.*, 2009; GARCIA; YAMAGUCHI, 2012).

Male and female non-obese (CON) animals demonstrated typical sexual dimorphism, with sex effects observed for BW gain ($F(1,26) = 376.6$; $p < 0.0001$; Fig. 2.b), final BW ($F(1,26) = 345.6$; $p < 0.0001$; Table 1), and NAL ($F(1,26) = 61,23$; $p < 0.0001$; Table 1). Thus, during their lifetimes, CON females of the NS and CO groups presented lower BW gain (approximately 42%; Fig 2.b), final BW (34%; Table 1) and NAL (11.7%; Table 1), when compared to male CONNS and CONCO ($p < 0.0001$) animals. These sex effects were also noted for the AUC for BW ($F(1,29) = 424.7$; $p < 0.0001$), with female CONNS rats presenting reduced AUC for FI, in relation to male CONNS animals (Fig. 2.c). The AUCs for FI ($F(1,26) = 11.44$; $p = 0.0023$) and FE ($F(1,26) = 69.88$; $p < 0.0001$) were also altered by sex in the non-obese groups. As such, the AUCs for FI (Fig. 2.e) and FE (Fig. 2.f) were significantly lower in the female CONNS and CO groups, in comparison to the male CONNS animals.

Figure 2 - Effect of supplementation with Copaiba-oil on body weight (BW) and food intake (FI) in male and female non-obese rats.



Body weight evolution (A); area under the curve for body weight (AUC) (B); body weight gain (C); food intake (D); area under the curve for FI (E); feed efficiency (F). Data are mean ±SEM; (n= 10 –15 rats per group). Capital letter above the bars indicates factor effects (I; interaction; S, sex; CO; CO-oil) on two-way Anova. *Tukey* post- hoc test ($p \leq 0.05$) differences are represented by lower letters above the bars: [a]CON_{NSm}: Control non-supplemented male; [b]CON_{COm}: Control Co-oil supplemented male; [c]CON_{NSf}: Control non-supplemented female and [d]CON_{COf}: Control Co-oil supplemented female.

Table 1- Effects of CO-oil supplementation on biometric and plasma biochemical parameters in male and female non-obese rats.

	Groups				(*p*-value)		
	CON_{NSm}	CON_{COm}	CON_{NSf}	CON_{COf}	I	Sex	CO
Final body weight (g)	$326.66 \pm 8.55^{c,d}$	$332.00 \pm 5.23^{c,d}$	$227.66 \pm 3.63^{a,b}$	$218.90 \pm 4.63^{a,b}$	0.2276	<0.0001	0.7659
NAL (cm)	$22 \pm 0.57^{c,d}$	$21.50 \pm 0.21^{c,d}$	$19.41 \pm 0.55^{a,b}$	$18.50 \pm 0.14^{a,b}$	0.5643	<0.0001	0.0577
Lee Index	0.31 ± 0.00	0.31 ± 0.00	0.31 ± 0.00	0.32 ± 0.00	0.4489	0.6773	0.3470
Inguinal fat (g/100g bw)	0.12 ± 0.03	0.07 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.1301	0.9816	0.5764
Perigonadal fat (g/100g bw)	0.34 ± 0.02	0.32 ± 0.01	0.41 ± 0.02^{d}	0.28 ± 0.04^{c}	0.0921	0.6076	0.0226
Cholesterol (mg/dL)	92.01 ± 4.04	86.23 ± 2.10	92.93 ± 6.90	91.85 ± 3.97	0.5916	0.4568	0.4345
Triglycerides (mg/dL)	53.72 ± 6.23	62.45 ± 3.83^{d}	47.25 ± 2.44	45.95 ± 3.02^{b}	0.2217	0.0081	0.3620
Fasting glucose (mg/dL)	87.33 ± 2.07	86.50 ± 2.57^{d}	96.00 ± 3.31	97.90 ± 4.44^{b}	0.7178	0.0125	0.8877
TyG	1.82 ± 0.02	1.86 ± 0.01	1.82 ± 0.01	1.82 ± 0.01	0.2384	0.1607	0.3908

Data are expressed as means ± standard error mean (SEM) after two-way Anova with *Tukey* post-test ($p \leq 0.05$); n = 10-15 rats/group; I, interaction; Sex; CO, Copaíba-oil supplementation effects. Letters above numbers show statistical differences between groups. [a]CON_{NSm}; [b]CON_{COm}; [c]CON_{NSf}; [d]CON_{COf}.

