Marina Galleazzo Martins

# MATERNAL HYPERGLYCEMIA AND OVERNUTRION: EFFECTS ON MATERNAL CARE AND OFFSPRING DEVELOPMENT AND BEHAVIOR ACROSS LIFE

# ASSOCIAÇÃO ENTRE HIPERGLICEMIA MATERNA E SUPERNUTRIÇÃO: EFEITOS NO CUIDADO MATERNO E NO DESENVOLVIMENTO E COMPORTAMENTO DOS DESCENDENTES EM DIFERENTES FASES DA VIDA

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Advisor: Ana Carolina Inhasz Kiss, PhD
Co-advisor: José de Anchieta de Castro e Horta Júnior, PhD

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To the ones who came before me:
Vô Lilo, Vó Eva, Vô Henrique, and Vó Teresa

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“(...) e, eu, rio abaixo, rio a fora, rio a dentro - o rio.”

*A terceira margem do rio,*
*João Guimarães Rosa*

# SUMMARY

## ABSTRACT

The present study evaluated if snack intake during pregnancy and lactation could aggravate previously established maternal hyperglycemia and its consequences to maternal care, as well as offspring development, metabolism, and behavior throughout life. Our hypothesis was that snack intake during pregnancy and lactation would trigger further impairments in maternal glycemia homeostasis, resulting in changes in maternal behavior as well on offspring development, metabolism, and behavior from birth to senescence. Chapter 1 describes how snack intake altered maternal food intake and decreased glucose tolerance in STZ-treated females. Birth weight classification was normalized in the offspring from hyperglycemic dams with access to snacks, which showed a patter similar to Control offspring. Moreover, hyperglycemic dams with access to snack were less anxious and had higher maternal motivation. Chapter 2 describes short and long-term consequences of this altered maternal nutrition and metabolism to both male and female offspring, analyzing glucose metabolism, behavior, and morphometric aspects in adolescence, adulthood, and senescence. Male offspring reproductive function was impaired in adulthood, while there was an increase in anxiety-like behavior in senescence, showing that consequences may only be evident in the long-term. No further compromises on offspring metabolism were observed. In conclusion, the present study showed that snack intake during pregnancy and lactation further impaired maternal hyperglycemia, leading to disruptions on maternal motivation during lactation and reduced the incidence of macrosomia in their offspring. In adulthood, the reproductive function was disrupted, and senescent offspring showed changes on anxiety-like behavior. Future studies will describe effects on offspring learning and memory, and its possible neural substrates. The experimental model used in this study is useful to study the consequences of maternal diabetes associated with inappropriate nutrition, since the glycemic levels resemble those most observed in pregnant women diagnosed with clinical or gestational diabetes. Although snack intake aggravated the glucose intolerance of mild hyperglycemic rats, glycemic levels were still within the mild range, which might explain why this condition did not lead to major impairments for both mother and offspring. However, even this mild maternal condition was enough to change maternal and offspring outcomes, reinforcing the importance of women sustaining target glucose levels and a healthy diet during pregnancy and lactation.

**Keywords:** pregnancy, lactation, glucose tolerance, offspring, rats

## RESUMO

O presente estudo avaliou se a ingestão de *snacks* durante a prenhez e lactação agravaria a hiperglicemia materna de ratas diabéticas e suas consequências para o cuidado materno, e o desenvolvimento, metabolismo e comportamento dos descendentes ao longo da vida. Nossa hipótese era que a ingestão de *snacks* durante a prenhez e lactação prejudicaria a homeostase glicêmica materna, resultando em alterações no comportamento materno, bem como desenvolvimento, metabolismo e comportamento dos descendentes do nascimento à senescência. O capítulo 1 descreve como a ingestão de *snacks* alterou a ingestão alimentar materna e reduziu a tolerância à glicose em fêmeas tratadas com STZ. A classificação do peso ao nascer foi normalizada nos descendentes de fêmeas hiperglicêmicas com acesso aos *snacks*, apresentando um padrão semelhante ao grupo Controle. Além disso, essas fêmeas eram menos ansiosas e apresentaram maior motivação materna. O capítulo 2 descreve consequências, a curto e longo prazo, do metabolismo e nutrição materna alterados nos descendentes machos e fêmeas, analisando o metabolismo da glicose, comportamento, e parâmetros morfométricos na adolescência, vida adulta e senescência. A função reprodutiva em descendentes machos foi alterada na vida adulta, assim como aumentaram os níveis de ansiedade na senescência, mostrando que essas consequências podem ser vistas apenas a longo prazo. Não houve comprometimento do metabolismo da glicose dos descendentes. Assim, o presente estudo mostrou que a ingestão de *snacks* durante a prenhez e lactação agrava a hiperglicemia materna, levando a alterações na motivação materna durante a lactação e reduzindo a incidência de macrossomia em seus descendentes. Na vida adulta, a função reprodutiva foi alterada, e animais idosos apresentaram maior ansiedade. Estudos futuros descreverão possíveis consequências para memória e aprendizagem dos descendentes, e as possíveis repercussões neurais. O modelo experimental utilizado nesse estudo é útil para a avaliação dos efeitos do diabete materno associado a uma nutrição inadequada, já que os níveis glicêmicos se assemelham aos observados em mulheres diagnosticadas com diabete clínico ou gestacional. Apesar dos *snacks* agravarem a hiperglicemia, os níveis glicêmicos ainda são moderados, o que poderia explicar porque não foram observadas alterações mais pronunciadas nas mães e seus descendentes. Entretanto, mesmo essa condição materna moderada foi suficiente para causar efeitos para mães e seus descendentes, reforçando a importância de mulheres manterem níveis glicêmicos adequados e uma dieta saudável durante a gestação e lactação.

**Palavras-chave:** prenhez, lactação, tolerância à glicose, descendentes, ratos

## BACKGROUND

Diabetes is a chronic disease characterized by hyperglycemia, either due to reduced insulin production by the pancreas or impaired insulin action in the body [1]. If not properly treated, hyperglycemia can lead to serious and lethal consequences to the organism, followed by a considerable burden in the health system and economic loss [1]. Diabetes can be assorted into three major categories: type 1, type 2, and gestational diabetes [2]. Besides those categories, genetic syndromes, exocrine pancreas diseases, drugs, and chemicals can be the cause of specific types of diabetes, which are rare in the population. Type 1 diabetes affects between 5-10% of all diabetic patients and is defined by β-cell loss and absolute insulin deficiency. Type 2 diabetes is the most frequent type of diabetes, affecting between 90-95% of the patients, and involves insulin resistance that can lead to progressive β-cell loss. Diabetes can also be present during pregnancy in the form of clinical diabetes, when women previously diagnosed with different types of diabetes become pregnant, or gestational diabetes, when it is first diagnosed during pregnancy. Gestational diabetes is a condition in which a woman without diabetes develops high blood sugar levels during pregnancy. It can affect 5-10% of pregnancies and it is diagnosed during the second or third trimester of pregnancy [2].

Gestational diabetes results from an exacerbation of a variety of physiological adjustments that happen in pregnancy to support offspring development. During pregnancy, the maternal organism is in a state of physiologically insulin resistance, which is pathologically exacerbated in women diagnosed with gestational diabetes [3]. In healthy pregnancies, maternal metabolic homeostasis is shifted towards lipids utilization, so glucose is readily available to the growing fetus [4]. In early pregnancy, insulin sensitivity increases, allowing fat deposition in this anabolic period [5]. However, in the third trimester, there is a peak in insulin resistance, with a decrease of 50% in insulin sensitivity [6]. Furthermore, pregnant women show a positive energy balance, essential for offspring development [6]. All these factors are considerably worsened in gestational diabetes.

Even with increased information and methods of diagnosis available, diabetes in women of childbearing age is still sub-diagnosed [2]. The American Diabetes Association (ADA) recommends that pregnant women not previously diagnosed with diabetes should be screened for gestational diabetes at 24 to 28 weeks of pregnancy through a 75-g oral glucose tolerance test. A diagnosis is made when the woman has any of the following glycemia: ≥ 92 mg/dL at fasting, ≥ 180 mg/dL 1 h after glucose load, and ≥ 153 mg/dL 2 h after glucose load [2]. The diagnosis is important, so proper treatment is provided to

avoid further consequences for both mother and offspring [7]. Both gestational and clinical diabetes have consequences to the mother and offspring. Since in clinical diabetes the uterine milieu is already altered by hyperglycemia in the first stages of pregnancy, offspring of women with clinical diabetes are at higher risk of congenital anomalies. On the other hand, gestational diabetes is mostly established during the fetal stage, compromising offspring growth and development. Changes in fetal growth and development are relevant since birth weight is a major predictor of long-term health [8, 9].

Women previously diagnosed with clinical diabetes or who develop gestational diabetes have a greater risk of obstetric complications, such as preterm birth, preeclampsia, c-section, and shoulder dystocia [3, 10]. Their offspring also show a greater risk of being premature, having severe malformations, Erb's palsy, and of being classified as macrosomic at birth [3, 10, 11], usually showing increased body fat regardless of their birth weight classification [11, 12]. The offspring of the diabetic mother are also at a greater risk of developing obesity, metabolic syndrome, insulin resistance, and of having a worse lipidic panel and higher fat deposition during childhood and adolescence [6, 13-15].

In addition to metabolic impairments, the offspring from diabetic mothers also may show deficits in intelligence, motor skills, attention, and hyperactivity. Six-month-old infants born to diabetic mothers show electrophysiological differences related to visual memory [16]. During primary school, this offspring has shown poorer fine and gross motor skills and higher levels of hyperactivity [17]. Moreover, maternal hyperglycemia severity was positively correlated with poorer offspring outcomes [17]. A systematic review and meta-analysis have shown that offspring of diabetic mothers have worse cognitive and behavioral outcomes, with an increased risk of being diagnosed with attention and hyperactivity disorder as well as autism spectrum disorders [18]. These data support the relevance of sustaining adequate glucose levels throughout pregnancy, reinforcing the importance of a healthy diet during this period to prevent offspring behavioral impairment later in life.

While most women with clinical diabetes are usually already under treatment before pregnancy and thus can prevent those outcomes, gestational diabetes is often undiagnosed, leading to a late hyperglycemia management and, consequently, to higher impact for the offspring. Diabetes criteria are constantly being reviewed to determine values that would better predict or indicate poor fetal outcomes, since different levels of maternal hyperglycemia and insulin resistance lead to different adverse offspring outcomes; the greater the maternal hyperglycemia, the greater the risks to mother and offspring [19]. However, it is important to highlight that there is a positive correlation

between maternal glycemia and offspring body fat during childhood even in non-diabetic mothers [20, 21]. It has also been shown a positive correlation between offspring hyperglycemia in childhood and $HbA_{1c}$ of mothers with no gestational diabetes [22]. Therefore, interventions during pregnancy are key to improve maternal and fetal outcomes. Notably, it has been shown that dietary interventions can improve gestational diabetes effects, resulting in a better maternal glucose management and fetal outcomes [23].

The development of clinical and gestational diabetes has been linked to several risk factors, such as ethnicity, overweight and obesity, smoking, being physical inactive, and an unhealthy diet. Dietary choices, such as an increased intake of foods rich in fat, sugar, and saturated fatty acids, are associated with an increased risk for Type 2 diabetes [24-27]. Healthy lifestyle habits during pregnancy, such as regular physical activity and adequate nutrition, are key to reduced diabetes effects both on the mother and their offspring. Notably, a healthy diet during pregnancy must be encouraged in these patients to reduce obesity and diabetes risks in future generations [28]. The impact of maternal diet independent of maternal obesity on offspring development should be addressed to promote intervention strategies that are more effective [21]. Lifestyle interventions are also crucial to separate the effects of maternal diabetes and overnutrition during pregnancy and lactation from the infant's nutritional environment in an “over-fed” house [12].

The EarlyNutrition Project recommendations highlight that pregnant women do not have to change macronutrient proportion in their diet, unless in cases of food deprivation [29]. A balanced diet should be maintained throughout pregnancy, with an increase in energy intake of no more than 10% of what should be consumed by non-pregnant women [29]. Nonetheless, poor food choices and overconsumption are related to the increase in obesity prevalence worldwide [30]. Maternal diet can also affect offspring birth weight, but studies have shown that the diet quality (proportion of macronutrients, for example) are more important than the total energy intake [31]. Researchers have shown an association between unhealthy diets during pregnancy and a higher risk of smaller birth weight, which is a predictor of long-term consequences [32-34]. Furthermore, a healthy lifestyle is associated with better hyperglycemia management during pregnancy [35, 36], while an unhealthy diet may worsen diabetes.

Cohort studies are valuable because they can identify multiple outcomes in the population, independently of case and control groups’ formation, as well as calculate incidence rate and relative risks [37]. However, most studies showing long-term consequences of maternal diabetes and an inappropriate nutrition in the human population show some limitations. Frequently, these studies are retrospective, and

mothers show a wide range of hyperglycemia and/or glucose intolerance. Further, it is not always easy to discern between patients with clinical and gestational diabetes. In addition, it is difficult to delimitated effects related to maternal metabolism or diet intake, as well as to propose possible interventions. In this context, animal models are valuable to study manipulations during pregnancy and lactation, their effects on offspring, and their possible mechanisms [38, 39].

A variety of experimental methods have been employed to model diabetes [38]. The administration of β-cytotoxic agents, such as streptozotocin (STZ), has been widely used to develop hyperglycemia in rodents. STZ administration can induce severe or mild hyperglycemia depending on rodents' lineage and age, dose, and administration route. When STZ is giving during adulthood, animals develop severe hyperglycemia, with fasting glycemia over 300 mg/dL, and frequently above 500 mg/dL. These glucose levels are rare in diabetic patients, so alternative models of mild hyperglycemia have been developed. The neonatal STZ administration leads to mild hyperglycemia in rats, with glucose levels between 120 and 300 mg/dL, due to partial regeneration of pancreatic β-cells in the first days after birth [40, 41]. Moreover, animals will display hypoinsulinemia and glucose intolerance during adulthood [42-45]. Apart from metabolic impairments, neonatal STZ administration also leads to behavioral impairments both in dams and their offspring. STZ-treated females show reduced exploratory behavior [44], while their male offspring, but not female, show higher immobility in the open-field arena [43], indicating sex-specific effects of maternal hyperglycemia in offspring development, as previously reported [46].

Dietary manipulations during pregnancy and lactation may worsen the metabolic impairment in previously hyperglycemic females [47, 48]. Several diet manipulations have been employed to study the consequences of an unhealthy diet in animal models, like hypercaloric or high-fat diets, and cafeteria diets, high-fat and high-sugar junk foods [49]. In all these models, a variety of deleterious effects have been observed in the offspring, including hyperphagia, increased body adiposity, elevated levels of triglycerides, hyperglycemia, and insulin resistance [50, 51], as well as increased anxiety-like behavior [52-55]. Recently, a snack intake model has been proposed [56], in consonance with the increased intake of snacks between meals in the population [57]. Snack intake in addition to regular meals leads to a higher caloric intake and, consequently, body weight gain [58, 59]. Snacks composed evenly by carbohydrate and fat, like potato chips, trigger a state of hedonic hyperphagia, in which food intake is disassociated to hunger [56, 60]. These snacks contribute little to satiety and their calories are not completely compensated by a reduced caloric intake in regular meals [58, 59], resulting in an increased daily caloric intake. Since an unhealthy diet is

associated with increased risk of developing diabetes, an inappropriate nutrition during pregnancy and lactation can trigger further impairments in an already established maternal hyperglycemia in animal models, with worse consequences to mother and offspring [61].

Although maternal hyperglycemia and inappropriate nutrition effects on offspring development have been extensively explored separately, their association and its impact on maternal metabolism and offspring development and behavior throughout life is less clear. Our hypothesis is that snack intake during pregnancy and lactation will trigger further impairments in maternal glycemia homeostasis, resulting in changes in maternal behavior as well as sex-specific effects on offspring development, metabolism, and behavior from weaning to senescence.

