



Maternal feeding associated to post-weaning diet affects metabolic and behavioral parameters in female offspring

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ABSTRACT

Genetic and environmental factors related to maternal diet may predispose offspring to serious diseases. However, consequences of a maternal diet intervention during gestation and lactation, and its association with caloric restriction after weaning on the progeny are not completely known. In this context, the goal of the present study was to investigate how different maternal diets, control (CONT), hypercaloric (HD) or restrictive (RD) diets during gestation and lactation, may affect the metabolism and behavior of the offspring that was also submitted to RD. Experimental groups were abbreviated accordingly maternal/offspring diets: CONT/CONT, CONT/RD, RD/CONT, RD/RD, HD/CONT, HD/RD. Our results showed that glucose serum concentration is increased in mice from dams fed a HD. However, offspring from RD-fed dams showed lower insulin and leptin levels than the other groups, indicating a maternal diet effect. Moreover, animals from RD/CONT group showed a higher adipocyte area in comparison to both HD/CONT and CON/CONT. Offspring from RD-fed dams exhibited a decrease in lateral area locomotion in the open field test. Evaluation of anxiety-like behavior and recognition memory showed no significant difference among groups. Thus, maternal RD provides a beneficial response in metabolic parameters, but its effects on behavior is not completely clarified.

1. Introduction

It is well known a variety of conditions such as unbalanced maternal nutrition, exposure to environmental insults, infection or stress occurring during fetal development can lead to health dysfunctions in the progeny [1,2]. In the past decade, there was an increase in the incidence of maternal obesity during pregnancy, which can be explained by lifestyle changes (high-caloric food intake and reduced physical activity). Studies with rodent models have demonstrated an association between hypercaloric diet exposition during gestation, and a higher risk to development of adverse outcomes in the offspring, for instance, congenital anomalies, and of developing obesity and metabolic syndrome [2–5]. On the other hand, maternal undernutrition during the periconceptional period and pregnancy also causes metabolic

dysfunction resulting in increased adiposity, reduced fetal capacity to modulate muscle glucose uptake, and alterations in fetal pancreatic function and insulin signaling [1,6,7].

Caloric restriction in adulthood has been described as a protective approach which contributes to promote longevity by providing antioxidant and anti-inflammatory pathways activation [8–10], improvement of glucose metabolism [11] and remodeling of adipose tissue [12]. However, it is poorly known whether caloric restriction during gestation and lactation is beneficial or detrimental for the development of the offspring.

Since fetal programming has been widely associated with nutritional interventions, we aimed to investigate whether behavior and metabolism of the progeny may be affected by different maternal diets (hypercaloric or restrictive diets) during gestation and lactation. Since the

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benefits of caloric restriction are still controversial, we also evaluated if caloric restriction in the offspring can be protective and able to reverse an altered metabolic programming caused by ingestion of hypercaloric diet by the dams. Thus, we administered three different diets during gestation and lactation: control, restrictive diet and hypercaloric diet. Then, we investigated metabolic and behavioral parameters on the offspring following control and restrictive diet.

2. Methods

2.1. Animals

Ten male and 30 female BALB/c albino mice (60 days old, weighing between 18 and 24 g) were obtained from the Animal Housing Facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) and properly maintained under controlled temperature ($22 \pm 2^\circ\text{C}$), humidity ($55 \pm 5\%$) and luminosity (12 h light/dark cycle; lights on at 6 a.m.) conditions. All procedures were performed accordingly to the ethical rules established by the guidelines of the *Brazilian Society for Neuroscience and Behavior* and the *Guide for the Care and Use of Laboratory Animals* of the Institute for Laboratory Animal Research. The Institutional Animal Care and Use Committee of UFCSPA (#388/15) approved this study and all efforts were made in order to minimize the number of animals used and its suffering.