These data suggest that CO-oil modulates energy metabolism. Consistent with this hypothesis, molecular in vitro studies have indicated that CO-oil is able to modulate neuronal pathways involved in energy metabolism (URASAKI *et al.*, 2020). The volatile compound, beta-caryophyllene, is one of the most frequent components of CO-oil and may regulate the endocannabinoid system (URASAKI *et al.*, 2020). In turn, the endocannabinoid system can modulate FI and energy expenditure (PULCINELLI *et al.*, 2022). Moreover, CO-oil also regulates PI3K/Akt/mTOR pathways in neurons (PARK; CHOI, 2017); these are well recognized pathways that are involved in the hypothalamic control of energy metabolism (JAIS; BRUNING, 2022).

Moreover, sex influenced the fasting values of glycemia and triglycerides (Table 1), where CON females presented augmented plasma glucose (11.49%) and reduced triglyceride (16.36%) levels, in comparison to male CON rats. There was no significant influence of CO-oil supplementation on BW gain ($p = 0.8821$) or final BW ($p = 0.7659$) in the male and female CON animals. However, an interaction effect (sex versus CO) was noted for the AUC for BW ($F(1,29) = 44.75$; $p < 0.0001$). Thus, male CONCO rats presented a reduced AUC for BW, in comparison to male CONNS animals ($p = 0.0013$; Fig. 2.c). In contrast, female CONCO rats presented an augmented AUC for BW, in relation to the female CONNS group ($p < 0.0001$; Fig. 2.c).

CO-oil supplementation altered FI ($F(1,26) = 35.80$; $p < 0.0001$) and FE ($F(1,26) = 29.63$; $p < 0.0001$) in the non-obese groups. An interaction between sex versus CO-oil supplementation was also verified for FI ($F(1,26) = 12.79$; $p = 0.0014$) and FE ($F(1,26) = 27.88$; $p < 0.0001$). As such, male CONCO rats presented a higher AUC for FI intake than the male CONNS group (130.56%; $p = 0.0007$). Consequently, male CONCO rats presented reduced FE (59.23%), in comparison to the male CONNS group ($p < 0.0001$).

Although no significant differences were observed after CO-oil supplementation for IL, NAL, inguinal WAT and plasma metabolic parameters, an isolated effect of CO-oil supplementation was verified on the perigonadal WAT depot ($F(1,23) = 5.973$; $p = 0.0266$). As such, female CONCL animals presented a 31.98% reduction in this fat depot, in relation to female CONNS rats ($p = 0.0354$). Neither sex nor CO-oil supplementation significantly modified IR in the non-obese groups, as shown by TyG index values (Table 1).

Effects of CO-oil supplementation on body weight, food intake, adiposity and the metabolic state of obese male and female animals

In our study, male and female MSG-treated rats developed obesity and metabolic abnormalities in adult life, confirming data previously published by us (GUARESCHI *et al.*, 2019; KUCHLER *et al.*, 2021) and others (MATYSKOVA *et al.*, 2008; NARDELLI *et al.*, 2011; LIU *et al.*, 2020). The administration of high doses of MSG caused hypothalamic lesions, resulting in disturbance of the HPA and metabolic energy imbalance (ROMAN-RAMOS *et al.*, 2011; OLNEY, 1969). Thus, reduced circulating levels of GH and slower growth are typical characteristics of MSG-obese rodents (de Paula *et al.*, 2021), as confirmed in the present study.

Typical sexual dimorphism was preserved in the MSG-obese group with a sex influence confirmed for final BW ($F(1,30) = 81.35$; $p < 0.0001$; Table 2), BW gain ($F(1,22) = 50.66$; $p < 0.0001$; Fig. 3.c) and NAL ($F(1,22) = 26.24$; $p < 0.0001$; Table 2). Thus, at 90 days of life, the females of the MSGNS and MSGCO groups were lighter and smaller than the male rats of both MSGNS and CO groups ($p < 0.0001$). In addition, female MSGNS and MSGCO rats gained less BW than male MSGNS and MSGCO rats (Fig. 3.c).

Male and female MSG-obese rats are prone to develop sexual hormone disturbances, contributing to fertility impairment, as a consequence of hypogonadism (RUDYK *et al.*, 2018; KAYODE *et al.*, 2020). However, despite this hypogonadism, we demonstrated herein that typical sexual dimorphism appears to be preserved in MSG-obese rats. As such, similarly to non-obese females, the MSG-female animals presented reduced BW, growth, and FI, compared to MSG-male rats.