Aligned with the Graduate Program of General Physiology research interests, this study investigated the relationship between physiological processes and the environment, particularly on how the maternal environment shapes offspring development and its survival in the long term. Furthermore, studies in animal models have the potential to be future applied to clinical settings and health improvement. The study of maternal hyperglycemia and inappropriate nutrition will be explored in chapters 1 and 2, and it may clarify window of opportunities where interventions during the perinatal period might be made to promote healthier pregnancies, that will lower the risk for the development of metabolic and cognitive disorders in the offspring throughout life. Chapter 1 describes how snack intake during pregnancy and lactation changes maternal food intake and worsens maternal hyperglycemia of STZ-treated females, as well as its impact on offspring birth weight and maternal behavior. This chapter resulted in a manuscript submitted to Physiology & Behavior in 20/04/2021 and is currently under review. Chapter 2 describes the postnatal life of male and female offspring born to those females, showing the consequences of an unbalanced maternal organism to offspring glucose metabolism, behavior, and biometric aspects in adolescence, adulthood, and senescence. This chapter has been also prepared as a manuscript according to Physiology & Behavior guideline and is yet to be submitted. Finally, a side study was conducted during an internship at the Department of Neuroscience at Carleton University that aimed to investigate the effects of maternal hyperglycemia in the offspring control of food intake after access to high-fat diet in the post-weaning period. However, these data are still under analysis and will not be presented at the moment.

# CHAPTER I: Snack intake further impairs maternal glucose tolerance and reduces the incidence of macrosomia in the offspring of mild hyperglycemic rats

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**Highlights**

- Snack intake further decreased glucose tolerance in STZ mild hyperglycemic rats
- Offspring birth weight classification of STZ-snack dams resembled the Control ones
- Caloric and macronutrient intake of STZ rats was alike to Controls on the same diet
- STZ-snack dams were less anxious and retrieved their litters faster
- Decreases in birth weight and litter growth were a function of snack intake alone

**Abstract**

Metabolic disorders, like diabetes, as well as maternal diet, alter nutrient availability in utero, inducing adaptations in the offspring. Whether the effects of maternal hyperglycemia can be modulated by diet, however, has yet to be explored. In the current study, we examined this issue by giving females rats, treated neonatally with STZ to induce mild hyperglycemia, and control littermates either *ad libitum* access to standard chow (Control n = 17; STZ n = 16) or standard chow and snacks (Control-snack n = 18; STZ-snack n = 19) (potato chips and a red fruit-flavored sucrose syrup solution 1.5%) throughout pregnancy and lactation. We hypothesized that the maternal glucose intolerance typically seen in female rats treated neonatally with STZ would be exacerbated by snack intake and, further, the combination of snack intake and STZ treatment would lead to alterations in maternal behavior and offspring development. Maternal body weight and food intake were measured daily through pregnancy and lactation and litter weight throughout lactation. At birth, litter size, offspring weight, body length, and anogenital distance were obtained and offspring were classified according to their weight. Measures of nursing and retrieval behavior, as well as exploration in the open field and the elevated plus-maze were also recorded. As expected, snack intake aggravated the glucose intolerance of STZ-treated rats during pregnancy. Both Control and STZ-treated females that had access to snacks ate more calories and had higher fat together with lower carbohydrate and protein intake than females having access to chow alone. Overall, STZ-treated dams gave birth to fewer pups. Chow-fed STZ females gave birth to a greater proportion of large for pregnancy age pups, whereas dams in the Control-snack group gave birth to a greater proportion of small pups. The birth weight classification of pups born to STZ-snack rats, however, resembled that of the Control group. Although all litters gained weight during lactation, litters from snack-fed dams, regardless of hyperglycemia, gained less weight and did not show catch-up growth by weaning. Overall, STZ rats spent more time nest building whereas the average inter milk

ejection interval was higher in snack-fed females. STZ-snack dams retrieved the complete litter faster than dams in the other groups and showed less anxiety-like behavior in the elevated plus-maze. Together, these data suggest that when mild hyperglycemic females are given access to snacks throughout pregnancy and lactation their intake is like that of Control females given similar access. The combination of hyperglycemia and snack access decreased glucose tolerance in pregnancy, but, except for normalizing birth weight classification, it produced few effects that were not seen as a function of snack intake or hyperglycemia alone. Since birth weight is a strong predictor of health issues, future studies will further investigate offspring behavioral and metabolic outcomes later in life.

**Keywords:** pregnancy, lactation, hyperglycemia, nutrition, birth weight, maternal behavior.

## 1. Introduction

The early nutritional environment can have profound influences on the adult metabolic phenotype [1] and there is evidence that both over and undernutrition in early life increases the risk for obesity and metabolic disorders in adulthood [2, 3]. Maternal diet is not the only factor that influences the nutrients available to the fetus. Maternal metabolic disorders, like diabetes, also alter nutrient availability in utero, inducing adaptations in the offspring [4]. Diabetic mothers are at increased risk for a variety of pregnancy complications including spontaneous abortion, increased fetal adiposity, and fetal hypoglycemia [5]. The offspring of diabetic mothers have a higher risk of being macrosomic at birth [6] and developing obesity during childhood and adulthood [5, 7]. As adults, these offspring are also at risk for impaired glucose tolerance and insulin resistance [7]. It is noteworthy that even in pregnant women not diagnosed with diabetes according to the current guidelines, fluctuations in maternal glucose levels are sufficient to affect offspring fat deposition [8].

A variety of experimental manipulations which produce varying degrees of hyperglycemia have been used to induce maternal hyperglycemia in rodents [9, 10]. Of these, neonatal streptozotocin (STZ) administration, which results in glucose levels between 120 and 300 mg/dL, reflects more broadly the glycemic state of the majority of women with diabetes during gestation. Similarly, female rats injected neonatally with STZ also show hypoinsulinemia and glucose intolerance, but no changes in fasting glycemia [11-14]. In addition to altering metabolism, neonatal STZ administration produces changes in behavior including impaired exploratory behavior in adolescence and adulthood [12]. These females show regular estrous cycles and mate readily, but fewer

of them carry pregnancies to term [13] and those that do give birth tend to have small litters, and heavier offspring. Mild hyperglycemic dams also show some changes in maternal behavior and retrieve their pups faster but have shorter nest bouts than controls [11]. The offspring of neonatal STZ mothers show changes in exploratory behavior during infancy and adulthood, spending less time immobile in the open field area [11, 15]. Whether the behavioral changes seen in the offspring of neonatal STZ-treated females reflect their mother's metabolic state or the changes in her maternal behavior are not clear.

The effects of maternal diabetes and diet manipulation have been thoroughly studied separately but there are currently no studies that explore the effect of their association on mothers and their offspring. It is possible, for example, that metabolic impairments caused by maternal hyperglycemia may be worsened by inappropriate nutrition [16]. To investigate this possibility in the current study, we utilized a paradigm in which neonatally STZ treated female rats were given access to snacks (1.5% flavored sugar water and potato chips) as well as standard chow throughout pregnancy and lactation and compared their glucose tolerance, maternal behavior, and offspring development with that of similarly treated rats that had access only to laboratory chow as well as untreated females given access to both snacks and chow or chow alone.

This approach was chosen because snack intake has increased in the human population over recent decades [17], and the availability of snacks leads to increased caloric intake and contributes to weight gain [18-20]. In addition, snacks do not induce long-lasting satiety and do not result in a compensatory reduction in caloric intake at regular meals in humans [18, 19]. Studies in rodents have shown that snacks with an equal proportion of carbohydrates and fat, like potato chips, trigger hedonic hyperphagia [20, 21], and in female rats, snack intake from weaning to adulthood leads to increased body weight and fat deposition [22]. These changes are not associated with alterations in fasting glycemia but led to a decrease in glucose clearance [22]. The effects of snack intake during pregnancy and lactation on the reproductive outcome and whether the pattern of intake would change across these reproductive states have not yet been studied in rodents. Thus, this, as well as the possibility that such patterns would differ between control females and those treated neonatally with STZ, were also investigated in the current study.

We hypothesized that, as in previous studies, neonatal STZ treatment would lead to maternal hyperglycemia as well as changes in maternal behavior and offspring development and that these would be exacerbated by intake of snacks, since diet manipulations such as high-fat and cafeteria diet have been shown to decrease glucose tolerance in normoglycemic rats [23, 24].

## 2. Materials and methods

### *2.1 Animals*

Male and female Wistar rats were obtained from the Multidisciplinary Center for Biological Research (CEMIB – State University of Campinas, SP) breeding facility. Animals were kept under controlled temperature (22 ± 2°C) and humidity (50 ± 10%), on a 12/12 h light/dark cycle (lights on 7 am, lights off 7 pm). All experimental procedures were approved by the local ethics committee of São Paulo State University (Unesp), Institute of Biosciences of Botucatu (Protocol number 919).

### *2.2 Experimental procedures*

#### *2.2.1 F1 Females*

##### *2.2.1.1 STZ administration and mating*

Female Wistar rats (n = 40) were mated to obtain the F1 generation. On postnatal day (PND) 1, litters were culled to 6-8 female pups and assigned to one of two treatments: 4 to 6 females/litter received 100 mg/kg of STZ (n = 192) (SIGMA Chemical Company, St. Louis, Millstone) diluted in citrate buffer (0,1 M, pH 4,5) subcutaneously; and 2 females/litter received the same volume of citrate buffer subcutaneously (n = 50) [11, 25, 26]. This method has been used previously to induce mild hyperglycemia in rats [27-30]. On PND 90, Control (n = 49) and STZ (n = 49) females were mated to control males to obtain F2 litters. Littermates were assigned to different experimental groups. Exceeding females were employed in other experiments.

##### *2.2.1.2 Pregnancy and lactation*

On pregnancy day (PD) 0, Control and STZ females were randomly assigned to receive either the standard chow (Control, n = 17; STZ, n = 16); or standard chow and snacks (Control-snack, n = 18; STZ-snack, n = 19). Snacks comprised potato chips (556 kcal/100 g) and a red fruit-flavored sucrose syrup solution 1.5% (Bacana®, 44 kcal/100 mL), without fructose. All rats received tap water and chow (382.5 kcal/100 g) *ad libitum* throughout the experiment. Females gave birth on PD 21 and litters were culled to 3 males and 3 females on PND 1. Dams receiving snacks and chow had access to the snacks only until lactation day (LD) 14 when pups began to eat independently [31], so that any effects of diet would only reflect those mediated by the metabolism or behavior of the dam. Food consumption and body weight were measured daily throughout pregnancy and lactation and dams weight gain was calculated. Data on daily food consumption was used to calculate total caloric intake, as well as the percentage of

calories coming from each diet fraction: chow, potato chips, and sucrose solution. The proportion of calories from specific macronutrients (carbohydrate, protein, and fat) was also calculated.

*2.2.1.3 Oral glucose tolerance test (OGTT)*

On PD15, dams from all experimental groups were given an oral glucose tolerance test (OGTT). After a 6h fast, dams received a 2g/kg glucose solution (200 g/L) by gavage. Blood samples were obtained from a tail snip 0, 15, 30, 60, and 120 min after glucose administration, and blood glucose was measured with a glucometer (OneTouch Ultra, Johnson & Johnson®). Overall blood glucose changes across the test were assessed by calculating the total area under the curve (AUC) using the trapezoid method [32].

*2.2.1.4 Maternal behavior*

Maternal behavior was assessed in two ways:

*Undisturbed*: On LD 5 and 10, undisturbed maternal behavior was recorded in the home cage for 6 h during the light phase of the light/dark cycle [33]. Mother and young interactions were later scored every minute during this period for the following behaviors: nursing, self-grooming, pup grooming, and nest building. The percentage of time away from pups was defined as the time the rat spent without any kind of interaction with pups, regardless of its position in the cage. The first 60 minutes of the longest nest bout was later used to assess latency to the first milk ejection, the number of milk ejections, and the inter-milk-ejection interval. A milk ejection was recorded each time a stretch response was observed for all the pups in the litter.

*Home-cage retrieval test:* On LD 6 and 11, mother-pup interaction was evaluated after maternal separation as previously reported [11]. On the test day, all pups were removed from the home cage and the nest destroyed. After 30 min, pups were returned to their cages and mother-pup interaction was recorded for 30 min. The latency to retrieve the first, fourth, and last pup of the litter was evaluated. All behavioral analyses were carried out with Countee [34].

*2.2.1.5 Maternal exploratory and anxiety-like behavior*

On LD 7, dams' exploratory and anxiety-like behavior were evaluated in the open field and elevated plus-maze respectively. The open-field test was conducted as described previously [11] and adapted from Broadhurst [35]. Briefly, animals were placed into a 100-cm-diameter arena divided into 25 sections for 5 minutes and the following parameters were recorded: number of sections entered (divided into 12 peripheral and

12 central sections), rearing, immobility, and self-grooming. Immediately after the open field test, rats were placed in the center platform of an elevated plus-maze with 50-cm-arms 40 cm above the ground, two closed and two open, and left to explore for 5 minutes. Time spent in the open arms and the center, the number of closed and open arms entries, and the total number of head dips were analyzed according to Walf and Frye [36]. All behavioral tests were recorded and later scored using Countee [34] by a researcher blind to experimental group membership.

*2.2.2 Offspring*

Females from all experimental groups delivered naturally, and litter size and sex ratio were recorded. All pups were weighed on PND 1 and classified as small for pregnancy age (SPA) (>1.7 standard deviations (SD) lower than average birth weight for controls); large for pregnancy age (LPA) (>1.7 SD higher than control average birth weight), or appropriate for pregnancy age (APA) (within ± 1.7 SD of Control group average birth weight) [37].

Body weight, naso-anal and anogenital distances were measured on PND 1. The anogenital index was calculated as the anogenital distance/body length ratio. During lactation, litter weight was recorded daily until LD 14 and then on LD 21. The total litter weight gain and feed efficiency (total litter weight gain/dam's total caloric intake) were also calculated.

*2.3 Statistical analysis*

Data are expressed as mean ± standard error of the mean. Data from AUC, litter size, female sex ratio, total caloric intake, specific macronutrient intake, feed efficiency, maternal weight gain in lactation, home-cage retrieval test, exploratory and anxiety-like behaviors, body weight at birth, naso-anal distance, and anogenital index were analyzed with a 2-way ANOVA, with maternal metabolic state (normo- or hyperglycemic) and diet (chow only or chow plus snack consumption) as between-subject factors. Data from glycemic curves, maternal weight gain in pregnancy and relative caloric intake, and undisturbed maternal behavior analysis parameters were analyzed with repeated measures two-way ANOVA, with time as the repeated measure and maternal metabolic state and diet as between-subject factors. F, p, and $\eta$ values are reported for all ANOVA analyses. Data from birth weight classification are expressed as frequency and were analyzed with Fisher's Exact Probability test. In all cases, statistical significance was set to alpha = .05. All statistical analyses were performed using SPSS (IBM, SPSS Statistics 22).

## 3. Results

### *3.1 STZ neonatal administration*

Of the 50 control rats, 49 reached adulthood and were mated; 45 of these had a positive pregnancy diagnosis, 39 carried their pregnancy to term, and 37 were included in the present study. From the 192 rats injected with STZ on PND 1, 49 reached adulthood and were mated. Of those, 46 had a positive pregnancy diagnosis, but only 36 dams carried their pregnancy to term and were followed throughout the experiment. The rate of survival to adulthood and the low rate of pregnancy completion (16%) is similar to that observed in previous studies using this model [11, 38]. Seven females (2 control, 5 STZ) gave birth to fewer than 4 pups and were withdrawn from the study. Two STZ-snack females gave birth to only 4 pups and were not followed through lactation. However, their food intake during pregnancy, litter size, and offspring birth weight were analyzed as they reflect an outcome of the experimental manipulation.