2.2. Experimental procedures

The adult females were divided into three groups ($n = 10$ per group) and individually housed with water *ad libitum*. The control group (CONT) received standard mice chow (Nuvital, Curitiba, Brazil) *ad libitum*, with a total energy content of 3.4 kcal/g (63% carbohydrate, 26% protein, 11% fat); the restrictive diet group (RD) had a 30% lower feed availability, adjusted according to the consumption of CONT group; and the hypercaloric diet group (HD) received a special chow (Pragsoluções Biociências, Jau, Brazil) *ad libitum*, with total energy content of 4.9 kcal/g (40.3% carbohydrate, 11.3% protein, 48.3% fat).

Females received the diets during 25 days for adaptation, after that, they were housed with males for mating during 7 days, in a ratio of 3 females:1 male. The mating was confirmed by checking if the copulatory plug was present in the vaginal smear. When pregnancy was confirmed, females were housed in separate cages until delivery. If pregnancy was not confirmed, females were re-housed with males. On the first post-partum day (PPD), the litters were standardized to 6 pups (3 males and 3 females, whenever possible).

2.2.1. Offspring

After weaning (21 days old), the female offspring of CONT, RD and HD dams were randomly divided into two groups: fed with standard chow (CONT) or a restrictive diet (RD). Then, six experimental groups were generated: CONT/CONT (maternal standard chow/offspring standard chow), CONT/RD (maternal standard chow/offspring restriction), RD/CONT (maternal restriction/offspring standard chow), RD/RD (maternal restriction/offspring restriction) HD/CONT (maternal hypercaloric/offspring standard chow), and HD/RD (maternal hypercaloric/offspring restriction). The offspring diet (CONT or RD) was maintained along all their lives. The behavioral test was assessed at 80 days old and euthanasia followed by tissue and blood collection was performed at around 100 days old.

2.3. Behavioral tests

Around 70 days of age, the estrous cycle of the offspring was assessed, by vaginal smear, in order to submit all animals to the behavioral tests in the diestrus period. The behavioral tests were performed and recorded during the light cycle (from 08:00 to 11:30). First the animals were submitted to the open field test, one week later they were

analyzed in the elevated plus maze and after another week of interval they were submitted to the novel object recognition test. After each animal submitted to the specific assay, the apparatus was washed with alcohol 50% and dried, in order to avoid any smell recognition. The recordings were analyzed using “The Observer” software (Noldus®, Holland).

2.3.1. Open field test (OF)

The test was performed in a wooden box with 100 cm^2 square of open-field, surrounded by walls 30 cm high, whose floor was divided into 25 squares marked on the floor, being 16 in the lateral zone and 9 in the central zone of the arena. The experimental procedure consisted of placing the animal in a corner of the open field (randomly chosen) and monitoring its movement. Then, parameters such as frequency and duration in the lateral or central zone of the arena were measured. The innate fear behavior was verified by the evaluation of the frequency of locomotion in the central zone of the OF [13,14].

2.3.2. Elevated plus maze test (EPM)

The EPM consists of an elevated apparatus above the floor (50 cm), with two open arms ($25 \times 5\text{ cm}$) across from each other and perpendicular to other two closed arms ($25 \times 5\text{ cm}$, with walls 13 cm high), with a central open area platform. The animals were individually tested, being placed in the center zone with its head facing to the open arm of the apparatus, to assess: the percentage of entries into the open arms [$100 \times \text{open}/(\text{open} + \text{enclosed})$], the percentage of time spent in the open arms [$100 \times \text{open}/(\text{open} + \text{enclosed})$], and the number of head-dipping, as previously described in literature [14,15].

2.3.3. Novel object recognition test (NOR)

Animals were habituated in a plastic box ($30.5\text{ cm} \times 19.8\text{ cm} \times 13.4\text{ cm}$) during 10 min. On the next day, they were individually introduced into the same arena containing two identical objects that they could freely explore for 5 min. Exploration was defined by the amount of time spent actively sniffing or interacting with the object at a distance no $> 2\text{ cm}$. Three hours later, mice were placed back into the same arena containing two objects, one of them being the same previously presented (familiar object), and the other being a new and unknown (novel object). This test session evaluated short-term memory. Recognition index was calculated by time spent exploring the new object/total exploration time multiplied by 100 [16].