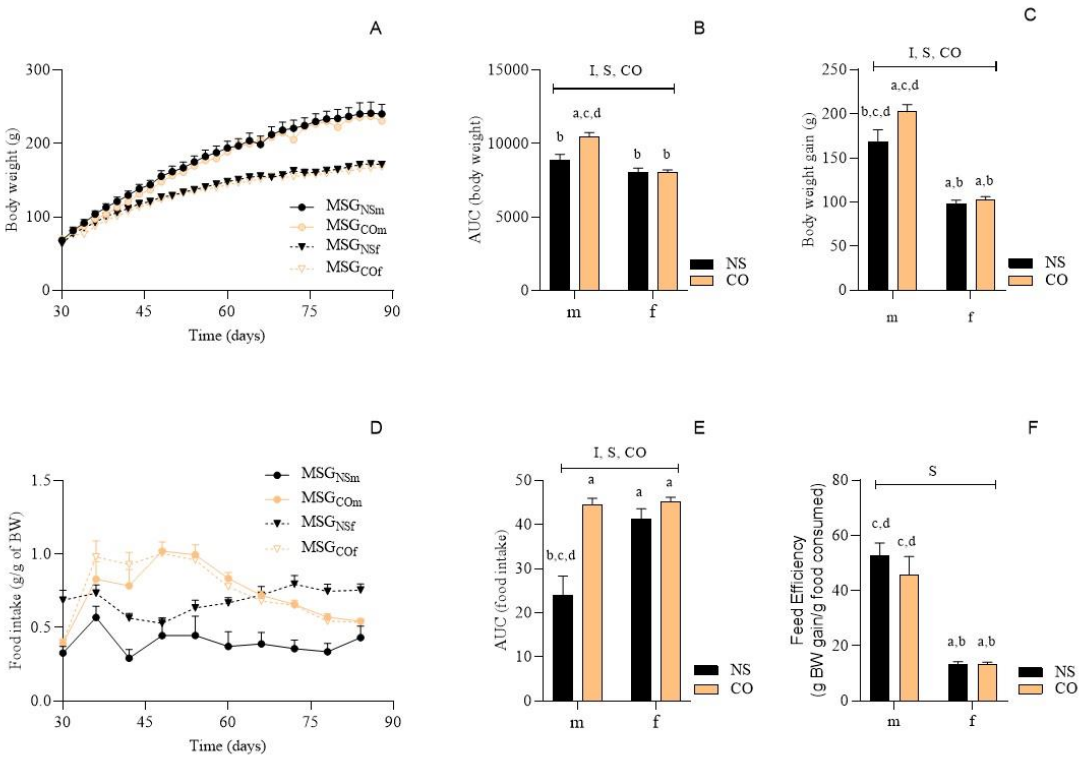
In association with these findings, the AUCs for BW ($F(1,28) = 23.09$; $p < 0.0001$), FI ($F(1,22) = 23.35$; $p < 0.0001$) and FE ($F(1,22) = 71.92$; $p < 0.0001$) were altered by sex. Thus, female MSGNS rats had an approximately 25% lower AUC for BW (Fig. 3.b), compared to male MSGNS ($p = 0.0066$) and MSGCO ($p = 0.0001$) rats, respectively. The AUC for FI (Fig. 3.e; $p < 0.05$) was approximately 21% lower in female MSGNS

Table 2 - Effects of CO-oil supplementation on biometric parameters and plasma biomarkers in male and female MSG-obese rats.

	Groups				(*p*-value)		
	MSG_{NSm}	MSG_{COm}	MSG_{NSf}	MSG_{COf}	I	Sex	CO
Final body weight (g)	$239.00 \pm 16.45^{c,d}$	$249.40 \pm 6.72^{c,d}$	$171.66 \pm 6.91^{a,b}$	$170.47 \pm 3.24^{a,b}$	0.4801	<0.0001	0.5746
NAL (cm)	17.66 ± 0.40^{d}	$18.50 \pm 0.34^{c,d}$	16.33 ± 0.35^{b}	$16.37 \pm 0.26^{a,c}$	0.2535	<0.0001	0.2084
Lee Index	0.34 ± 0.00	0.34 ± 0.00	0.33 ± 0.00	0.34 ± 0.00	0.5106	0.2228	0.3467
Inguinal fat (g/100g bw)	0.38 ± 0.02	0.24 ± 0.03^{d}	0.35 ± 0.06	0.42 ± 0.04^{b}	0.0317	0.0973	0.5161
Perigonadal fat (g/100g bw)	0.77 ± 0.07	0.84 ± 0.06	1.17 ± 0.24	0.77 ± 0.04	0.0563	0.1714	0.1679
Cholesterol (mg/dL)	92.02 ± 6.17	97.81 ± 2.62	89.30 ± 5.54	93.34 ± 3.22	0.8452	0.4275	0.2809
Triglycerides (mg/dL)	$94.71 \pm 4.13^{b,d}$	$132.36 \pm 12.66^{a,c,d}$	$88.43 \pm 10.11^{b,d}$	$54.30 \pm 3.99^{a,b,c}$	0.0002	<0.0001	0.8290
Fasting glucose (mg/dL)	103.16 ± 3.94	97.83 ± 3.26	104.83 ± 3.48	98.87 ± 2.42	0.9241	0.6802	0.0955
TyG	1.99 ± 0.01^{d}	2.01 ± 0.02^{d}	1.99 ± 0.02^{d}	$1.86 \pm 0.01^{a,b,c}$	0.0007	0.0012	0.0117