### *3.2 Pregnancy oral glucose tolerance test (OGTT)*

Neonatal STZ treatment impaired glucose tolerance in the OGTT carried out on PD 15 in the F1 females (significant effect of metabolic state, $F(1,23)=30.259$, $p<.001$, $\eta=0.568$ (see Figure 1). This effect was modified by a significant time x metabolic state effect ($F(4,92)=13.548$, $p<.001$, $\eta=0.371$), showing a significant metabolism effect on glycemia levels at 15 ($F(1,23)=30.198$, $p<.001$, $\eta=0.568$), 30 ($F(1,23)=37.48$, $p<.001$, $\eta=0.62$), and 60 minutes ($F(1,23)=14.626$, $p<.01$, $\eta=0.389$) after glucose administration, with glycemia in STZ dams always higher than in Control dams. As expected, neonatal STZ treatment increased fasting glycemia (Mean STZ = 95.55±6.60mg/dL, Mean Control = 84.83±4.06mg/dL, trend towards metabolism effect, $F(1,23)=3.374$, $p=.079$, $\eta=0.128$) as measured in the oral glucose tolerance test. As these data show, there was also a main effect of time ($F(4,92)=29.177$, $p<.001$, $\eta=0.559$) and a trend towards a diet effect $F(1,23)=3.574$, $p=.071$, $\eta=0.135$) which was apparently driven by the effects of diet in the STZ females, although neither the time x diet, metabolic state x diet nor metabolic state x time x diet interaction effects were significant. Consistent with these data, AUC was higher in STZ than Control females (metabolic state effect, $F(1,23)=24.782$, $p<.001$, $\eta=0.519$; and there was a trend towards a diet effect, $F(1,23)=2.986$, $p=.097$, $\eta=0.115$) that again appears to be greater in the STZ group than in Control although the metabolic state x diet interaction did not reach significance.

### *3.3 Maternal body weight and food intake during pregnancy*

There was no significant effect of neonatal STZ treatment on any measure of chow or snack intake. Overall, caloric intake was higher in snack-fed rats (diet effect, $F(1,50)=126.426$, $p<.001$, $\eta=0.717$) (Figure 2C) which was associated with a significant decrease in the proportion of calories from carbohydrates and protein (Carb $F(1,50)=5.793$, $p<.05$, $\eta=0.104$; protein $F(1,50)=3344.562$, $p<.001$, $\eta=0.985$) and an increase in the proportion of calories from fat intake ($F(1,50)=94.326$, $p<.001$, $\eta=0.654$) in snack-fed x chow-fed females (Figure 2D-2F). Pattern of intake varied across pregnancy (time x diet fraction effect, $F(38,1577)=12.567$, $p<.001$, $\eta=0.232$) (Figure 2A). Most calories came from potato chips and sucrose solution at the beginning of pregnancy ($F(2,87)=33.629$, $p<.001$, *post hoc* $p<.001$), there was a switch to more calories coming from chow towards the end ($F(2,87)=13.044$, $p<.001$, *post hoc* $p<.001$, PD 1 compared to PD 20). As expected all rats gained weight across pregnancy (significant effect of time $F(1,50)=332.386$, $p<.001$, $\eta=0.869$), and snack-fed rats gained more weight than chow-fed ones in the first half of pregnancy (time x diet effect, $F(1,50)=10.428$, $p<.01$, $\eta=0.173$) (Figure 2B).

*3.3 Offspring outcomes*

STZ rats gave birth to fewer pups than Controls (metabolic state effect, $F(1,66)=7.228$, $p<.01$, $\eta=0.099$) (Table 2), but sex ratio did not differ between groups (Fisher's test, $p>.05$). There was also a trend towards increased body length (metabolic state effect, $F(1,61)=2.859$, $p=.096$, $\eta=0.045$) and decreased female anogenital index (metabolic state effect, $F(1,61)=2.944$, $p=.091$, $\eta=0.046$) in offspring of STZ rats (Table 2). Overall, offspring born to snack-fed rats were lighter (diet effect, $F(1,61)=11.104$, $p<.01$, $\eta=0.154$), shorter (diet effect, $F(1,61)=7.995$, $p<.01$, $\eta=0.116$), and had increased anogenital index in both males (diet effect, $F(1,61)=8.583$, $p<.01$, $\eta=0.123$) and females (diet effect, $F(1,61)=5.888$, $p<.05$, $\eta=0.088$) than offspring of chow fed females.

Figure 3 shows birth weight classification for pups born to normo- and hyperglycemic mothers on PND 1. There was an increase in the number of pups classified as SPA in both Control-snack and STZ-snack rats and an increase in the number of pups classified as LPA in STZ rats (Figure 3, Fisher's test $p<.05$).

*3.4 Maternal and offspring body weight and food intake during lactation*

Overall, caloric intake was higher in snack-fed rats (diet effect, $F(1,41)=6.36$, $p<.05$, $\eta=0.134$) (Figure 4C) and, as in pregnancy, protein and carb intakes were lower and fat intake was higher in snack-fed rats than in controls (protein: $F(1,41)=1454.397$, $p<.001$, $\eta=0.973$; carbs: $F(1,41)=26.292$, $p<.01$, $\eta=0.391$ (Fat, $F(1,41)=235.566$, $p<.001$,

$\eta$=0.852) (Figure 4D-F). A higher proportion of calories came from chow than from snacks in both snack-fed groups (diet fraction effect, F(2,60)=48.145, p<.001, $\eta$=0.616, *post hoc* p<.001 and time x diet fraction effect, F(26,780)=18.011, p<.001, $\eta$=0.375) (Figure 4A). On LD 1, there was a higher intake of chow and sucrose solution compared to potato chips (F(2,63)=9.618, p<.001, *post hoc* p<.05), but there was a switch to more calories coming from chow rather than snacks on LD 7 (F(2,63)=80.786, p<.001, *post hoc* p<.001) that persisted until LD 14 (F(2,63)=122.738, p<.001, *post hoc* p<.001). Despite their increased caloric intake, snack-fed rats lost weight during the first two weeks of lactation whereas chow-fed rats gained weight (diet effect, F(1,41)=34.148, p<.001, $\eta$=0.454) (Figure 4B).

As expected, all litters gained weight during lactation (time effect: F(12,492)=1839.633, p<.001, $\eta$=0.978), but snack-fed litters gained less weight than chow-fed ones (F(1,41)=26.614, p<.001, $\eta$=0.394 and F(12,492)=18.067, p<.001, $\eta$=0.306, diet and time x diet interaction effects respectively) (Figure 5A). Feed efficiency (total litter weight gain/dam's total caloric intake) was lower in snack-fed rats (F(1,41)=99.218, p<.001, $\eta$=0.708) (Figure 5B) but higher in STZ-treated females (F(1,41)=5.1, p<.05, $\eta$=0.111) (Figure 5B). It is noteworthy that offspring from snack-fed dams were still lighter at weaning (females = 26.37±0.96, males = 39.90±2.04) than chow-fed offspring (females = 33.76±1.32, males = 48.66±2.86; significant effect of diet, F(1,40)=12.808, p<.01, $\eta$=0.243). As expected male pups were heavier than female pups at weaning (F(1,40)=51.032, p<.001, $\eta$=0.561) regardless of maternal metabolic state or diet condition.

*3.5 Maternal behavior*

Undisturbed maternal behavior was analyzed on LD 5 and 10 and the results are shown in Table 3. As expected, time spent nursing (F(1,41)=18.852, p<.001, $\eta$=0.315), self-grooming (F(1,41)=17.031, p<.001, $\eta$=0.293) and pup grooming (F(1,41)=3.935, p=.054, $\eta$=0.088) decreased from LD 5 to LD 10, with no significant effect of maternal metabolism or snack consumption. Accordingly, time away from pups increased over time in all experimental groups (F(1,41)=31.404, p<.001, $\eta$=0.434).

Nest building also decreased over time in all experimental groups (F(1,41)=22.179, p<.001, $\eta$=0.351), with dams spending less time building nests on LD 10 compared to LD 5, but overall, STZ females spent more time building nests, regardless of their diet (F(1,41)=4.797, p<.05, $\eta$=0.105). There was also a trend towards a time x metabolic state interaction (F(1,41)=3.807, p=.058, $\eta$=0.085) (Table 3).

The latency to the first milk ejection decreased between LD 5 and LD 10 in all experimental groups ($F(1,39)=8.589$, $p<.01$, $\eta=0.18$), and the average inter milk ejection interval was higher in snack-fed females regardless of their metabolic state ($F(1,39)=4.315$, $p<.05$, $\eta=0.10$) (Table 3).

There was no difference in the latency to retrieve the 1st and 4th in the home cage retrieval test on LD 6 (Figures 6A-6B). However, STZ-snack dams retrieved the whole litter faster than dams in the other groups, resulting in a metabolism x diet effect on the latency to retrieve all the pups ($F(1,34)=4.208$, $p<.05$, $\eta=0.11$) (Figure 6C). Since 11-day-old pups are highly mobile and tend to seek their mother instead of being retrieved, data for latency to retrieve pups was missing for several females for this test and these data were not analyzed.

*3.6 Maternal exploratory and anxiety-like behavior*

Neither metabolism nor snack consumption had any effect on the number of squares entered, rearing, or time spent in self-grooming in the open field ($p>.05$). In STZ, but not Control females, snack consumption increased the number of open arms entries (metabolic state x diet effect, $F(1,38)=6.633$, $p<.05$, $\eta=0.149$; diet effect, $F(1,38)=6.633$, $p<.05$, $\eta=0.149$ ) and the time spent in them (metabolic state x diet effect, $F(1,38)=5.099$, $p<.05$, $\eta=0.118$, and a trend towards diet effect, $F(1,38)=3.404$, $p=.073$, $\eta=0.082$) (Figure 7).

## 4. Discussion

The goal of the current study was to investigate the effect of the exposure to high-calorie palatable snack foods on reproductive outcome in neonatal STZ-treated and Control female rats. Consistent with previous research using this experimental model, neonatal STZ administration impaired maternal glucose tolerance during pregnancy. This effect was exacerbated when dams had access to snacks (Figure 1A and B). Thus, although this paradigm gave the females the opportunity to choose their intake of macronutrients, they did not adjust this as a function of their metabolic state. Mild hyperglycemic females given access to snacks throughout pregnancy and lactation had similar caloric and macronutrient intake of Control females given similar access. The combination of hyperglycemia and snack access normalized birth weight classification (Figure 3). Plus, dams were less anxious and retrieved their litters faster. However, several effects were a function of snack intake or hyperglycemia alone. Hyperglycemic rats, either receiving chow or snacks, gave birth to smaller litters with a greater proportion of macrosomic pups and increased time spent on nest building during maternal behavior

observations. Access to snacks, regardless of maternal metabolic state, reduced offspring birthweight and litter growth, and increased inter milk ejection interval.

Diet-induced exacerbated glucose intolerance has been observed in other studies using diet manipulations, such as high fat and cafeteria diet [23, 24], but this is the first study to address this effect in STZ-treated and Control females during pregnancy. The current data suggest that the pattern of snack intake did not significantly increase glucose intolerance in Control rats, while it did exacerbate it in STZ-treated rats. Although previous studies have found that hyperglycemic cycling rats are hyperphagic [39], we found no differences in caloric intake between Control and STZ snack-fed females in pregnancy, suggesting that this did not contribute to any differential effects on glucose tolerance. Differences in results between studies may reflect different degrees of hyperglycemia.

Under the current experimental conditions, both STZ and Control females showed a similar pattern of snack and chow intake across pregnancy. Overall, caloric intake was higher in snack-fed dams than in chow-fed ones (Figure 2C), with a greater proportion of calories coming from fat at the expense of carbohydrates and especially proteins. At the beginning of pregnancy, when dams first had access to snacks, there is a high intake of sucrose solution and potato chips, presumably because of their novelty [22]. However, there is a switch towards a higher chow intake from pregnancy day 14 on (Figure 2A). This effect might reflect the increased insulin resistance of late pregnancy [40] and the need for more protein intake during this stage [31]. Consequently, snack-fed dams gained more weight in the first half of pregnancy, but not in the second half (Figure 2B).

Previous research suggests that both a high-fat diet and hyperglycemia have opposing effects on offspring birth weight. Pregnancies complicated by mild diabetes usually result in macrosomia in the offspring [4, 11, 41], whereas dams fed with a high-fat diet often give birth to growth-restricted pups [42-46]. The results of the current study are consistent with both these outcomes. Overall, STZ-treated females gave birth to a greater proportion of LPA pups than Controls and snack intake increased the number of SPA pups both in Control-snack and STZ-snack groups. Thus, in the presence of both maternal hyperglycemia and a high-fat diet, the hyperglycemia effect on birthweight is counterbalanced by the diet effect, and offspring birth weight classification resembles the Control group. It is possible that excess of maternally-derived glucose and fetal hyperinsulinemia in hyperglycemic mothers, which stimulates fetal metabolism and neonatal growth [4], is offset, at least in part, by the reduced placental flow associated with abnormal placental function, which is a trait of dams fed with a high-fat diet [43, 47]. Additionally, although some studies with diet manipulations show a catch-up effect on

offspring weight [43, 46], such an outcome was not observed in the present study, since offspring from snack-fed rats were lighter throughout lactation.

The similarity of diet choice between STZ-treated and Control females persisted throughout the first two weeks of lactation. Snack-fed dams had a higher caloric intake as a result of higher fat and lower carbohydrate and protein intake. Interestingly, however, the proportion of calories coming from chow was higher than that coming from snacks in the second week of lactation. Although dams in both snack-fed groups decreased their protein intake, they sustained approximately 11% of protein intake during pregnancy and lactation, which has been previously described in control females that were given the choice between protein and carbohydrate diets [48]. Additionally, those dams lost weight in the first two weeks of lactation, while chow-fed dams gained weight despite a lower caloric intake. Body weight loss in snack-fed dams might reflect a lower protein intake during lactation, which could lead to mobilization of protein supply in addition to fat storage to sustain milk production [49]. This could explain the fact that snack-fed dams presented a higher caloric intake, even though their offspring was lighter than the ones from chow-fed dams from birth to weaning.

Besides maternal metabolism, maternal care also contributes to offspring outcomes [50] and was evaluated in the present study. Changes in maternal motivation were found in the home-cage retrieval test, where STZ-snack females retrieved all their pups faster. Other parameters of maternal behavior were not altered by hyperglycemia and snack consumption association. STZ-treated dams spent more time building nests compared to Control ones, which has been described in previous studies [11]. Since nest building is a thermoregulatory behavior [51], this difference could be partially explained by the fact that STZ rats are hypoinsulinemic and insulin plays an important role in hypothalamic thermoregulation [52]. Previous studies from our group have shown that when lactating rats are given insulin centrally the opposite is observed, with decreasing time in nest building [53], reinforcing our hypothesis.

In addition, snack consumption led to a decrease in anxiety-like behavior in hyperglycemic dams during lactation, with no changes in exploratory behavior. Several studies show that rats with hyperglycemia are more anxious [12, 54]. However, these studies only evaluated virgin females and it is known that anxiety behavior is reduced during lactation, as part of the preparation for maternal care [55]. Furthermore, snacks themselves seem to affect anxiety-like behavior in dams. Hedonic diets are associated with dopamine release in the mesolimbic pathway, which is highly stimulated by high-fat and high-sugar foods [56]. This pathway is known for its role in reinforcing behaviors, through an increase in dopamine levels in the nucleus accumbens [57, 58]. Those diets also mitigate the behavioral effects controlled by this system [59]. Therefore, it is

suggested that, in the presence of maternal hyperglycemia, snacks can act as a comfort food [60], reducing anxiety-like behavior.

In summary, the current study shows that snack intake during pregnancy and lactation increases maternal glucose intolerance established by neonatal STZ administration, but that this increase by itself, despite reducing the incidence of macrosomia in the offspring and changing some aspects of maternal motivation, does not lead to major effects on offspring growth and development. This may be because, although the glucose tolerance of STZ-snack rats was decreased, it was still within the mild hyperglycemic range, resulting in only subtle changes. The experimental model used in this study, while not reproducing all aspects of the diabetic syndrome, is useful to study the consequences of maternal diabetes associated with inappropriate nutrition, since the glycemic levels observed resemble those most observed in pregnant women diagnosed with clinical or gestational diabetes. Thus, this combination produced few effects that were not seen as a function of snack intake or hyperglycemia alone. Consistent with previous studies, changes in maternal behavior and the increased incidence of large for pregnancy age offspring reflected the STZ treatment whereas decreases in offspring birth weight and litter growth were a function of snack intake alone. Given their low birth weight and slow growth one might expect, based on previous work [7, 43, 61], that the offspring of snack-fed dams, either control or STZ, would show an obese phenotype as adults as well as other behavioral and metabolic impairments. Due to the relevance of the lactational period, being nursed by an STZ-treated female could affect these outcomes. Whether this prediction stands remains to be investigated in future studies.