2.4. Tissue and blood collection

Around 100 days old, the females were anesthetized and euthanized with ketamine (90 mg/kg, DOPALEN, Vetbrands, São Paulo, Brasil) and xilazine (10 mg/kg, ANASEDAN, Ceva, São Paulo, Brasil), for the collect of biological material. The trunk blood was collected in sterile tubes, centrifuged for 10 min, at 1500g and 4°C . Serum was separated and stored at -80°C for posterior analysis.

Hepatic and visceral adipose tissues (from stomach, spleen, pancreas, small and large intestines, and reproductive tract) were dissected, weighed and formalin-fixed for histological analysis.

2.4.1. Serum analysis

Insulin and leptin serum concentrations were measured in duplicate using mouse-specific ELISA commercial kits (SIGMA-ALDRICH™ Saint Louis, MO, USA), according to the manufacturer. Glucose level was determined using standard enzymatic method with an automatic analyzer (BS120 Chemistry Analyzer, Mindray, Shenzhen, China).

2.4.2. Histological analysis and scoring system definition

After tissue collection, samples were fixed in 4% buffered paraformaldehyde for at least 24 h, histologically processed with ethanol/xylene overnight embedded in paraffin. Tissue sections of $4\mu\text{m}$ were stained with hematoxylin-eosin. For morphometric analysis of adipose

tissue, micrographs of three independent fields of histological section were obtained with a 20× objective, using light microscopy (Olympus BX-61, Japan) for each animal. Images were treated using the open free access Fiji software [17] to better define all limits of adipocytes, and the area of each cell was measured using Image Pro Plus 6.0 software (IPP6 – Media Cybernetics, Silver Spring, MD, USA) in a blinded manner to the experimental groups. Around 1000 to 1500 cells were assessed in each experienced group, followed by an exclusion filtering of adipocytes presenting area below 350 μm^2 , once they may be a mixture of adipocytes and stromal cells [18]. The average area \pm standard deviation was firstly calculated among filtered adipose cells of each animal, followed by an average calculation among all average area of animals of each tested group.

The hepatic tissues were analyzed manually and classified according a semi-quantitative analysis by an experienced pathologist, blinded to experimental groups, using light microscopy (Olympus BX-61, Japan). In accordance with the histological features, they were evaluated about three broad categories: steatosis, inflammatory lymphocyte infiltration and ballooning. The evaluation system was based on [19].

2.5. Statistical analysis

Data were expressed as mean \pm S.E.M. The Shapiro-Wilk test was used to assess data normality. The results were analyzed by two-way (ANOVA), followed by Bonferroni *post hoc* test. Statistical analyses were made using GraphPad Prism 6, (La Jolla, USA). In all cases, differences were considered significant when $p < 0.05$.

3. Results

An effect of maternal diet was found on the glucose serum concentration [$F_{(2,24)} = 13.64$; $p = 0.001$, Fig. 1A], indicating that maternal HD induces a hyperglycemic profile in the offspring, irrespective of its diet pattern (CONT or RD). There was an increase in glucose concentration in HD/CONT when compared to CONT/CONT ($p < 0.01$) and RD/CONT ($p < 0.05$). HD/RD glycemia was also higher than CONT/RD ($p < 0.01$) and RD/RD ($p < 0.01$).

Fig. 1B shows the insulin levels in the serum of CONT and RD-fed offspring. An effect of maternal diet was observed on insulin levels [$F_{(2,27)} = 6.76$; $p = 0.004$]. Post-hoc test showed that HD/RD had a higher insulin serum concentration than RD/RD ($p < 0.05$).

Leptin serum concentration (Fig. 1C) showed a maternal diet [$F_{(2,27)} = 9.07$; $p = 0.001$] and also an offspring diet [$F_{(1,27)} = 5.75$; $p = 0.02$] effect. Leptin was increased in HD/CONT in comparison to CONT/CONT ($p < 0.05$) and RD/CONT ($p < 0.001$). RD-fed offspring showed an increase in leptin levels in CONT/RD compared to RD/RD ($p < 0.01$).