Data are expressed as mean ± standard error mean (SEM) after two-way Anova with Tukey post-test ($p \leq 0.05$); n = 10-15 rats/group; I, interaction; Sex; CO, Copaíba-oil supplementation effects. Letters above numbers show statistical differences between groups. $^{a}MSG_{NSm;}$ $^{b}MSG_{COm;}$ $^{c}MSG_{NSf;}$ $^{d}MSG_{CO.}$

Figure 3 - Effec of supplementation with Copaiba-oil on body weight (BW) and food intake (FI) of male and female non-obese rats in male and female hypothalamic-obese rats.



Graphs show body weight evolution (A); area under the curve for body weight (AUC) (B); body weight gain (C); food intake (D); area under the curve for FI (E); feed efficiency (F). Data are mean ±SEM; (n= 10 –15 rats per group). Capital letter above the bars indicates factor effects (I; interaction; S, sex; CO; CO-oil) by two-way Anova. *Tukey* post- hoc test ($p \leq 0.05$) differences are represented by lower letters above the bars: $^{a}MSG_{NSm;}$ MSG-obese non-supplemented male; $^{b}MSG_{COm}$; MSG-obese supplemented with Copaíba-oil male; $^{c}MSG_{NSf;}$ MSG-obese non-supplemented female and $^{d}MSG_{COf;}$ MSG-obese supplemented with Copaíba-oil female.

and CO animals, in comparison to male MSGNS and MSGCO rats. Moreover, the female MSGNS and MSGCO groups presented reductions of 79.67% ($p < 0.0001$) and 76.82% ($p < 0.0001$), respectively, in FE, compared to male MSGNS rats.

CO-oil supplementation influenced the AUC for BW ($F(1,28) = 4.787$; $p = 0.0372$; Fig. 3.b) and FE ($F(1,22) = 30.83$; $p < 0.0001$; Fig. 3.f) in MSG-obese animals. Furthermore, a sex versus CO-oil supplementation interaction was also observed for FE ($F(1,22) = 36.90$; $p < 0.0001$). Therefore, female MSGCO rats demonstrated reductions of 16.53% ($p = 0.0168$) and 64%, respectively, in the AUCs for BW and FE (63.64%; $p < 0.0001$), in comparison to male MSGNS rats. Male MSGCO animals had a higher AUC for BW, in relation to females, in the MSGNS (37.68%; $p<0.0001$) and MSGCO (19.81%; $p<0.0001$) groups. Furthermore, animals from the male MSGCO group had a lower (63.64%) FE, in relation to male MSGNS animals.

Interestingly, MSG-obese females had high subcutaneous WAT, in association with reduced TG and IR, compared to MSG-obese males, suggesting that hypothalamic obesity is worse in males. In the MSG-obese group, sex did not alter either LI ($F(1,21) = 1.578$; $p = 0.2228$) or the perigonadal WAT content ($F(1,20) = 2.012$; $p = 0.1714$). However, the interaction between CO-oil supplementation and sex ($F(1,21) = 5.297$; $p = 0.0317$) modified the inguinal WAT depot weight. Thus, the inguinal WAT weight was reduced by 48.18% in male MSGCO rats, compared to female MSGCO rats ($p = 0.0388$; Table 2).

Fasting values of GLU ($F(1,22) = 0.1745$; $p = 0.6802$) and TC ($F(1,20) = 0.6560$; $p = 0.4275$) were not affected by sex in obese animals. However, plasma levels of TGL were influenced by sex ($F(1,21) = 27.46$; $p < 0.0001$; Table 2), as well as by the sex versus CO-oil supplementation interaction ($F(1,21) = 19.89$; $p = 0.0002$). Thus, the female MSGNS and MSGCO groups presented lower TGL levels (33.20% and 58.98%, respectively) than the male MSGCO rats. Moreover, male MSGCO animals presented higher (39.78%) triglyceride levels, compared to male MSGNS animals ($p = 0.0209$). Consequently, TyG (a marker for IR) was modulated by sex ($F(1,22) = 13.81$; $p = 0.0012$) and CO-oil supplementation ($F(1,22) = 7.567$; $p = 0.0117$), as well as by the interaction of these factors ($F(1,22) = 15.34$; $p = 0.0007$). Thus, female MSGCO rats had lower TyG values, compared to the other groups (MSGNS and MSGCO males and MSGNS females). More recent findings have shown that oral CO-oil administration can reduce adiposity, BW gain, oxidative stress, and inflammatory processes in the diet-induced obesity model (Telles *et al.*, 2022; de Paula *et al.*,2021). Herein, we demonstrated, for the first time, that oral and chronic CO-oil ingestion can alter adiposity, BW, FI and the metabolic state in rodents, and that its effects are influenced by obesity and sex.