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## Table's legends

**Table 1.** Offspring outcomes on PND 1. Values expressed as mean ± standard error of the mean (Two-way ANOVA, $p<.05$). The female ratio is expressed as a percentage (Fisher's test, $p<.05$).

**Table 2.** Maternal behavior and milk ejections on LD 5 and 10. Values expressed as mean ± standard error of the mean (Three-way ANOVA, $p<.05$). Data with no significant time effect were collapsed. * Control, n=8; Control-snack, n=13; STZ, n=10; STZ-snack, n=12.

**Table 1**

| | Control | Control-snack | STZ | STZ-snack | Statistical significance |
|---|---|---|---|---|---|
| Litter size | 12.41±0.40 | 11.44±0.48 | 10.43±0.73 | 10.15±0.71 | Control > STZ p <.05 |
| Female ratio (%) | 51.16 | 55.20 | 46.72 | 49.97 | n.s. |
| Birth weight (g) | 6.72±0.12 | 6.21±0.14 | 6.86±0.14 | 6.45±0.12 | Chow > snack p<.05 |
| Body length (cm) | 5.21±0.04 | 5.03±0.05 | 5.27±0.05 | 5.15±0.05 | Chow > snack p<.05 |
| Female anogenital index | 0.03±0.002 | 0.03±0.002 | 0.02±0.001 | 0.03±0.002 | Chow < snack p<.05 |
| Male anogenital index | 0.06±0.003 | 0.07±0.003 | 0.06±0.002 | 0.07±0.003 | Chow < snack p<.05 |

**Table 2**

| | | Control (n=9) | Control-snack (n=13) | STZ (n=10) | STZ-snack (n=13) | Statistical significance |
|---|---|---|---|---|---|---|
| Nursing (% time) | LD5 | 70.92±2.91 | 76.16±1.53 | 70.08±6.88 | 74.61±3.08 | LD5 > LD10 p<.05 |
| | LD10 | 57.71±6.13 | 66.32±3.13 | 60.16±3.49 | 61.94±4.54 | |
| Pup grooming (% time) | LD5 | 7.31±0.65 | 7.84±0.61 | 8.58±2.19 | 8.56±0.84 | n.s. |
| | LD10 | 7.46±0.57 | 6.68±0.93 | 7.25±0.62 | 6.23±1.02 | |
| Self-grooming (% time) | LD5 | 1.23±0.16 | 1.64±0.18 | 1.50±0.34 | 1.36±0.27 | LD5 > LD10 p<.05 |
| | LD10 | 0.74±0.13 | 1.34±0.21 | 0.44±0.10 | 0.81±0.31 | |
| Nest building (% time) | LD5 | 0.49±0.17 | 0.51±0.20 | 1.38±.0.34 | 0.76±0.27 | LD5 > LD10 Control < STZ p<.05 |
| | LD10 | 0.18±0.06 | 0.19±0.07 | 0.36±0.10 | 0.27±0.10 | |
| Time away from pups (% time) | LD5 | 20.03±3.09 | 13.80±1.56 | 18.44±5.01 | 14.67±3.11 | LD5 < LD10 p<.05 |
| | LD10 | 33.88±6.53 | 25.44±3.32 | 31.77±3.42 | 30.72±4.62 | |
| Latency to 1st milk ejection (s)* | LD5 | 662.75±85.11 | 630.38±47.39 | 613.10±86.70 | 702.58±56.99 | LD5 > LD10 p<.05 |
| | LD10 | 624.75±60.35 | 548.46±53.13 | 484.30±44.51 | 517.08±42.01 | |
| Inter milk ejection interval (s)* | | 271.64±9.22 | 304.14±17.60 | 290.16±17.28 | 321.85±26.11 | Chow < snack-fed p<.05 |
| Total # milk ejections* in 1 hour | | 10.64±0.49 | 10.02±0.58 | 10.31±0.63 | 9.47±0.68 | n.s. |

**Figure's legends**

**Figure 1.** Oral glucose tolerance test (OGTT) on PD 15. Values expressed as mean ± standard error of the mean. OGTT (A) significant effects: time, metabolic state, time x metabolic state interaction, and a trend towards diet effect (Three-way ANOVA, $p<.05$). AUC (B) significant effects: metabolic state and a trend towards diet effect (Univariate ANOVA, $p<.05$).

**Figure 2.** Maternal food intake and weight gain during pregnancy. Values expressed as mean ± standard error of the mean. Only significant main effects and interactions are shown. Relative caloric intake by diet fraction in snack-fed dams (A) significant effects: time x diet fraction interaction. Maternal weight gain in the first and second half of pregnancy (B) significant effects: time and time x diet interaction (Three-way ANOVA, $p<.05$). Total caloric intake (C) significant effect: diet. Carb intake (D) significant effect: diet. Protein intake (E) significant effect: diet. Fat intake (F) significant effect: diet (Two-way ANOVA, $p<.05$).

**Figure 3.** Newborns birth weight classification on PND 1. Values expressed as % of pups classified as small (SPA), appropriate (APA), or large (LPA) for pregnancy age. a = statistically significant difference compared to the Control group; b = statistically significant difference compared to the Control-snack group; c = statistically significant difference compared to the STZ group (Fisher's test, $p<.05$).

**Figure 4.** Maternal food intake and weight gain during lactation. Values expressed as mean ± standard error of the mean. Only significant main effects and interactions are shown. Relative caloric intake by diet fraction in snack-fed dams (A) significant effects: diet fraction and time x diet fraction interaction (Three-way ANOVA, $p<.05$). Maternal weight gain (B) significant effect: diet. Total caloric intake (C) significant effects: diet and metabolism x diet interaction. Carb intake (D) significant effects: diet and a trend towards metabolism and metabolism x diet interaction. Protein intake (E) significant effect: diet. Fat intake (F) significant effect: diet (Two-way ANOVA, $p<.05$).

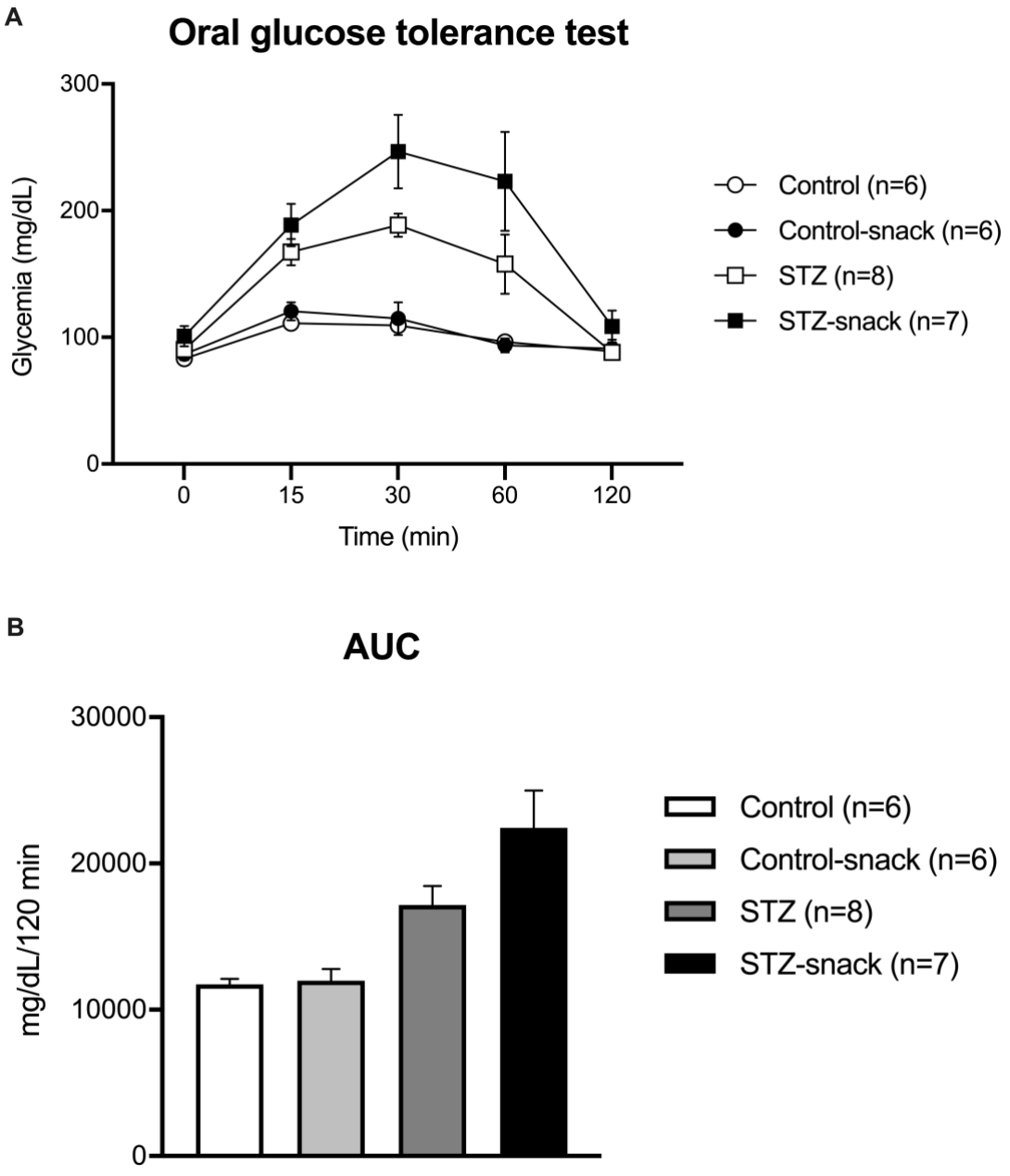
**Figure 5.** Litter weight gain and feed efficiency during lactation. Values expressed as mean ± standard error of the mean. Only significant main effects and interactions are shown. Litter weight gain (A) significant effect: lactation day; diet; and lactation day x diet

interaction (Three-way ANOVA, $p<.05$). Feed efficiency (B) significant effects: metabolism and diet (Two-way ANOVA, $p<.05$).

**Figure 6.** Latency to retrieve pups in the home-cage test on LD 6. Values expressed as mean ± standard error of the mean. Only significant main effects and interactions are shown. Latency to retrieve 1$^{st}$ pup (A). Latency to retrieve 4$^{th}$ pup (B). Latency to retrieve the last pup (C) significant effect: metabolism x diet interaction (Two-way ANOVA, $p<.05$).

**Figure 7.** Maternal anxiety-like behavior in the elevated plus-maze on LD 7. Values expressed as mean ± standard error of the mean. Total open arms entries (A) significant effects: diet and maternal metabolism x diet interaction. Time spent in open arms (B) significant effect: maternal metabolism x diet interaction (Two-way ANOVA, $p<.05$).

**Figure 1**


A
Oral glucose tolerance test
Glycemia (mg/dL)
300
200
100
0
0
15
30
60
120
Time (min)
Control (n=6)
Control-snack (n=6)
STZ (n=8)
STZ-snack (n=7)
B
AUC
mg/dL/120 min
30000
20000
10000
0
Control (n=6)
Control-snack (n=6)
STZ (n=8)
STZ-snack (n=7)

## Figure 2

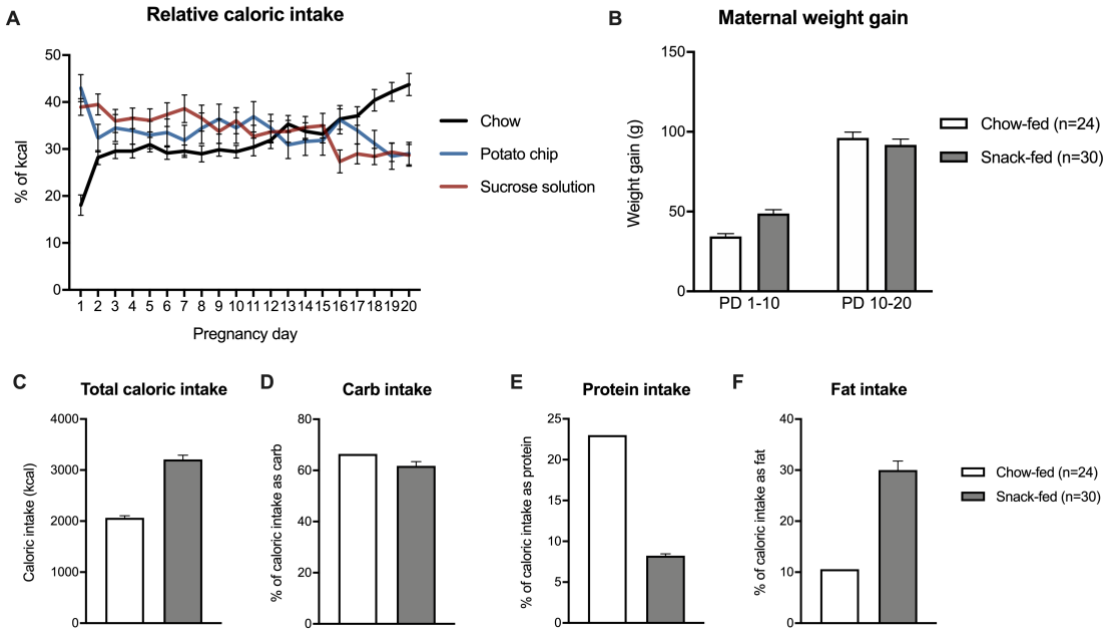
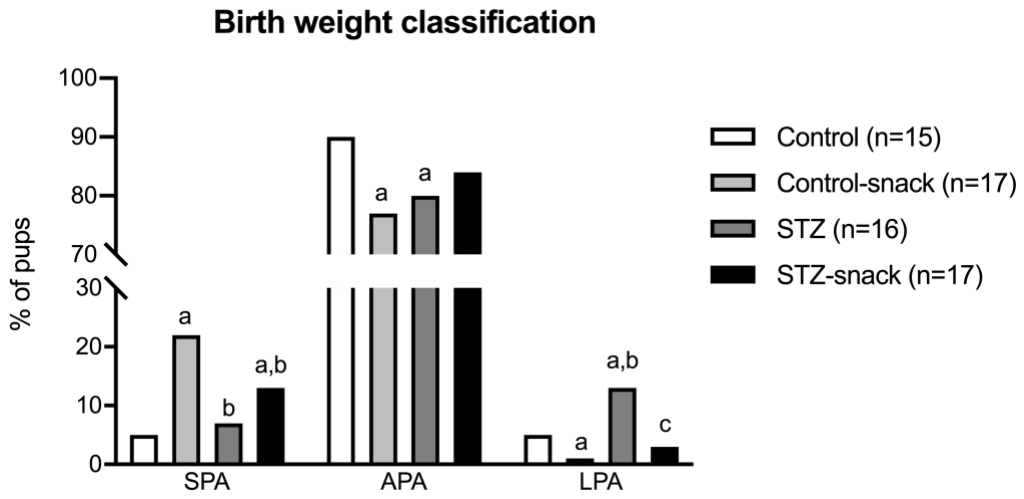

A
Relative caloric intake
% of kcal
Pregnancy day
Chow
Potato chip
Sucrose solution
B
Maternal weight gain
Weight gain (g)
PD 1-10
PD 10-20
Chow-fed (n=24)
Snack-fed (n=30)
C
Total caloric intake
Caloric intake (kcal)
D
Carb intake
% of caloric intake as carb
E
Protein intake
% of caloric intake as protein
F
Fat intake
% of caloric intake as fat
Chow-fed (n=24)
Snack-fed (n=30)