In the histological analysis of the adipose tissue (Fig. 2), it was found a maternal diet [$F_{(2,66)} = 3.83$; $p = 0.02$] and an offspring diet [$F_{(1,66)} = 8.32$; $p = 0.005$] effect on the adipocyte area. RD/CONT showed a higher adipocyte area in comparison with HD/CONT ($p < 0.05$) and CON/CONT ($p < 0.001$). HD/CONT was also higher than CONT/CONT ($p < 0.05$).

On the other hand, histological analysis of the liver did not show pathological hepatic alterations such as steatosis, inflammatory lymphocyte infiltration and ballooning (data not shown).

In the evaluation of the locomotor activity and innate fear behavior in the OF test, it was observed a maternal diet effect on the frequency of locomotion in the lateral area [$F_{(2,64)} = 10.75$; $p < 0.0001$, Fig. 3A]. In the post-hoc test, we found a decrease in this frequency in RD/CONT compared to CONT/CONT ($p < 0.05$) and HD/CONT ($p < 0.001$). RD/RD also showed a decrease in lateral locomotion in comparison to HD/RD ($p < 0.05$). Regarding frequency of locomotion in the central area, there was also an effect of maternal diet [$F_{(2,68)} = 4.61$; $p = 0.013$, Fig. 3B], showing that RD-fed dams had decreased this parameter. However, no differences between groups were found in the