Accordingly, female MSG-obese mice demonstrated better insulin sensitivity than male MSG-obese mice (Matyskova *et al.*, 2008). In humans, women tend to accumulate higher subcutaneous WAT content, whereas men present lower total body fat, but with more visceral WAT content (Nicholson *et al.*, 2012). The higher visceral WAT found in men is associated with elevated circulating levels of cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), compared to women (Palmer; Clegg, 2015). The role of these pro-inflammatory cytokines in IR is well recognized (Li *et al.*, 2022). Furthermore, female sexual hormones, particularly estrogen, exert inhibitory effects on the expression of pro-inflammatory cytokines, which could explain the better metabolic state in females (Sung *et al.*, 2012). However, the mechanism by which estrogen acts in female MSG-rats with hypogonadism is unclear.

Insulin sensibility is dependent upon the correct stimulation of the PI3K/AKT pathway, which is negatively modulated by pro-inflammatory cytokines, thereby inducing IR. Chronic inflammation is associated with activation of the JAK-STAT pathways, in association with high levels of pro-inflammatory cytokines (Li *et al.*, 2022). Oral ingestion of CO-oil had anti-inflammatory, anti-oxidative and anti-adiposity effects in obese animals. Furthermore, in vitro studies have demonstrated that CO-oil regulates PI3K/AKT and Jak/STAT (Cardinelli *et al.*, 2023). In addition, the major CO-oil component, beta-caryophyllene, selectively binds to the cannabinoid receptor 2 beta (CB2), in turn reducing inflammatory processes in WAT (Urasaki *et al.*, 2020; Pulcinelli *et al.*, 2022). Sexual dimorphism has been observed in the regulation of IR pathways (Goosens; Jocken; Blaak, 2021),

and this could explain why only female MSG-obese rats are responsive to the anti-adiposity effects of CO-oil.

Obesity and its deleterious effects on metabolism are directly associated with T2DM and cardiovascular diseases (HORTON; BARRET, 2021). Recent data suggest that 38% of people worldwide have excess weight and, according to the World Obesity Federation (WOB, 2023), in 2035 more than half of the world´s population is expected be above their adequate body weight. Thus, the finding of appropriate strategies to avoid obesity progression and its deleterious metabolic effects is urgently needed. In this context, many plants contain natural biological compounds that have positive effects on health, including anti-adiposity actions (VEIGA *et al.*, 2007; HORÁCIO *et al.*, 2017). CO-oil contains several compounds with anti-inflammatory, analgesic, anti-oxidant, and antiseptic properties (DIAS *et al.*, 2014; HORÁCIO *et al.*, 2017; AMES-SIBIN *et al.*, 2018).