**Figure 3**


Birth weight classification
% of pups
100
90
80
70
30
20
10
0
a
b
a,b
a
a
a
a,b
c
SPA
APA
LPA
Control (n=15)
Control-snack (n=17)
STZ (n=16)
STZ-snack (n=17)

## Figure 4

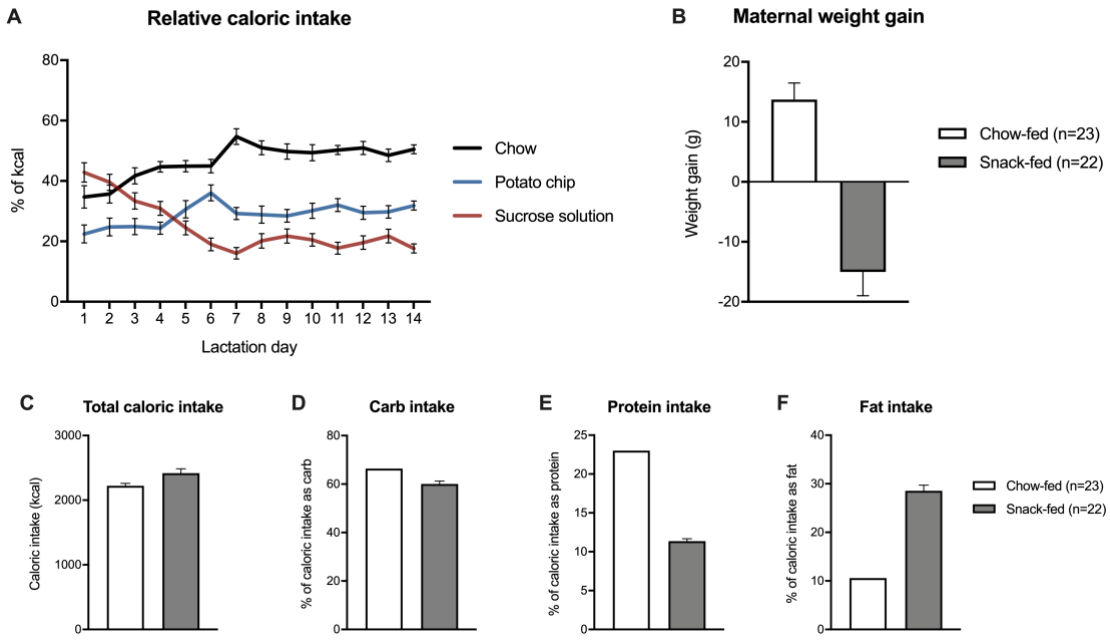

A
Relative caloric intake
% of kcal
Lactation day
Chow
Potato chip
Sucrose solution
B
Maternal weight gain
Weight gain (g)
Chow-fed (n=23)
Snack-fed (n=22)
C
Total caloric intake
Caloric intake (kcal)
D
Carb intake
% of caloric intake as carb
E
Protein intake
% of caloric intake as protein
F
Fat intake
% of caloric intake as fat
Chow-fed (n=23)
Snack-fed (n=22)

**Figure 5**

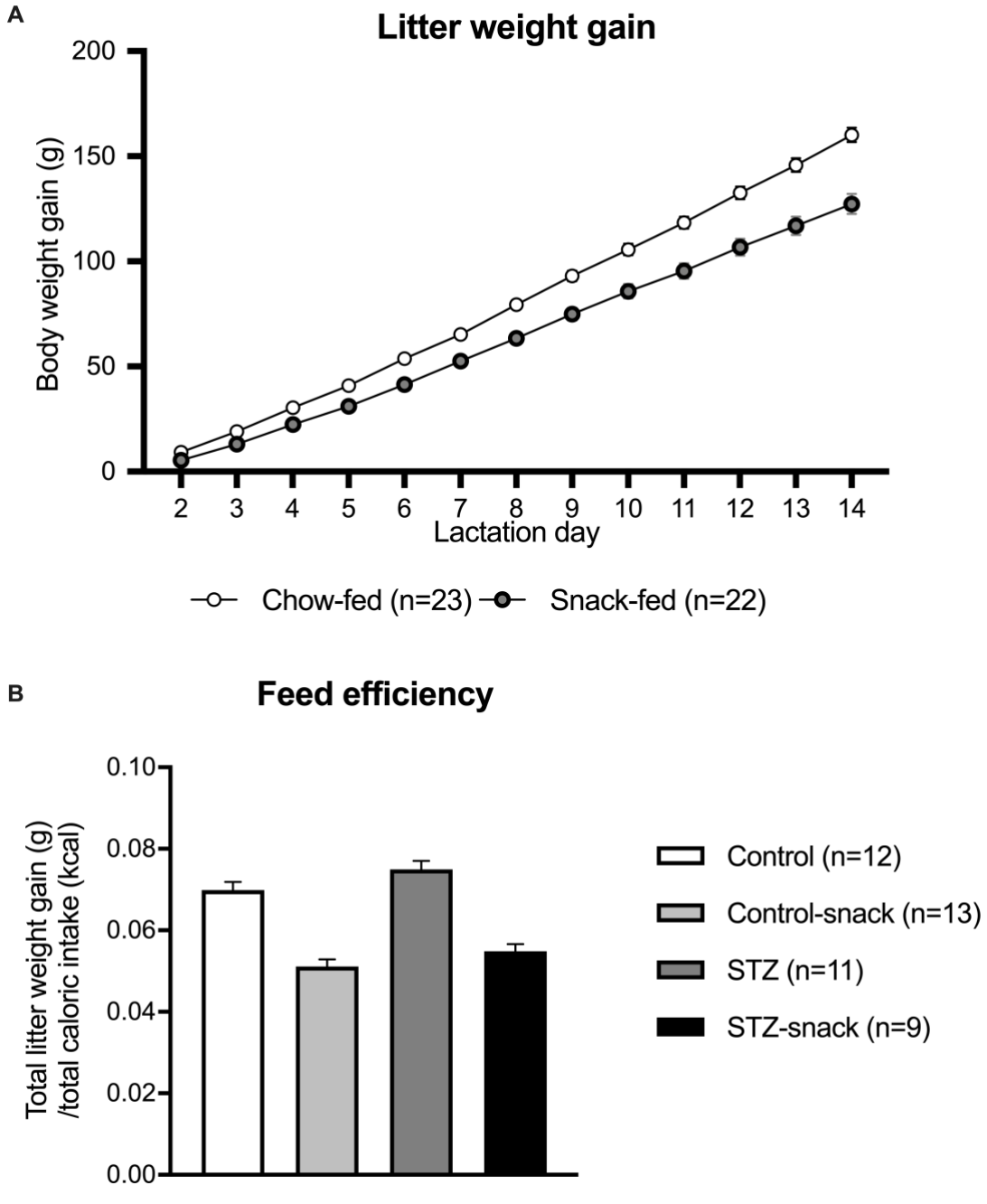

A
Litter weight gain
Body weight gain (g)
200
150
100
50
0
2
3
4
5
6
7
8
9
10
11
12
13
14
Lactation day
Chow-fed (n=23)
Snack-fed (n=22)
B
Feed efficiency
Total litter weight gain (g) /total caloric intake (kcal)
0.10
0.08
0.06
0.04
0.02
0.00
Control (n=12)
Control-snack (n=13)
STZ (n=11)
STZ-snack (n=9)

**Figure 6**

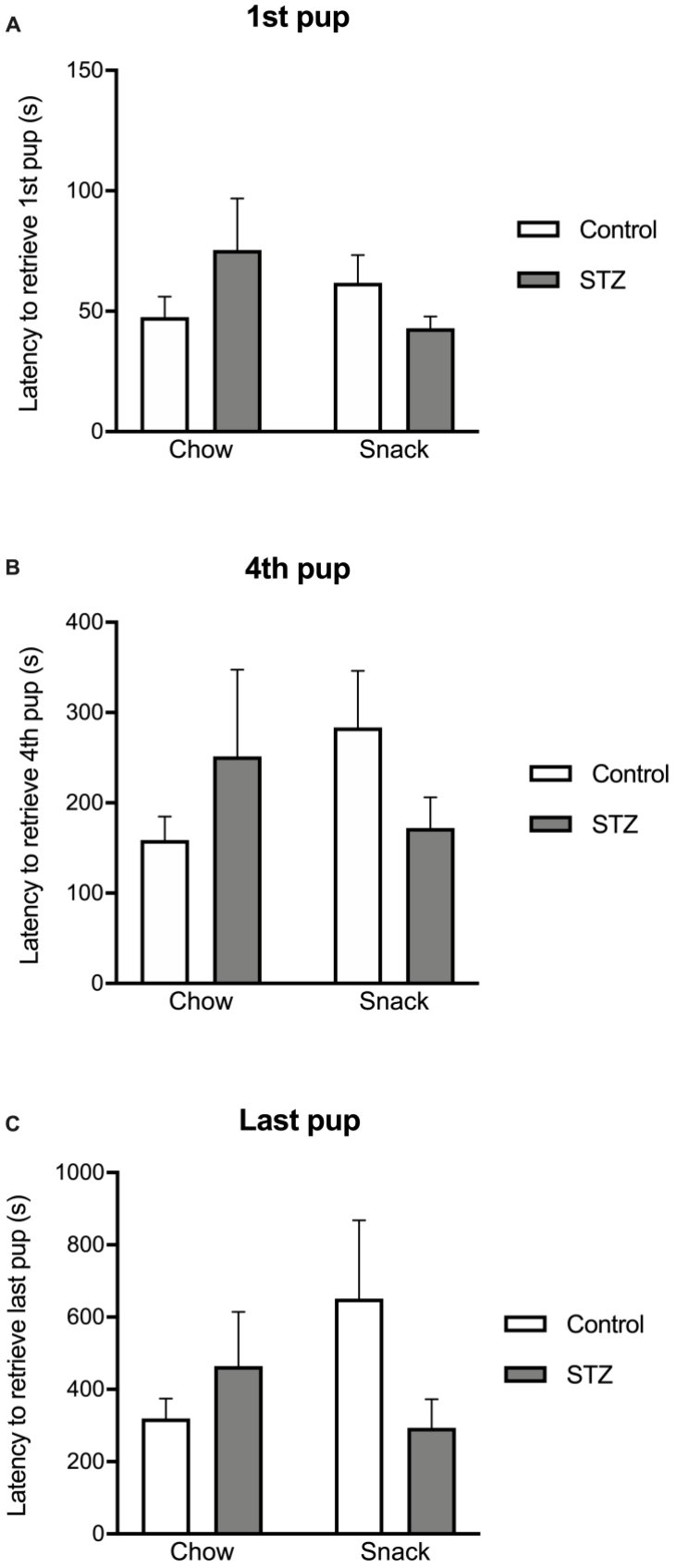
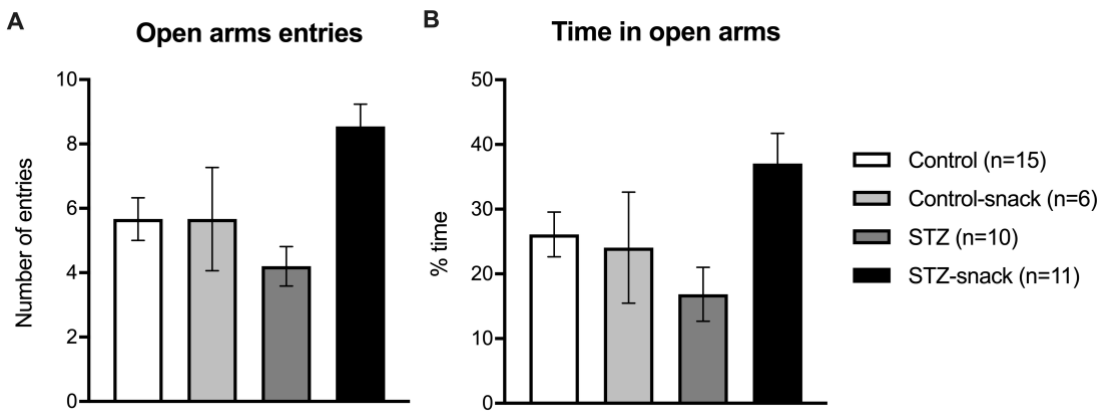

A
1st pup
Latency to retrieve 1st pup (s)
150
100
50
0
Chow
Snack
Control
STZ
B
4th pup
Latency to retrieve 4th pup (s)
400
300
200
100
0
Chow
Snack
Control
STZ
C
Last pup
Latency to retrieve last pup (s)
1000
800
600
400
200
0
Chow
Snack
Control
STZ

**Figure 7**


A
Open arms entries
Number of entries
10
8
6
4
2
0
B
Time in open arms
% time
50
40
30
20
10
0
Control (n=15)
Control-snack (n=6)
STZ (n=10)
STZ-snack (n=11)

# CHAPTER II: Effects of maternal mild hyperglycemia associated with snack consumption on offspring metabolism and behavior in different life stages

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**Highlights**

- Adult offspring from hyperglycemic dams with access to snacks had reproductive impairments
- Changes in anxiety behavior were seen in senescent offspring
- Maternal hyperglycemia associated with snack intake did not further compromise offspring metabolism
- Maternal hyperglycemia impaired return to baseline glycemia in adulthood
- Behavioral differences in adolescence and adulthood were related to maternal diet alone

**Abstract**

The incidence of diabetes is increasing worldwide, which is concerning especially considering women of childbearing age. Appropriate nutrition during pregnancy and lactation is critical to ensure offspring will thrive and an unhealthy diet may worsen the metabolic impairments caused by a previously established maternal hyperglycemia. In the present study, we evaluated the effects of maternal hyperglycemia associated with snack intake, which can aggravate maternal glucose intolerance, on offspring metabolism and behavior in different life stages. In order to achieve these goals, maternal hyperglycemia was induced in female Wistar rats by neonatal streptozotocin (STZ) administration. During pregnancy and lactation, these females received standard chow or standard chow plus snacks (potato chips and sucrose solution), and thus four experimental groups were formed: Control (n=17), Control-snack (n=18), STZ (n=16), and STZ-snack (n=19). Dams gave birth naturally and litters were culled to three male and three female pups. Male and female offspring were evaluated in adolescence (postnatal day (PND) 30), adulthood (PND 90), and senescence (PND 360). Behavioral paradigms to evaluate general activity, anxiety and anhedonia were employed. Plus, effects on glucose homeostasis were evaluated. Finally, possible reproductive impairments were evaluated on male adult offspring. Maternal hyperglycemia associated with snack intake changed aspects of offspring reproduction and anxiety-like behavior in adult and senescent offspring, without further compromising offspring glucose metabolism. However, no major impairments were observed on current employed behavioral tasks. Further, several effects were a function of snack intake or hyperglycemia alone. Despite not affecting glucose tolerance, glycemic return to baseline in adult offspring of hyperglycemic dams, regardless of maternal diet, was compromised. Plus, behavioral differences observed on adolescent and adult offspring were related to maternal snack intake alone. Despite aggravating maternal

hyperglycemia, the snack intake experimental model still leads to glucose levels within the mild hyperglycemic range. However, even this mild maternal condition was enough to change some aspects of offspring reproductive and general behavior, reinforcing the importance of women sustaining target glucose levels and a healthy diet during pregnancy and lactation.

**Keywords:** glucose tolerance, nutrition, offspring, pregnancy, reproduction

## 1. Introduction

The incidence of diabetes is increasing worldwide, which is concerning especially considering women of childbearing age [1]. The presence of diabetes during pregnancy, either clinical diabetes, diagnosed before pregnancy, or gestational diabetes, first detected during pregnancy, has consequences to the offspring [2]. Since mammalian fetuses are completely dependable on the maternal nutrient supply, maternal metabolic status can promote or inhibit fetal growth and development [3]. About 15-45% of babies born to diabetic mothers can have large birth weight [4], thus macrosomia is one of the commonest complications for pregnancies affected by diabetes [5]. Both low and high birth weight are related to increased risk of metabolic and cardiovascular disorders [6, 7].