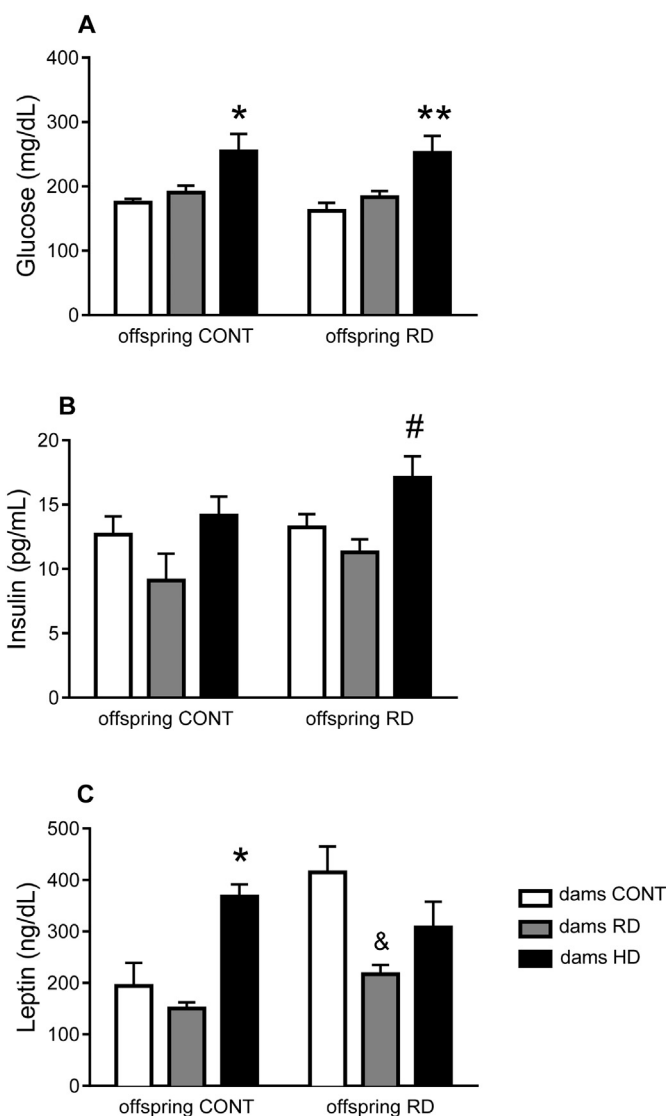


Fig. 1. Metabolic markers. (A) Glucose serum concentration (mg/dL) in offspring CONT/CONT ($n = 6$), RD/CONT ($n = 6$), HD/CONT ($n = 6$), CONT/RD ($n = 4$), RD/RD ($n = 5$) and HD/RD ($n = 5$). (B) Insulin serum concentration (pg/mL) in offspring CONT/CONT ($n = 6$), RD/CONT ($n = 6$), HD/CONT ($n = 7$), CONT/RD ($n = 6$), RD/RD ($n = 6$) and HD/RD ($n = 7$); (C) Leptin serum concentration (ng/dL) in offspring CONT/CONT ($n = 6$), RD/CONT ($n = 7$), HD/CONT ($n = 8$), CONT/RD ($n = 7$), RD/RD ($n = 8$) and HD/RD ($n = 6$). Data expressed as mean \pm SEM ($p < .05$). * indicate significance in relation to CONT/CONT and RD/CONT; ** indicate significance in relation to CONT/RD and RD/RD; # indicate significance in relation to RD/RD; & indicate significance in relation to CONT/RD.

post-hoc test.

Table 1 shows the results of anxiety-like behavior and recognition memory, evaluated using EPM and NOR tests, respectively. No significant difference was found in the percentage of entries into the open arms and in the percentage of time spent in the open arms, as well as, in the number of head-dipping in all groups. Besides, data from the NOR also showed no significant differences in the recognition index at 3 h after habituation among the analyzed offspring groups.

4. Discussion

Previous studies using animal models have reported that consumption of a high fat diet during pregnancy and lactation may cause adverse metabolic consequences in the progeny (males and females),