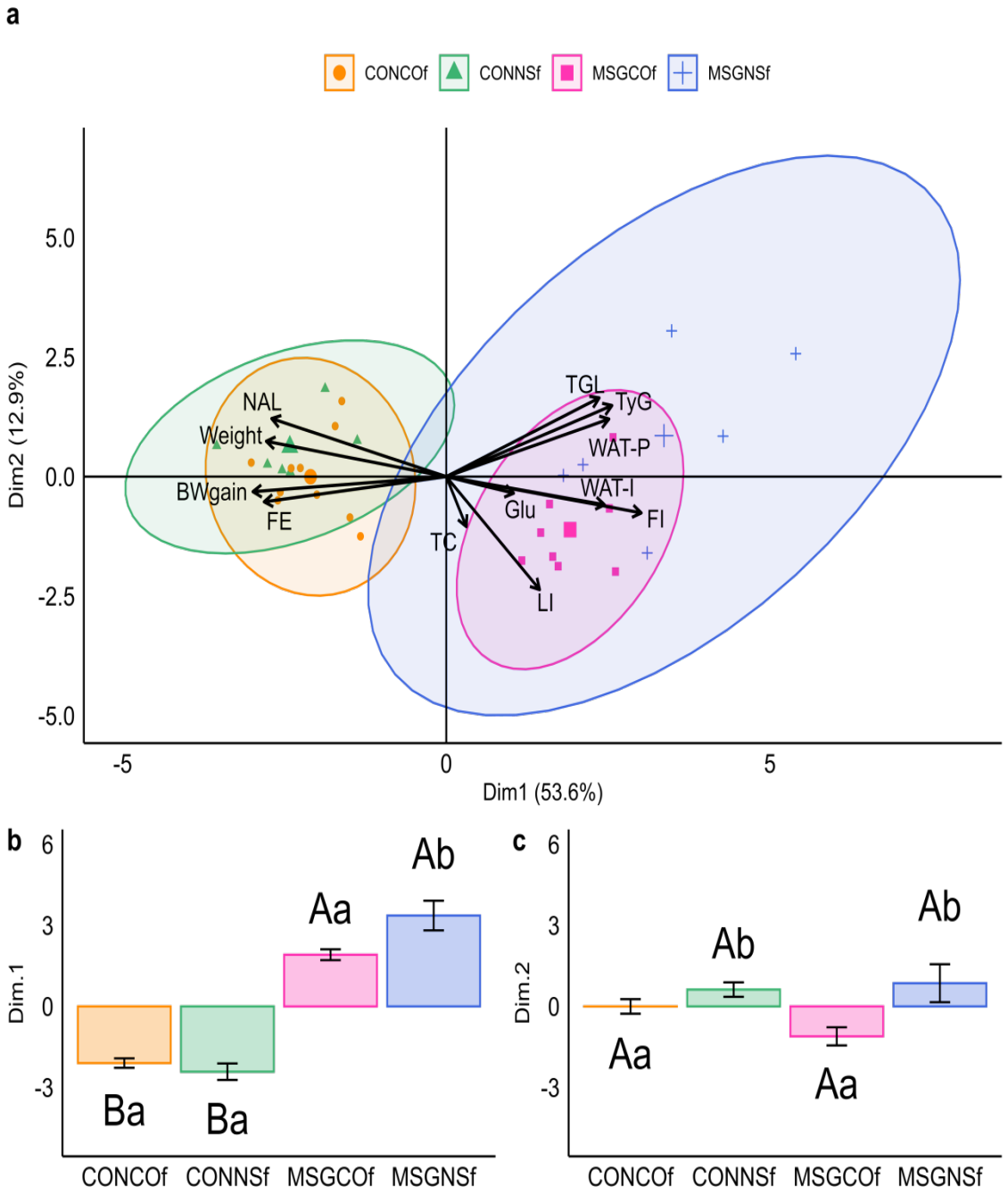
The PCA shows the metabolic and biometric profiles for male and female non-obese (CON) and MSG-obese rodents that were supplemented with CO-oil. In the CON and MSG female groups (Fig. 4), the variables were grouped into Dim1 and Dim2, representing 66.53% of data variation. For Dim1 the BW gain, FI, FE, BW, NAL, WAT-P, WAT-I and GLU variables were determined, representing 53.64% of the total variability of the data. Thus, in Dim1, the variables, BW gain, BW, FE and NAL, were higher in female CON rats than in female MSG-obese animals. In contrast, FI, adiposity (WAT-P and WAT-I) and fasting GLU were higher for female MSG-obese rats, compared to the female CON group (Fig. 4a). As such, the MSGNS and MSGCO groups were different to the non-obese animals ($F(1,26) = 259.669$; $p < 0.0001$; Fig. 4b). However, when analyzing the effects of CO-oil supplementation on obese females, we noted significant differences in female MSGCO rats, compared to female MSGNS animals ($F(1,26) = 8,647$; $p = 0.007$). Thus, the female MSGCO group demonstrated reduced adiposity and metabolic abnormalities, with values that were closer to those of the non-obese female CON group.

We also noted that female rats appear to be more responsive to the anti-adiposity effects of oral CO-oil supplementation, as female MSG-obese and non-obese groups supplemented with CO-oil presented reductions in WAT, an effect that was not observed in males. These findings confirm a recent study published by us, which showed a reduction in WAT mass in female MSG-CO supplemented rats, which was not observed in male MSG-CO supplemented rats (KAILER *et al.*, 2024). Accordingly, Silva Lima *et al.* (2017) observed that female rats, but not male rats, drank more water during CO-oil-resin supplementation; the authors suggested that this might be related to a "greater specific susceptibility of females" in responding to the intervention (SILVA LIMA *et al.*, 2017). Importantly, our results also showed that female MSG-obese rats supplemented with CO-oil displayed a reduction in BW gain.

The TyG, TGL and TC variables were grouped for the Dim2 group and accounted for only 12.89% of the data. Again, the MSGNS and MSGCO groups presented higher values for these variables, when compared to the non-obese female CON group. CO-oil supplementation exerted a significant influence on Dim2 ($F(1,26) = 10.0198$; $p = 0.004$), indicating that the female CONCO and MSGCO groups are different from their respective female NS groups (Fig. 4a).