Besides macrosomia, the offspring of diabetic mothers show a greater risk of developing diabetes, obesity, metabolic syndrome, insulin resistance, and higher fat deposition during childhood and adolescence [8-11]. In addition to metabolic impairments, these offspring may show cognitive deficits, poorer motor skills, and an increased risk of being diagnosed with attention, hyperactivity, or autism spectrum disorders [12, 13]. The consequences of maternal diabetes to the offspring also go beyond those evident at birth and early childhood and include long-lasting metabolic and behavioral impairments [3]. Plus, the development of diabetes itself is related to long-term consequences, such as reproductive impairment and cognitive function decline [14, 15].

Maternal hyperglycemia severity is positively correlated to poorer offspring outcomes [13], reinforcing the relevance of sustaining adequate glucose levels throughout pregnancy to prevent offspring metabolic and behavioral impairment later in life. However, even with increased information and methods of diagnosis available, diabetes during pregnancy is still sub-diagnosed [16] and growing at alarming rates [17]. Diet is a great ally to help to keep glucose homeostasis [18]. Unhealthy dietary choices have been related to the increased risk of developing type 2 and gestational diabetes [19-22]. Appropriate nutrition during pregnancy and lactation is critical to ensure offspring

will thrive and an unhealthy diet may worsen the metabolic impairments caused by maternal hyperglycemia [23]. Nonetheless, although the combination of metabolic issues and an inadequate diet in pregnant women is common, there are currently no studies that explore the effect of their association on the offspring.

Animal models are valuable to study how maternal metabolic and diet manipulations affect offspring development. A recent study [24] has shown that when pregnant mild hyperglycemic rats (glycemic levels between 120 - 300 mg/dL achieved by neonatal STZ administration) given a choice between regular chow or snacks, comprised of potato chips and sucrose solution, their glucose intolerance is exacerbated, leading to changes on maternal behavior and reducing the incidence of macrosomia. Since birth weight is a strong predictor of health issues, one might expect changes in offspring behavioral and metabolic outcomes later in life. However, the effects on offspring metabolism and behavior have not been explored yet. Therefore, the present study aimed to investigate the impact of mild hyperglycemia and inappropriate nutrition on male and female offspring behavior and metabolism at different life stages. Our hypothesis is that the offspring of hyperglycemic dams given access to snacks during pregnancy and lactation will show worse metabolic and behavioral outcomes than the offspring of dams on a standard diet and that those effects would change across life.

## 2. Materials and methods

### *2.1 Animals*

Male and female Wistar rats were obtained from the Multidisciplinary Center for Biological Research (CEMIB – State University of Campinas, SP) breeding facility. Animals were kept under controlled temperature (22 ± 2°C) and humidity (50 ± 10%), on a 12/12 h light/dark cycle (lights on 7 am, lights off 7 pm). All experimental procedures were approved by the local ethics committee of São Paulo State University (Unesp), Institute of Biosciences of Botucatu (Protocol number 919).

### *2.2 General procedures and experimental groups*

An experimental model of mild hyperglycemia, achieved by STZ neonatal administration, associated with snack intake, was employed as described previously [24] (Figure 1A). Briefly, female Wistar rats were mated (n = 40), and on postnatal day (PND) 1, litters were culled to 6-8 female pups and assigned to one of two treatments: 4 to 6 females/litter received 100 mg/kg of STZ (SIGMA Chemical Company, St. Louis, Millstone) diluted in citrate buffer (0,1 M, pH 4,5) subcutaneously; and 2 females/litter received the same volume of citrate buffer subcutaneously [25-27].

On PND 90, Control (n = 49) and STZ (n = 49) were mated to control males and randomly assigned to receive either standard chow (Control, n = 17; STZ, n = 16); or standard chow plus snacks (Control-snack, n = 18; STZ-snack, n = 19). Snacks comprised potato chips (556 kcal/100 g) and a red fruit-flavored sucrose syrup solution 1.5% (Bacana®, 44 kcal/100 mL), without fructose. All rats received tap water ad libitum throughout the experiment. Dams receiving snacks plus chow had access to the snacks only until lactation day (LD) 14, since around this day pups begin to eat independently [28] so that any effects of diet would only reflect those mediated by the metabolism or behavior of the dam. Females gave birth on PD 21, and litters were culled to 3 males and 3 females offspring on PND 1, which were further evaluated in the present study.

Therefore, four experimental groups were formed: Control, offspring from normoglycemic dams which had access only to chow during pregnancy and lactation; Control-snack, offspring from normoglycemic dams which had access to chow and snacks; STZ, offspring from hyperglycemic dams which had access only to chow; and STZ-snack, offspring from hyperglycemic dams which had access to chow and snacks.

In order to evaluate the effects of maternal hyperglycemia associated with snack intake on offspring behavior and metabolism, several analyses were performed. Classical behavioral paradigms to evaluate general activity, anxiety and anhedonia were employed. Plus, effects on glucose homeostasis were evaluated. Finally, possible reproductive impairments were evaluated (Figure 1B).

To evaluate if the effects of maternal metabolic and diet manipulations would change across life, tests were conducted on different sets of animals on PND 30, 90, and 360, representing different life stages: infancy, adulthood, and senescence, respectively [29]. Only one male and one female from each litter were evaluated at each age, thus the litter was the experimental unit.

To avoid further variability on metabolic and behavioral analysis due to female cyclic hormonal changes, females evaluated during their reproductive age, on the PND90, were previously ovariectomized on PND 70 after a ketamine (5.7 mg/100 g body weight) and xylazine (0.86 mg/100 g body weight) anesthesia. Briefly, incisions were made on each flank, ovaries were removed, and incisions were sutured. Procedures on PND 30 and PND 360 were conducted with intact females.

## 2.3 Behavioral analysis

All behavioral procedures were carried out in the light phase of the light/dark cycle, between 9 a.m. and 1 p.m., and were recorded. Video analyses were carried out with the Countee app [30].

2.3.1 Marble-burying test

This test aimed to evaluate aversion to new objects, often expressed by burying behavior, which is innate in rodents and could be useful to predict anxiety-like behavior [31, 32], as well as assess stereotyped behaviors [33]. Twenty marble spheres were equally distributed in a home cage and the animal was left to explore it for 30 min. After that, buried spheres were counted (adapted from [32]).

2.3.2 Open-field test

The open-field test was conducted to analyze exploratory behavior as described previously [25], and adapted from Broadhurst [34]. Briefly, animals were placed into a 100-cm-diameter arena divided into 25 sections for 5 minutes and the following parameters were recorded: number of sections entered (divided into 12 peripheral and 12 central sections) and rearings, as well as the time of immobility and self-grooming.

2.3.3 Elevated plus-maze test

The elevated plus-maze is widely used to evaluate anxiety-like behavior in rodents [35]. Rats were placed in the center platform of an elevated plus-maze with 50-cm-arms 40 cm above the ground, two closed and two open, and left to explore for 5 minutes. Time spent in the open arms and the center, the number of closed and open arms entries, and the total number of head dips, defined as each time the animal moves its head towards the floor in the border of the open arms, were analyzed according to Walf and Frye [36].

2.3.4 Sucrose preference test

The sucrose preference test aimed to evaluate sucrose preference and anhedonia-like behavior. For 48 h, animals had free access to tap water and a 4% sucrose solution. Standard chow was available throughout the test. Bottles changed places after 24 h to reduce place preference. Body weight, total water, and sucrose solution intake were measured, and the preference index was analyzed (sucrose solution intake/total intake) [37].

*2.4 Metabolic and morphometric analysis*

After the behavioral tests, animals were given an oral glucose tolerance test (OGTT) to evaluate if glucose metabolism was impaired by maternal metabolic and/or diet manipulations. After a 6h fast, animals received a 2g/kg glucose solution (200 g/L) by gavage. Blood samples were obtained from a tail snip 0, 15, 30, 60, and 120 min after glucose administration, and blood glucose was measured with a glucometer (OneTouch

Ultra, Johnson & Johnson®). Overall blood glucose changes across the test were assessed by calculating the total area under the curve (AUC) using the trapezoid method [38]. Return to baseline glycemia was evaluated in all experimental groups comparing fasting glucose levels with the 120 minutes time point.

The day after the OGTT, male and female offspring were weighted and killed following lethal anesthesia with ketamine and xylazine. Nasoanal and anogenital distances were recorded. Retroperitoneal and visceral fat pads were dissected and weighted. The anogenital index was calculated as anogenital distance / nasoanal distance.

*2.5 Reproductive analysis*

Male copulatory behavior was evaluated in sexually naïve male offspring from all experimental groups. The test was conducted before previously described behavioral and metabolic analysis and only in the adulthood group (PND 90). At least 2 hours after the onset of the dark phase, males were allowed to interact with sexually receptive ovariectomized females in which estrous was induced by estradiol (50 μg/kg subcutaneously, 54 h before evaluation) and progesterone (2 mg/kg subcutaneously, 6 h before evaluation) administration [39]. Briefly, each male acclimated for five minutes in a Plexiglas cage, the female was introduced, and their interaction was evaluated for 40 minutes. The following parameters were analyzed: latencies to first mount, intromission, and ejaculation; the number of mounts and intromissions; postejaculatory intromission latency; the number of postejaculatory intromissions; and the total number of ejaculations [40, 41]. A male was considered sexually inactive if it did not show any mount or intromission in the first ten minutes [42]. Two weeks after the copulatory behavior analysis, males had their reproductive organs and fat pads dissected at the moment of euthanasia, in addition to previously described morphometric analysis. Testis, epididymis, prostate, full seminal vesicle (as well as seminal content), and perigonadal fat pad were weighted. The gonadosomatic index was calculated as total testis weight / body weight.

*2.6 Statistical analysis*

Data are expressed as mean ± standard error mean. Before statistical analysis, z scores were calculated for all variables to explore possible outliers. Animals showing Z scores below -3 and above 3 in a parameter were considered outliers and excluded from the following analysis. Data from the marble-burying test, exploratory and anxiety-like behaviors, sucrose preference, and AUC were analyzed with a 3-way ANOVA, with maternal metabolic state (normo- or hyperglycemic), diet (chow only or chow plus snack

consumption), and sex as between-subject factors. Data from glycemic curves were analyzed with repeated measures 4-way ANOVA, with time as the repeated measure and maternal metabolic state, diet, and sex as between-subject factors. Data from AUC were analyzed with a 3-way ANOVA, with maternal metabolic state, diet, and sex as between-subject factors. Return to baseline glycemic levels was analyzed with a paired t-test. Morphometric data were analyzed with a 3-way ANOVA with maternal metabolic state, diet, and sex as between-subject factors and body weight as a covariant. Reproductive organ weight was analyzed similarly, but with a 2-way ANOVA since only male subjects were evaluated. Data from male copulatory behavior were analyzed with a 2-way ANOVA with maternal metabolic state and diet as between-subject factors. F, p, and $\eta$ values are reported for all ANOVA analyses. In all cases, statistical significance was set to alpha = .05. All statistical analyses were performed using SPSS (IBM, SPSS Statistics 22).

## 3. Results

### *3.1 Behavioral analysis*

Offspring behavior was evaluated with the burying test, the open field, elevated plus maze, and sucrose preference test in adolescence (PND 30), adulthood (PND 90), and senescence (PND 360). Maternal hyperglycemia and snack intake did not disrupt burying behavior in the offspring in any age group ($p>.05$, data not shown). Plus, no major effects of maternal hyperglycemia and/or snack intake were observed on exploratory behavior in the open field ($p>.05$, Figure SM 1).

Regarding the elevated plus-maze, adolescent female offspring from snack-fed dams spent more time in the open arms (diet x sex interaction, $F(1,67)=6.395$, $p<.05$, $\eta=.087$) and had more head dips (diet x sex interaction, $F(1,67)=4.521$, $p<.05$, $\eta=.063$), while in male offspring the effect was the opposite (Figure 2A-B). On PND 90, there were no significant differences in time spent on open arms or number of head dips regarding maternal metabolism and/or diet ($p>0.05$). There was only a sex difference in the number of head dips, with adult female offspring having more head dips than males (sex effect, $F(1,91)=5.488$, $p<.05$, $\eta=.057$). Regarding PND 360, as already observed in adulthood, females had more head dips than males (sex effect, $F(1,90)=11.71$, $p<.01$, $\eta=.115$). Plus, there was a metabolism and diet interaction on the number of head dips ($F(1,90)=16.253$, $p<.001$, $\eta=.153$) that, because of a sex effect, was further explored separately for males and females. Female offspring from all experimental groups had more head dips than Controls (metabolism x diet interaction, $F(1,44)=6.333$, $p<.05$, $\eta=.126$). Regarding the males, maternal hyperglycemia tended to reduce the number of

head dips (metabolism effect, F(1,46)=3.191, p=.081, η=.065) and this reduction was more expressive when maternal hyperglycemia was associated with snacks (metabolism x diet interaction, F(1,46)=10.736, p<.01, η=.189). Plus, there was a trend towards reduced time spent in the open arms on STZ-snack offspring (metabolism x diet interaction, F(1,90)=2.877, p=.093, η=.031) (Figure 2E).

Anhedonia was assessed through the sucrose preference test. During adulthood, sucrose preference was reduced in both male and female offspring of snack-fed dams (diet effect, F(1,84)=5.156, p<.05, η=.058) (Figure 3B). In adolescence, sucrose preference showed a metabolism x diet x sex interaction (F(1,50)=4.721, p.05, η=.086). However, when this interaction was further explored by analyzing each sex separately, the differences associated with maternal metabolism and snack intake were not sustained (p>.05). No differences in sucrose preference were seen in the PND360 group (p>.05).

*3.2 Metabolic analysis*

As expected, glycemic levels changed across the OGTT test in all groups in adolescence (time effect, F(4,132)=80.573, p<.001, η=.709) (Figure 4A), adulthood (time effect, F(4,144)=91.115, p<.001, η=.717) (Figure 4B), and senescence (time effect, F(4,244)=161.196, p<.001, η=.725). Neither maternal metabolism nor snack intake affected glucose tolerance in young and adult animals (p>.05). However, on PND 360 offspring glucose tolerance was affected by maternal snack intake (time x diet interaction, F(4,244)=2.686, p<.05, η=.042) in a sex-specific manner, since female offspring showed a higher glycemic curve than males (sex effect, F(1,61)=4.054, p<.05, η=.062). Since there was a sex effect, a further analysis was performed and showed that female offspring of chow-fed dams have a greater degree of glucose intolerance than snack-fed ones (time x diet interaction, F(4,124)=2.849, p<.05, η=.084 (Figure 4C) with no effect on male offspring (p > 0.05).

Besides the OGTT curve, glucose levels return to baseline after 2h were also evaluated by comparing fasting glycemia with the 120 minutes OGTT time point. All adolescent animals from different experimental groups presented a proper return to baseline glycemic levels (p > 0.05). However, adult offspring from STZ dams, both chow or snack fed, did not return to baseline glycemic levels (t(9)=2.547, p<.05 and t(9)=4.31, p<.01, respectively). None of the older animals returned to fasting glycemia regardless of experimental group, an expected outcome due to metabolic impairment related to aging (Control: t(18)=8.234, p<.001; Control-snack: t(20)=6.14, p<.001; STZ: t(15)=6.397, p<.001; STZ-snack: t(12)=6.016, p<.001).

*3.3 Morphometric analysis*

The analysis of morphometric parameters described below on adolescence (PND30), adulthood (PND 90), and senescence (PND 360) are presented in Tables 1, 2, and 3, respectively.

As expected, adolescent males were heavier (sex effect, $F(1,40)=25.036$, p.001, $\eta=.385$) and had a higher anogenital index than females (sex effect, $F(1,40)=587.346$, $p<.001$, $\eta=.936$). Offspring from STZ-treated dams were heavier (metabolism effect, $F(1,40)=4.584$, $p<.05$, $\eta=.103$) and larger (metabolism effect, $F(1,39)=4.971$, $p<05$, $\eta=.113$) than Control ones, regardless of maternal diet. On the other hand, male offspring from snack-fed dams showed a reduced anogenital index (diet effect, $F(1,20)=4.914$, $p<.05$, $\eta=.197$) and retroperitoneal fat pads (diet effect, $F(1,39)=5.555$, $p<.05$, $\eta=.125$), without further effects due to maternal metabolic condition.