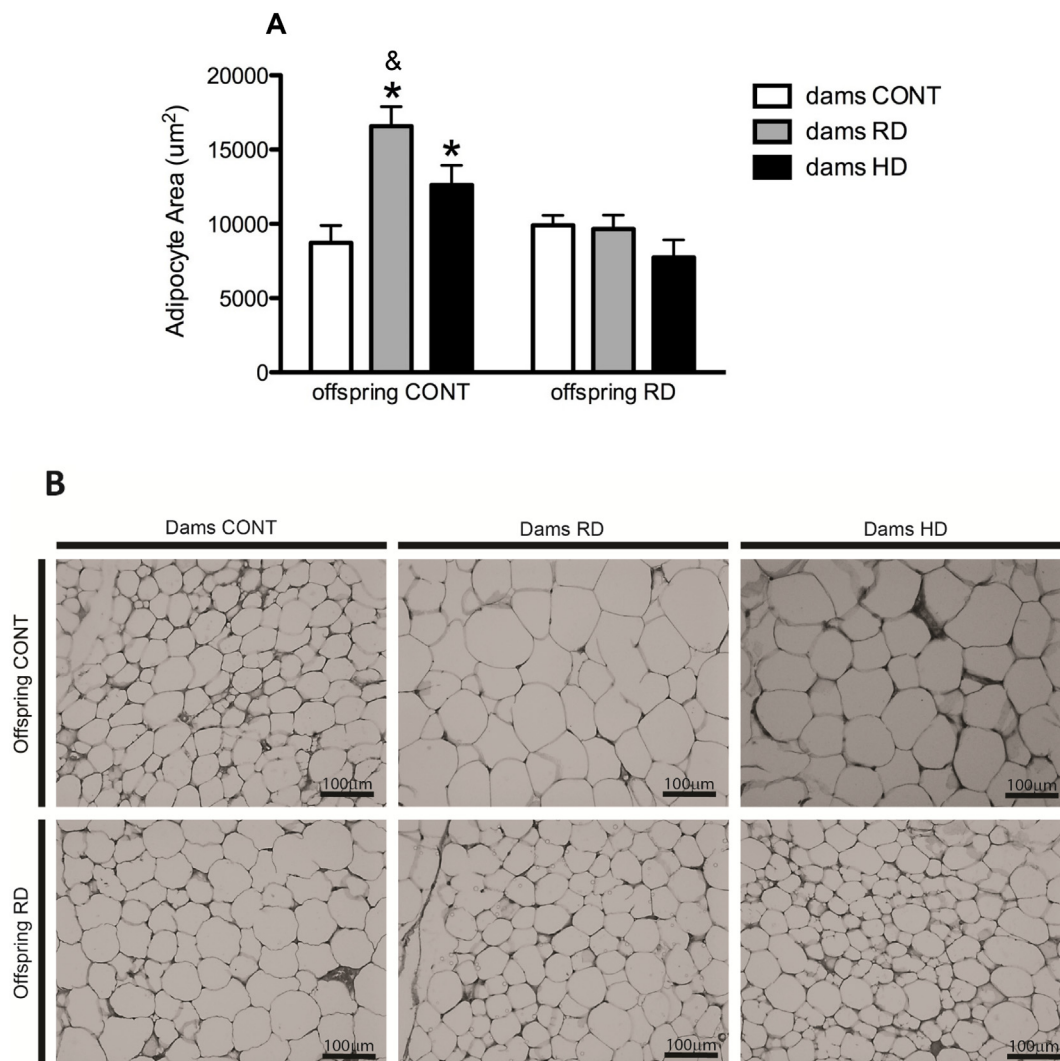


Fig. 2. Morphometric analysis of adipose tissue. (A) Adipocyte area (μm^2) was measured in offspring CONT/CONT ($n = 6$), RD/CONT ($n = 7$), HD/CONT ($n = 6$), CONT/RD ($n = 6$), RD/RD ($n = 5$) and HD/RD ($n = 6$). (B) Representative micrographs of adipose tissue from each experimental group. Data expressed as mean \pm SEM. * indicate significance in relation to CONT/CONT; & indicate significance in relation to HD/CONT.

which can arise from weaning to adulthood [20,21]. In the present study, we showed that glycemia and insulin levels in the offspring are increased by the maternal HD. In addition, the RD feeding intervention in the offspring starting soon after weaning was not sufficient to improve these parameters in the adulthood. On the other hand, leptin levels and adipocyte size are influenced for both maternal and offspring diets. Behavioral assessment showed again a maternal diet effect, in this case, maternal RD exposure led to lower locomotion activity and increased innate fear. Thus, despite many studies have reported the effects of maternal diet on the offspring physiology, here we also submitted the progeny to a diet intervention, i.e. RD.

Caloric restriction is able to reduce body weight and ameliorates insulin and glucose levels in male mice and human beings [11,22]. The main outcome of caloric restriction is the increase in longevity, and this anti-aging effect is associated with enhanced insulin sensitivity [11,23]. Several molecular mechanisms might explain the beneficial effects of RD on aging, including a decrease in free radical production and in the inflammatory processes, induction of cytoprotective responses to cellular stress, and stimulation of growth factors production [11,24]. However, in the present study, RD was not able to revert the HD maternal imprints on glucose and insulin plasma levels. These findings corroborate with similar studies showing that the progeny of dams fed obesogenic diets during pregnancy and lactation showed insulin

resistance and glucose intolerance [3,4,25]. It is worth to mention that these studies did not evaluate RD in the offspring. Thus, we corroborate with previous findings showing that metabolism is highly susceptible to intrauterine programming, which reinforces the idea that epigenetic modifications exert a robust effect on the fetal environment, influencing the adult phenotype in multiple generations [3,26,27].