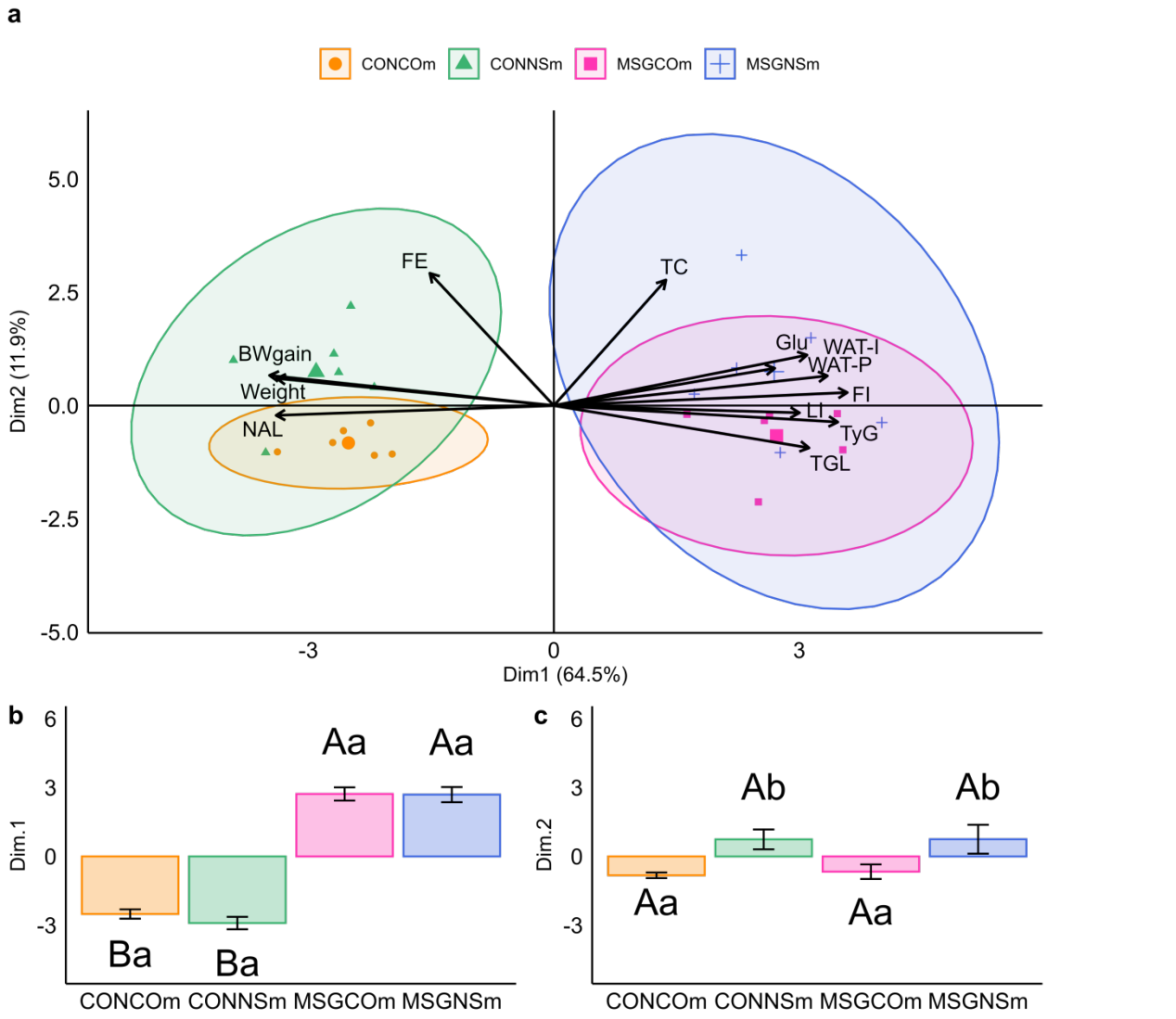
In males (Fig. 5), Dim1 and Dim2 explained 76.75% of data variation. The variables, FI, BW, BW gain, TyG, NAL, WAT-P and WAT-I, TGL, LI and GLU were clustered in Dim1, which represented 64.82% of data variability. Dim1 analysis showed that the male CON groups had higher values of BW gain, BW and NAL, in comparison to the MSG groups. In contrast, the variables, FI, TyG, adiposity (WAT-P and WAT-I), TGL, LI and GLU characterized the obese groups, with the MSG-obese males presenting higher values than those of non-obese CON males ($F(1,22) = 421.71$; $p<0.0001$; Fig. 5b). CO-oil supplementation did not significantly alter these variables in the male groups. Few studies have evaluated the effects of CO-oil supplementation on WAT in rodents or humans. Using diet-induced obesity (high sucrose), Telles and collaborators (2022) demonstrated that CO-oil supplementation (oral, 200 mg/kg/day/for 8 weeks) of male obese rats resulted in WAT reduction, accompanied by reduced inflammatory and oxidative stress. Moreover, as mentioned above, compounds found in CO-oil could modulate molecular pathways (e.g., AKT, Jak/Stat and CB2) that may regulate adipogenesis