In adulthood, male offspring were also heavier (sex effect, $F(1,43)=134.411$ $p<.001$, $\eta=.758$), larger (sex effect, $F(1,42)=14.274$, $p<.001$, $\eta=.254$), had a higher anogenital index (sex effect, $F(1,43)=899.714$, p<,001, $\eta=.954$), and more visceral fat pads than females (sex effect, $F(1,42)=16.40$, p<,001, $\eta=,281$). Furthermore, offspring from snack-fed dams were lighter, regardless of sex or maternal diet (diet effect, $F(1,43)=6.193$, $p<.05$, $\eta=.126$). Additionally, while maternal snack intake increased male offspring anogenital index, the association of maternal hyperglycemia and snack intake resulted in an anogenital index in male offspring that resembles the Control group (metabolism x diet interaction, $F(1,21)=13.693$, $p<.01$, $\eta=.395$).

Senescent males were still heavier (sex effect, $F(1,35)=319.133$, $p<.001$, $\eta=.901$), larger (sex effect, $F(1,34)=10.954$, $p<.01$, $\eta=.244$), had an increased anogenital index (sex effect, $F(1,35)=454.538$, $p<.001$, $\eta=.929$), and visceral fat pads (sex effect, $F(1,34)=11.862$, $p<.01$, $\eta=.259$) than females. The increased anogenital index on male offspring from snack-fed dams was also observed at this age (diet effect, $F(1,18)=6.768$, $p<.05$, $\eta=.273$). Plus, female offspring from STZ-treated females showed an increased anogenital index (metabolism effect, $F(1,17)=4.715$, $p<.05$, $\eta=.217$). Offspring from hyperglycemic dams showed a reduction in visceral fat pads (metabolism effect, $F(1,34)=5.836$, $p<.05$, $\eta=.146$).

*3.4 Reproductive analysis*

Data from male copulatory behavior evaluated during adulthood (PND 90) is described in Table 4. Maternal snack intake and hyperglycemia influenced offspring sexual behavior. Offspring from both Control-snack and STZ groups presented reduced

latency to first ejaculation (metabolism x diet interaction, $F(1,28)=4.478$, $p<.05$, $\eta=.138$) and increased number of ejaculations (metabolism x diet interaction, $F(1,27)=4.493$, $p<.05$, $\eta=.143$) while sexual behavior from the STZ snack offspring resembles the Control group.

The weight of reproductive organs was also analyzed and is presented in Table 5. STZ offspring were heavier than Control ones, regardless of maternal snack intake (metabolism effect, $F(1,25)=4.392$, $p<.05$, $\eta=.149$). Maternal hyperglycemia and snack intake reduced seminal vesicle content, as well as the weight of the full seminal vesicle, plus snack intake affected Control and STZ offspring differently (seminal vesicle content: diet effect, $F(1,23)=5,124$, $p<.05$, $\eta=.182$, and metabolism x diet interaction, $F(1,23)=6,442$, $p<.05$, $\eta=.219$; full seminal vesicle: metabolism x diet interaction, $F(1,23)=10.231$, $p<.01$, $\eta=.308$).

## 4. Discussion

In the present study, we evaluated the effects of maternal hyperglycemia associated with snack intake, which can, among other effects, aggravate maternal glucose intolerance, on offspring metabolism and behavior in different life stages. This association led to changes in offspring reproductive function and general behavior in adulthood and senescence, respectively, showing that consequences may only be evident in the long-term. There were also metabolic and behavioral effects related to hyperglycemia or snack intake alone, with most findings in agreement with previous studies, thus reinforcing the validity of the chosen experimental model.

In adolescence, the association between maternal hyperglycemia and snack intake did not lead to further impairments on offspring metabolism or behavior. Most effects were related to maternal hyperglycemia or snack intake alone. Offspring from hyperglycemic mothers, which are often born macrosomic [4], is still heavier and longer at this age, consistent with previous studies in macrosomic offspring [43, 44]. Fat pads were reduced in the offspring of snack-fed dams, which might be explained by their lower birth weight and body weight at weaning [24], although no differences were seen in body weight in adolescence. Regarding behavioral observations, maternal snack intake changed offspring behavior in a sex-specific manner, with female offspring showing a decreased anxiety-like behavior while males were the opposite. Maternal high-fat and cafeteria diet intake have been previously shown to reduce anxiety-like behavior in adolescent [45] and adult offspring [46], although most studies describe the maternal high-fat intake as being anxiogenic to the offspring [47]. Based on the evidence of the effects of both maternal diet and hyperglycemia on offspring metabolism at weaning [44,

48, 49], one might expect an effect on glucose tolerance of those offspring. However, the lack of metabolic impairments at this age agrees with previous studies showing metabolic impairments due to maternal hyperglycemia [43] as well as maternal diet [50] are usually seen later in life.

Although during infancy there were no major effects of altered maternal metabolism and nutrition, in adulthood changes in reproductive function were observed. Seminal content was reduced on offspring of hyperglycemic mothers treated with snacks. This reduction was also observed in offspring from normoglycemic dams that received snacks and hyperglycemic dams on regular chow compared to Control ones. Plus, STZ-snack offspring presented a pattern of copulatory behavior like Control males, while Control-snack STZ offspring ejaculated more often and faster and had increased anogenital index. The anogenital index is dependent on androgen action during perinatal development [51] and changes in it may be related to sexual development and reproductive disorders [52]. Taking less time to ejaculate and having more ejaculations are not necessarily an improvement to sexual behavior, since several phases must adequately happen in a timely manner to lead to successful female fertilization and, thus, reproductive success [53]. Studies have shown that changes in sexual behavior in the offspring of hyperglycemic dams are dependent on glucose levels severity, ranging from decreases in the number of intromissions and ejaculations on male offspring of mothers with severe diabetes [54] to no differences in offspring of mild hyperglycemic dams [25]. The fact that offspring from Control dams treated with snacks and hyperglycemic dams on regular chow show a degree of reproductive impairment while offspring exposed to both maternal altered metabolism and nutrition presents a reproductive outcome similar to the Control group, which could be related to maternal hyperglycemia and snack intake having opposing effects during offspring development. A similar effect was previously described for birth weight classification, in which the hyperglycemia effect was counterbalanced by the diet effect [24]. Birth weight can predict a series of outcomes on offspring health [55-57], and there is a correlation between birth weight and sexual intercourse reduction in the human population [58]. However, this effect needs to be further addressed.

There were also behavioral and metabolic differences as a function of maternal hyperglycemia or diet alone in adulthood. Despite previous studies showing adult offspring from severe hyperglycemic dams have higher levels of exploratory and anxiety-like behavior [59], no such differences were observed in the present study. A study using the same experimental model also showed limited impairments in male offspring [25], showing that maternal hyperglycemia severity is related to offspring behavior impairments. Previous studies also did not find differences in anxiety-like behavior in the

offspring of hyperglycemic dams [60, 61], but some have reported learning impairments in the female offspring [61]. Thus, other behavioral approaches, involving memory and learning assessment, could be employed in future studies to explore this possibility, especially since maternal diabetes is often related to learning disorders [62]. However, differences in offspring metabolism were observed. Although there were no differences in the OGTT glycemic curve, glucose levels did not return to baseline in the offspring of hyperglycemic dams, showing that these offspring have an impairment in glucose metabolism at this age. Previous studies have shown impaired glucose tolerance in the adult offspring of hyperglycemic dams [43], although Han et al. [63] did not find changes in glucose tolerance in the offspring of severe hyperglycemic females up to 15 weeks of age. It is important to reinforce that, although the experimental model employed aggravated maternal hyperglycemia, glucose levels after snack intake are still within the mild hyperglycemic range (120-300 mg/dL) [24], which may explain why further impairments were not seen in offspring metabolism. Insulin levels and glycated hemoglobin analysis could help to better understand metabolic impairments in the animals and could be performed on future studies. Regarding maternal diet, differences in sucrose preference were observed, with offspring from snack-fed dams presenting lower sucrose preference, regardless of maternal metabolic state, as previously reported for offspring of high-fat-fed females [64], and which might be an indicator of higher anhedonia, a common trait in mental disorders [65]. Future studies should evaluate depressive-like behavior in these animals, to clarify if this decreased sucrose preference is related to major impairments.

While in early life no differences related to maternal metabolism and diet were observed in offspring general behavior, senescent offspring from STZ-snack dams showed increased anxiety-like behavior, showing the effects of maternal metabolism and diet manipulations may manifest in the offspring only in the long term. Anxiety-related changes have already been described in the adult offspring of hyperglycemic dams [66] and those which had access to high-fat or cafeteria diet [47], but no studies explore these effects in older animals. Although metabolic impairments related to maternal hyperglycemia were described in adult offspring [43], this effect was not evident in senescent animals. This might be due to age-related changes in glucose tolerance [67] and, at this life stage, all experimental groups were impaired, as can be noted by the lack of return to baseline glucose levels on all of them, making differences related to maternal metabolism less evident. However, although no changes were observed related to maternal metabolism, there was a sex-specific difference in glucose tolerance in the offspring of chow-fed dams, with females showing a slightly increased glucose intolerance. This could be due to differences in adiposity, which might lead to differences

in insulin sensitivity and, therefore, glucose metabolism [68]. However, since no significant differences in fat were observed related to maternal diet, this difference in glucose tolerance should be further investigated.

In conclusion, maternal hyperglycemia associated with snack intake changed aspects of offspring reproduction and anxiety-like behavior in a different manner than hyperglycemia or snack intake alone, without further compromising offspring metabolism. Plus, most were long-term effects, during adulthood and senescence. However, no major impairments were observed on current employed behavioral tasks. Since both maternal diabetes and inappropriate nutrition have been linked to offspring cognitive impairments [47, 66], future studies that evaluate deficits in learning and memory may clarify further behavioral impairments in the offspring of hyperglycemic dams fed with snacks. It is noteworthy that, despite aggravating maternal hyperglycemia, the snack intake experimental model still leads to glucose levels within the mild hyperglycemic range in pregnant females [24], which may explain why there were no more pronounced effects in the offspring. Further, some findings may not be a direct consequence of maternal manipulations themselves, but they may occur as a reflection of these manipulations on offspring birth weight, which is a strong predictor of future outcomes [55-57] and could be better investigated in future studies. Still, it has to be considered that even this mild maternal condition was enough to change some aspects of offspring reproductive and general behavior, reinforcing the importance of women sustaining target glucose levels and a healthy diet during pregnancy and lactation.

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**Tables - Legends**

**Table 1.** Morphometric parameters in PND30 age group. Values expressed as mean ± standard error of the mean (Three-way ANOVA, $p<.05$). Data with no significant sex effect were collapsed.

**Table 2.** Morphometric parameters in PND90 age group. Values expressed as mean ± standard error of the mean (Three-way ANOVA, $p<.05$). Data with no significant sex effect were collapsed.

**Table 3.** Morphometric parameters in PND360 age group. Values expressed as mean ± standard error of the mean (Three-way ANOVA, $p<.05$). Data with no significant sex effect were collapsed.

**Table 4.** Male copulatory behavior on PND 75. Values expressed as mean ± standard error of the mean (Two-way ANOVA, $p<.05$).

**Table 5.** Reproductive organs weight in male offspring in PND90 age group. Values expressed as mean ± standard error of the mean (Two-way ANOVA, $p<.05$).

**Table 1**

| | **Control (n=12)** | **Control-snack (n=12)** | **STZ (n=10)** | **STZ-snack (n=14)** | **Statistical significance** |
|---|---|---|---|---|---|
| Body weight (g) | | | | | |
| *Females* | 162.35±2.09 | 143.62±1.73 | 162.16±5.79 | 154.51±4.91 | Sex and metabolism effects |
| *Males* | 181.13±10.13 | 168.43±9.48 | 192.90±10.67 | 195.16±11.61 | |
| Nasoanal distance (cm) | 18.20±0.20 | 17.85±0.22 | 18.11±0.22 | 18.22±0.22 | Metabolism effect |
| Anogenital index | | | | | |
| *Females* | 0.06±0.00 | 0.06±0.00 | 0.06±0.00 | 0.07±0.00 | Sex and diet effects |
| *Males* | 0.13±0.00 | 0.12±0.00 | 0.13±0.00 | 0.12±0.00 | |
| Visceral fat pads (g) | 1.55±0.09 | 1.45±0.10 | 1.62±0.12 | 1.66±0.13 | n.s. |
| Retroperitoneal fat pads (g) | 1.04±0.13 | 0.59±0.09 | 1.43±0.16 | 1.00±0.18 | Diet effect |

**Table 2**

| | Control (n=14) | Control-snack (n=11) | STZ (n=14) | STZ-snack (n=12) | Statistical significance |
|---|---|---|---|---|---|
| Body weight (g) | | | | | |
| *Females* | 315.84±17.92 | 286.17±11.79 | 316.86±11.18 | 293.18±9.34 | Sex and diet effects |
| *Males* | 417.99±8.27 | 388.66±15.52 | 443.29±17.30 | 426.32±16.67 | |
| Nasoanal distance (cm) | | | | | |
| *Females* | 22.19±0.26 | 21.73±0.23 | 22.17±0.21 | 21.72±0.22 | Sex effect |
| *Males* | 24.57±0.20 | 23.96±0.41 | 24.60±0.25 | 24.35±0.16 | |
| Anogenital index | | | | | |
| *Females* | 0.07±0.00 | 0.07±0.00 | 0.07±0.00 | 0.07±0.00 | Sex, metabolism x diet, and metabolism x diet x sex effects |
| *Males* | 0.13±0.00 | 0.15±0.00 | 0.15±0.00 | 0.14±0.00 | |
| Visceral fat pads (g) | | | | | |
| *Females* | 4.41±0.33 | 4.06±0.41 | 4.28±0.20 | 3.82±0.36 | Sex effect |
| *Males* | 4.33±0.37 | 3.82±0.25 | 5.25±0.43 | 4.43±0.47 | |
| Retroperitoneal fat pads (g) | 6.21±0.93 | 5.30±0.53 | 7.65±1.04 | 5.60±1.04 | n.s. |

**Table 3**

| | Control (n=11) | Control-snack (n=13) | STZ (n=10) | STZ-snack (n=9) | Statistical significance |
|---|---|---|---|---|---|
| Body weight (g) | | | | | |
| *Females* | 335.83±11.83 | 300.10±12.46 | 330.25±5.27 | 321.33±8.59 | Sex effect |
| *Males* | 566.40±18.49 | 521.83±16.20 | 557.17±32.32 | 553.60±7.28 | |
| Nasoanal distance (cm) | | | | | |
| *Females* | 22.83±0.28 | 22.31±0.25 | 22.90±0.29 | 22.63±0.32 | Sex effect |
| *Males* | 26.12±0.26 | 25.68±0.16 | 25.98±0.19 | 26.32±0.26 | |
| Anogenital index | | | | | |
| *Females* | 0.06±0.00 | 0.07±0.00 | 0.07±0.00 | 0.08±0.00 | Sex and diet effects |
| *Males* | 0.14±0.00 | 0.16±0.00 | 0.14±0.00 | 0.16±0.01 | |
| Visceral fat pads (g) | | | | | |
| *Females* | 6.33±0.81 | 5.44±0.52 | 5.83±0.70 | 4.53±0.26 | Sex and metabolism effects |
| *Males* | 7.44±0.46 | 7.30±0.73 | 6.81±0.59 | 6.97±0.39 | |
| Retroperitoneal fat pads (g) | 10.16±1.72 | 9.26±1.48 | 10.38±1.27 | 7.92±1.28 | n.s. |