Leptin is an anorexigenic hormone produced by adipose tissue, thus its blood levels are decreased in food-restricted animals and usually increased when the adipose depots are elevated [28]. In the current study, leptin levels were influenced by both maternal and offspring diets, but it is clearly shown that offspring from RD-fed dams had a decreased leptin level. It was previously demonstrated that offspring from dams that received junk food showed higher leptin and insulin concentrations compared with their chow-fed counterparts [29]. Here, we not only addressed the maternal impact of the HD but also the capacity of RD-fed offspring to revert the epigenetic influence of maternal HD. In this context, we showed that HD/CONT offspring had a higher leptin concentration than CONT/CONT and RD/CONT, which highlights the importance of this programming to modulate metabolic responses of the progeny. A similar finding was showed by Gali et al. (2018) that evaluated in male the effect of maternal high-fat diet immediately after weaning. They demonstrated a hypermethylation in the anorexigenic *Pomc* gene in the hypothalamus, which interferes in leptin

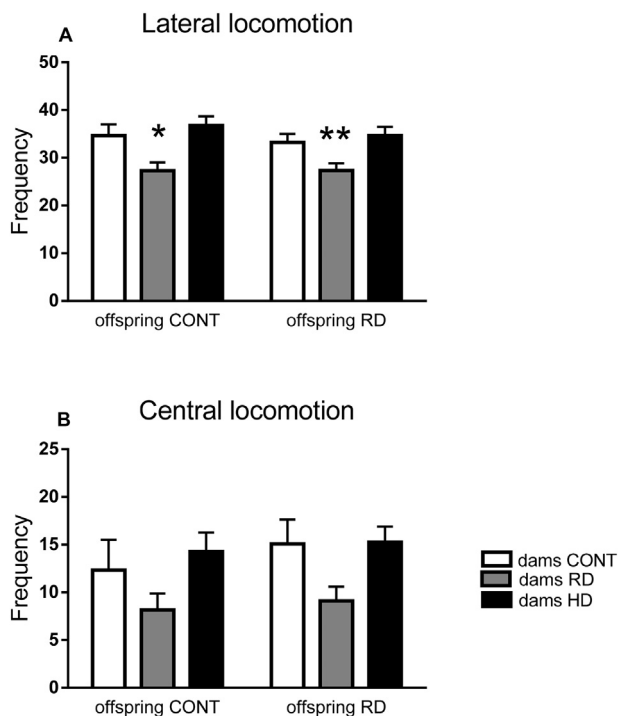


Fig. 3. Locomotor activity in the open field test. (A) Frequency in lateral locomotion was analyzed in offspring CONT/CONT ($n = 12$), RD/CONT ($n = 13$), HD/CONT ($n = 14$), CONT/RD ($n = 9$), RD/RD ($n = 9$) and HD/RD ($n = 14$). (B) Frequency in central locomotion. Data expressed as mean \pm SEM. * indicates significance in relation to CONT/CONT and HD/CONT; ** indicates significance in relation to CONT/RD.

signaling. Thus, the increase in leptin levels is not correlated with a satiety response. These results reinforce that maternal perinatal environment programs long-term changes in metabolic homeostasis [30].

In the present study, histological analysis of the adipose tissue showed a significant increase in the adipocyte area of RD/CONT and HD/CONT offspring compared to CONT/CONT group, showing that maternal diet interventions affect adipocyte area in the adulthood. Narita et al. (2018) showed in male Wistar rats that caloric restriction has a different impact on adipose tissue stores distributed throughout the body, including adipocyte size, lipid metabolism, signaling pathways, adipocytokine secretion profile. Differently from the study cited, we analyzed the visceral adipose tissues of the females [12]. A possible explanation is that not only HD leads to increased adiposity in the offspring, but maternal undernutrition during the periconceptional period and pregnancy, also results in increased adiposity in the progeny as described elsewhere [1,6,7]. However, when offspring was submitted

to RD, adipocyte area did not change irrespective of the maternal diet. Taken together, these findings show that adipocyte area is influenced by maternal diet, but RD in the offspring is able to overcome the effect of hyper or hypocaloric nutrition during pregnancy and lactation.

It is known that HD promotes liver dysfunction such as Nash (for review see [31]). Thus, in the present study, the lack of hepatic pathological findings may be related to the fact that the offspring did not receive HD, and hepatic dysfunction is less influenced by epigenetic mechanisms in comparison to other metabolic parameters. It was previously demonstrated that caloric restriction protects the liver from oxidative stress and inflammation [32]. However, caloric restriction during pregnancy does not cause hepatic steatosis in the male Wistar offspring [33] which is in accordance with our results.