Figure 4 - Principal Component Analysis (PCA) of CO-oil supplemented female obese and non-obese rats.



and lipogenesis (PARK; CHOI, 2017; YEN *et al.*, 2015). Further studies are necessary to clarify this mechanism in the WAT of females. Interestingly, a decrease in TG and improvement in IR, as indicated by the TyG index, were only observed in the MSG CO-oil supplemented females. CO-oil has previously been reported to reduce hyperglycemia and regulate glucose tolerance (YEN *et al.*, 2015; CARVALHO *et al.*, 2018). Consistent with this observation, Basha and Sankaranarayanan (2015) showed that sesquiterpene (β-caryophyllene) could decrease glycemia in diabetic mice by increasing insulin production (BASHA; SANKARANARAYANAN, 2015). Furthermore, signaling for insulin release can be influenced by β-caryophyllene, which can activate

Figure 5 - Principal Component Analysis (PCA) of male obese and non-obese CO-oil supplemented rats.



5a: Ordering diagram of eigenvalues and eigenvectors, showing the ellipses that separate the groups; CONCOm, non-obese CO-oil supplemented male; CONNSm, non-obese non-supplemented male; MSGCOm, MSG-obese CO-oil supplemented male; and MSGNSm, MSG-obese non-supplemented male. 5b: Dim 1 represented by means and standard deviations. 5c: Dim2 represented by means and standard deviations. The uppercase letters at the top of the error bars indicate the MSG factor, and the lowercase letters indicate the copaiba factor in the Tukey test for multiple comparisons between groups. NAL: naso-anal length; BW gain: body weight gain; FE: feed efficiency; TGL: triglycerides; TyG: triglycerides-glucose index; WAT-P: white adipose tissue perigonadal; WAT-I: white adipose tissue inguinal; Glu: glucose; FI: food intake; TC: total cholesterol; LI: Lee Index.

Rac1, a G-protein involved in the regulation of type-4 glucose transporter (GLUT-4) vesicle translocation. Subsequent translocation of glucose from the intracellular space to the plasma membrane enhances glucose uptake in insulin-trigger tissues (CARDINELLI *et al.*, 2023). Taken together, our findings suggest that obese rats that are female are more responsive to the anti-adiposity and metabolic effects of CO-oil, as confirmed by our PCA data.

TC and FE variables were grouped in Dim2 and represented only 11.93% of variability. In this Dim, MSGNS males had higher TC values in relation to non-obese CONNS animals, confirming classical metabolic abnormalities found in MSG-treated

rodents (von diemen; trindade; trindade, 2006; dolnikoff *et al.*, 2011; guareschi *et al.*, 2019). In contrast, CONNS males had higher FE than MSGNS males. CO-oil supplementation altered Dim2 ($F(1,22) = 15.9852$; $p < 0.001$), with the male MSGCO and CONCO groups differing from their respective NS animals (Fig.5). Studies have demonstrated that Co-oil inhibits inflammatory responses (NF-kB, IL-1β, IL-6, IL-17, TNF-α, and IFN-γ) in rats (veiga junior *et al.* 2007; pulcinelli *et al.*, 2022). These cytokines and their associated pathways play roles in food intake, energy expenditure and insulin sensitivity, which may contribute to these effects (meng; kautz, 2022). Moreover, a sex difference has been observed for hypothalamic food intake control (ruigrok *et al.*, 2021).

Our study presents some limitations. Firstly, we used commercially available CO-oil and we did not perform any specific oil composition analysis. The composition data provided by Amazon Oil (https://amazonoil.com.br/produtos-da-floresta/oleo-essencial-de-copaiba/) indicates that CO-oil resin (copaifera officinalis) is constituted of 72 sesquiterpenes (hydrocarbons) and 28 diterpenes (carboxylic acids), of which beta-caryophyllene is the most prevalent compound (40 – 70 %) (carvalho; milke, 2014; paula *et al.*, 2023). The beneficial effects of oral CO-oil observed in this study occurred in rodents, whose molecular mechanisms are not yet fully understood. Furthermore, complementary studies should be performed to assess the suitability of the use of copaiba oil in humans, via clinical dosage studies, and toxicity, pharmacokinetic and pharmacodynamic analyses.

CONCLUSION

In conclusion, chronic treatment with oral CO-oil reduces feed efficiency in both obese and non-obese male rats, suggesting potential effects of this oil in modulating energy metabolism. However, anti-adiposity, coupled with improved IR, were observed only in female obese rats that were supplemented with CO-oil, suggesting a sex-dependent influence on the beneficial health effects of oral CO-oil.

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