It is well demonstrated that obesity promotes cognitive decline and it is associated with the development of psychiatric disorders, such as depression [34]. On the other hand, caloric restriction ameliorates neurological function [9,35]. Substantial evidences indicate that diet-induced obesity leads to deficits in learning and memory processes in male rodents [36,37]. Almeida-Suhett et al. (2017) found that high-fat diet-fed mice presented an impairment in working memory. Here, we used object recognition test to assess recognition memory. Neither maternal or offspring diet intervention interfered in recognition memory. However, a previous study demonstrated that caloric restriction during pregnancy leads to a worse performance of the offspring in the object recognition test. Nevertheless, it was seen only at 3 weeks of age, and adult mice did not show significant differences, which is in accordance with our results [38]. Thus, even the absence/lack of memory impairment seen in adult mice does not exclude the possibility of a detrimental effect of the maternal diet on cognition in animals of different ages.

Based on the need to conserve energy, it would be expected that caloric restriction promotes a reduction in the physical activity of animals. Unexpectedly, many studies have reported that caloric restriction actually causes increased levels of activity [39,40]. However, we have found a maternal diet effect on the locomotor activity in the OF test, and the offspring of the RD-fed dams showed decreased locomotion in the lateral and central area of the apparatus. Even 1 week of caloric restriction prior to conception caused a decrease in the time spent in the central zone of the OF, showing an anxiogenic effect on adult male offspring [41] which is in agreement with our data. It was also shown that adult male offspring of dams subjected to RD exhibited increased fear reactivity to a predator odor, with no alteration in anxiety-like behavior as measured in the EPM and OF [42]. We found similar results in the EPM, with no differences between groups. It is interesting to note that paternal diet can also modify the behavioral responses in the adulthood. The offspring of males Wistar that received RD exhibited a reduction in anxiety-like behavior, as evidenced by the increase in time spent in the open arms of the EPM and a higher frequency of entries into the central area of the OF [43].

Table 1
Anxiety-like behavior and recognition memory in adult female offspring.

	CONT/CONT	RD/CONT	HD/CONT	CONT/RD	RD/RD	HD/RD
Elevated Pluz Maze						
% time spent into the open arms	43.00 \pm 8.69	46.00 \pm 7.04	41.10 \pm 7.79	57.10 \pm 8.88	44.62 \pm 5.21	52.90 \pm 8.34
% entries into the open arms	47.88 \pm 1.70	44.78 \pm 3.12	45.10 \pm 2.83	49.50 \pm 3.47	50.10 \pm 5.05	48.83 \pm 2.20
Number head-dipping	15.14 \pm 2.95	9.70 \pm 1.41	8.90 \pm 1.66	9.6 \pm 2.00	14.75 \pm 1.91	10.78 \pm 1.47
Object Recognition						
Recognition index (3 h)	63.67 \pm 3.02	62.00 \pm 3.77	62.46 \pm 1.49	56.10 \pm 3.89	57.63 \pm 3.03	62.90 \pm 2.33

Data expressed as mean \pm S.E.M.

Two-way ANOVA, followed by Bonferroni post-hoc test.

Abbreviations: CONT/CONT (maternal standard chow/offspring standard chow), CONT/RD (maternal standard chow/offspring restriction), RD/CONT (maternal restriction/ offspring standard chow), RD/RD (maternal restriction/offspring restriction) HD/CONT (maternal hypercaloric/offspring standard chow), and HD/RD (maternal hypercaloric/offspring restriction).

Considering the findings in this study, we conclude that the maternal diet, during gestation and lactation, influences metabolic and behavioral responses of the offspring in a long-term. In addition, maternal RD appears to exert a protective metabolic effect. However, in the OF test the locomotion activity was decreased in the offspring of RD-fed dams. Thus, despite RD provides a beneficial response in metabolic parameters, the effects of RD on behavior need more investigation.

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