**Table 4**

| | **Control (n=17)** | **Control-snack (n=12)** | **STZ (n=10)** | **STZ-snack (n=9)** | **Statistical significance** |
|---|---|---|---|---|---|
| *Latency to 1st intromission (s)* | 78.00±13.08 | 72.00±29.40 | 30.00±5.11 | 105.00±37.10 | n.s. |
| *# Intromissions* | 38.35±3.19 | 40.42±6.12 | 39.40±9.09 | 39.67±4.16 | n.s. |
| *Latency to 1st ejaculation (s)* | 1348.67±113.89 | 947.40±139.76 | 1059.17±211.74 | 1341.14±193.29 | Metabolism x diet effect |
| *Latency to postejaculatory intromission (s)* | 1724.89±108.42 | 1322.90±153.92 | 1466.33±224.81 | 1586.50±154.11 | n.s. |
| *# Postejaculatory intromissions* | 16.89±4.65 | 21.70±138 | 15.67±3.64 | 17.17±2.47 | n.s. |
| *# Ejaculations* | 1.67±0.29 | 2.50±0.37 | 2.50±0.34 | 1.83±0.31 | Metabolism x diet effect |

**Table 5**

| | Control (n=7) | Control-snack (n=9) | STZ (n=7) | STZ-snack (n=6) | Statistical significance |
|---|---|---|---|---|---|
| Body weight (g) | 417.99±8.27 | 394.42±11.34 | 443.29±17.30 | 426.32±16.67 | Metabolism effect |
| Testis (g) | 1.67±0.06 | 1.73±0.08 | 1.80±0.04 | 1.73±0.11 | n.s. |
| Epididymis (g) | 0.66±0.02 | 0.62±0.02 | 0.72±0.02 | 0.66±0.03 | n.s. |
| Prostate (g) | 0.38±0.02 | 0.40±0.04 | 0.39±0.03 | 0.38±0.03 | n.s. |
| Full seminal vesicle (g) | 1.91±0.11 | 1.49±0.07 | 1.55±0.07 | 1.71±0.12 | Metabolism x diet effect |
| Seminal vesicle content (g) | 1.23±0.11 | 0.79±0.05 | 0.86±0.10 | 0.89±0.09 | Diet and metabolism x diet effects |
| Perigonadal fat (g) | 6.72±0.34 | 6.21±0.64 | 8.52±1.15 | 7.61±1.23 | n.s. |
| Gonadosomatic index (%) | 0.80±0.03 | 0.89±0.06 | 0.81±0.03 | 0.81±0.05 | n.s. |

**Figures - Legends**

**Figure 1.** Experimental design.

**Figure 2.** Anxiety-like behavior on PND 30, 90, and 360. Values expressed as mean ± standard error of the mean. Only significant main effects and interactions are shown. Time in open arms on PND 30 (A) significant effect: diet x sex interaction. Head dips on PND 30 (B) significant effect: diet x sex interaction. Time in open arms in PND 90 (C). Head dips on PND 90 (D) significant effect: sex. Time in open arms on PND 360 (E). Head dips on PND 360 (F) significant effects: sex and metabolism x diet interaction (Three-way ANOVA, $p<.05$).

**Figure 3.** Sucrose preference on PND 30, 90, and 360. Values expressed as mean ± standard error of the mean. Only significant main effects and interactions are shown. Sucrose preference on PND 30 (A). Sucrose preference on PND 90 (B) significant effect: diet (Three-way ANOVA, $p<.05$). Sucrose preference on PND 360 (C).

**Figure 4.** Oral glucose tolerance test on PND 30, 90, and 360. Values expressed as mean ± standard error of the mean. Only significant main effects and interactions are shown. Glycemic curves on PND 30 (A), 90 (B), and 360 (C). Significant effects on PND 360: time, sex, and time x diet (Four-way ANOVA, $p<.05$). Glycemic return at 120 minutes in each experimental group on PND 30 (D), 90 (E), and 360 (F). * $p<.05$, t test. ** $p<.01$, t test. *** $p<.001$, t test.

## Figure 1

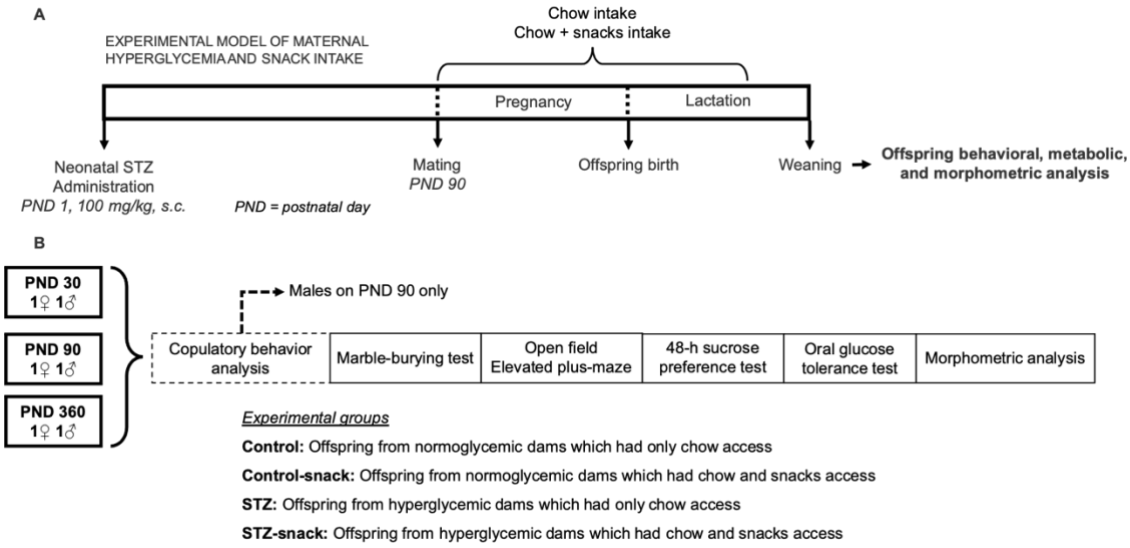

A
EXPERIMENTAL MODEL OF MATERNAL HYPERGLYCEMIA AND SNACK INTAKE
Chow intake
Chow + snacks intake
Pregnancy
Lactation
Neonatal STZ Administration
PND 1, 100 mg/kg, s.c.
Mating
PND 90
Offspring birth
Weaning
Offspring behavioral, metabolic, and morphometric analysis
PND = postnatal day
B
PND 30
1♀ 1♂
PND 90
1♀ 1♂
PND 360
1♀ 1♂
Males on PND 90 only
Copulatory behavior analysis
Marble-burying test
Open field
Elevated plus-maze
48-h sucrose preference test
Oral glucose tolerance test
Morphometric analysis
Experimental groups
Control: Offspring from normoglycemic dams which had only chow access
Control-snack: Offspring from normoglycemic dams which had chow and snacks access
STZ: Offspring from hyperglycemic dams which had only chow access
STZ-snack: Offspring from hyperglycemic dams which had chow and snacks access

**Figure 2**

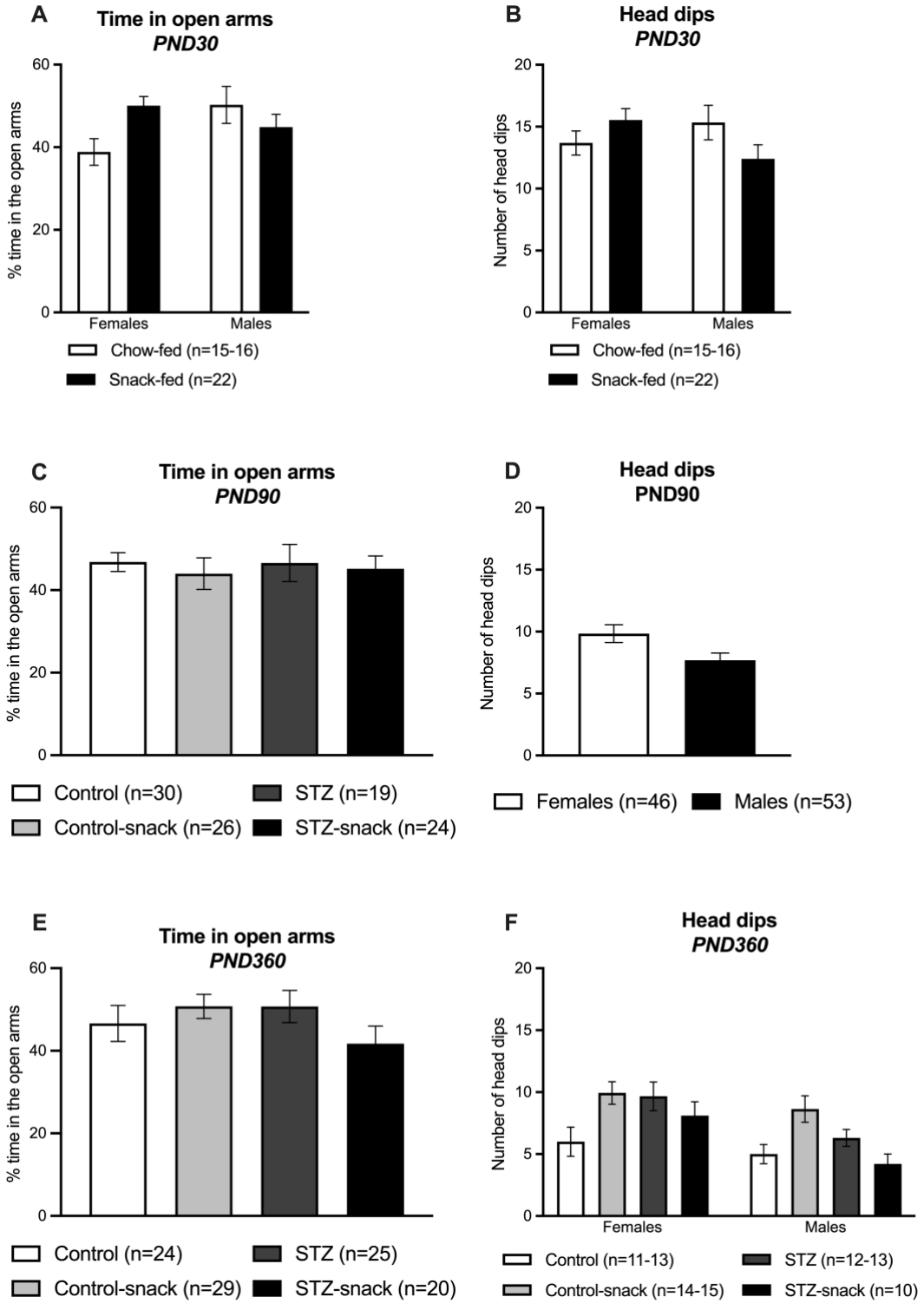

A
Time in open arms
PND30
% time in the open arms
Females
Males
Chow-fed (n=15-16)
Snack-fed (n=22)
B
Head dips
PND30
Number of head dips
Females
Males
Chow-fed (n=15-16)
Snack-fed (n=22)
C
Time in open arms
PND90
% time in the open arms
Control (n=30)
STZ (n=19)
Control-snack (n=26)
STZ-snack (n=24)
D
Head dips
PND90
Number of head dips
Females (n=46)
Males (n=53)
E
Time in open arms
PND360
% time in the open arms
Control (n=24)
STZ (n=25)
Control-snack (n=29)
STZ-snack (n=20)
F
Head dips
PND360
Number of head dips
Females
Males
Control (n=11-13)
STZ (n=12-13)
Control-snack (n=14-15)
STZ-snack (n=10)

**Figure 3**

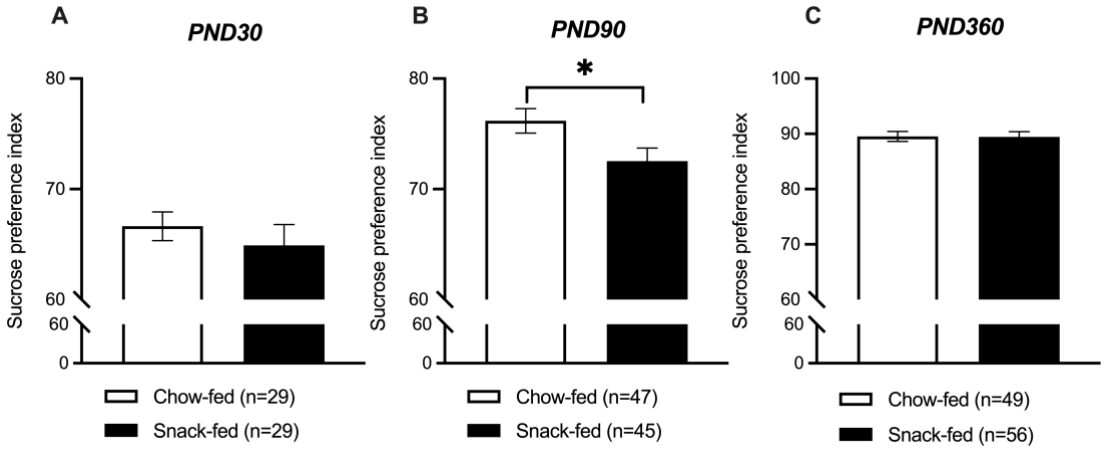
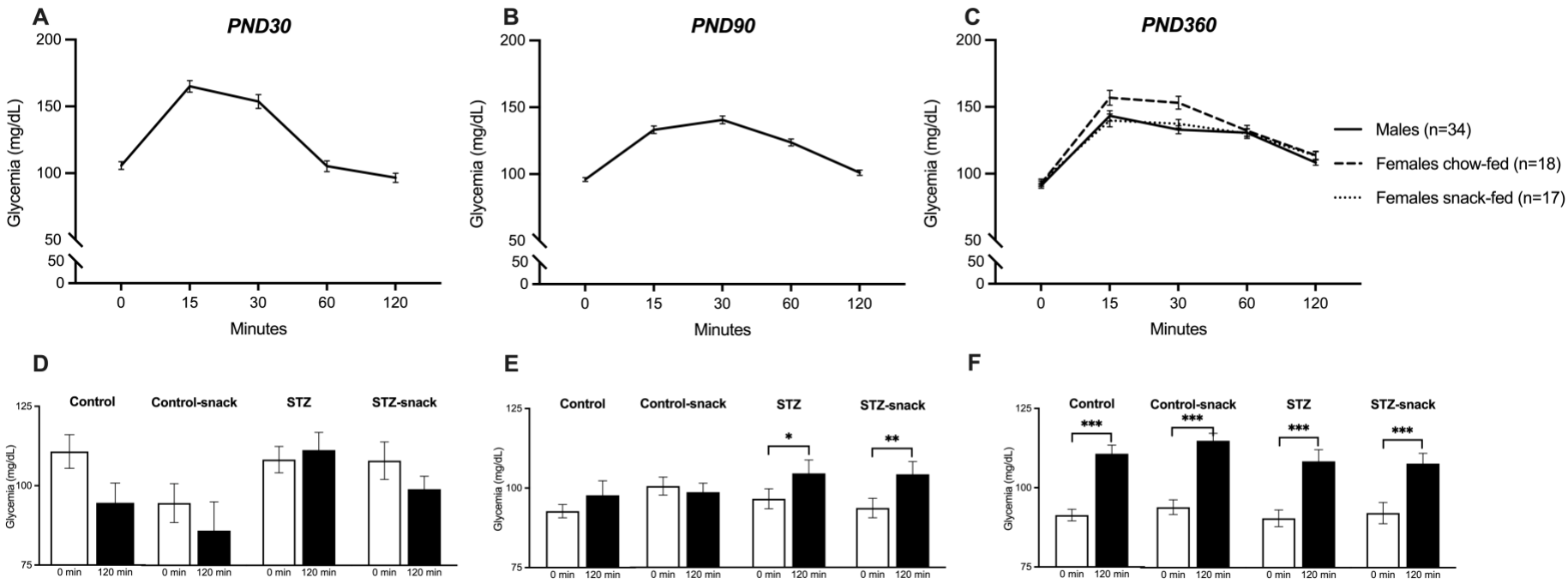

A
PND30
B
PND90
C
PND360
*
Sucrose preference index
80
70
60
0
100
90
Chow-fed (n=29)
Snack-fed (n=29)
Chow-fed (n=47)
Snack-fed (n=45)
Chow-fed (n=49)
Snack-fed (n=56)

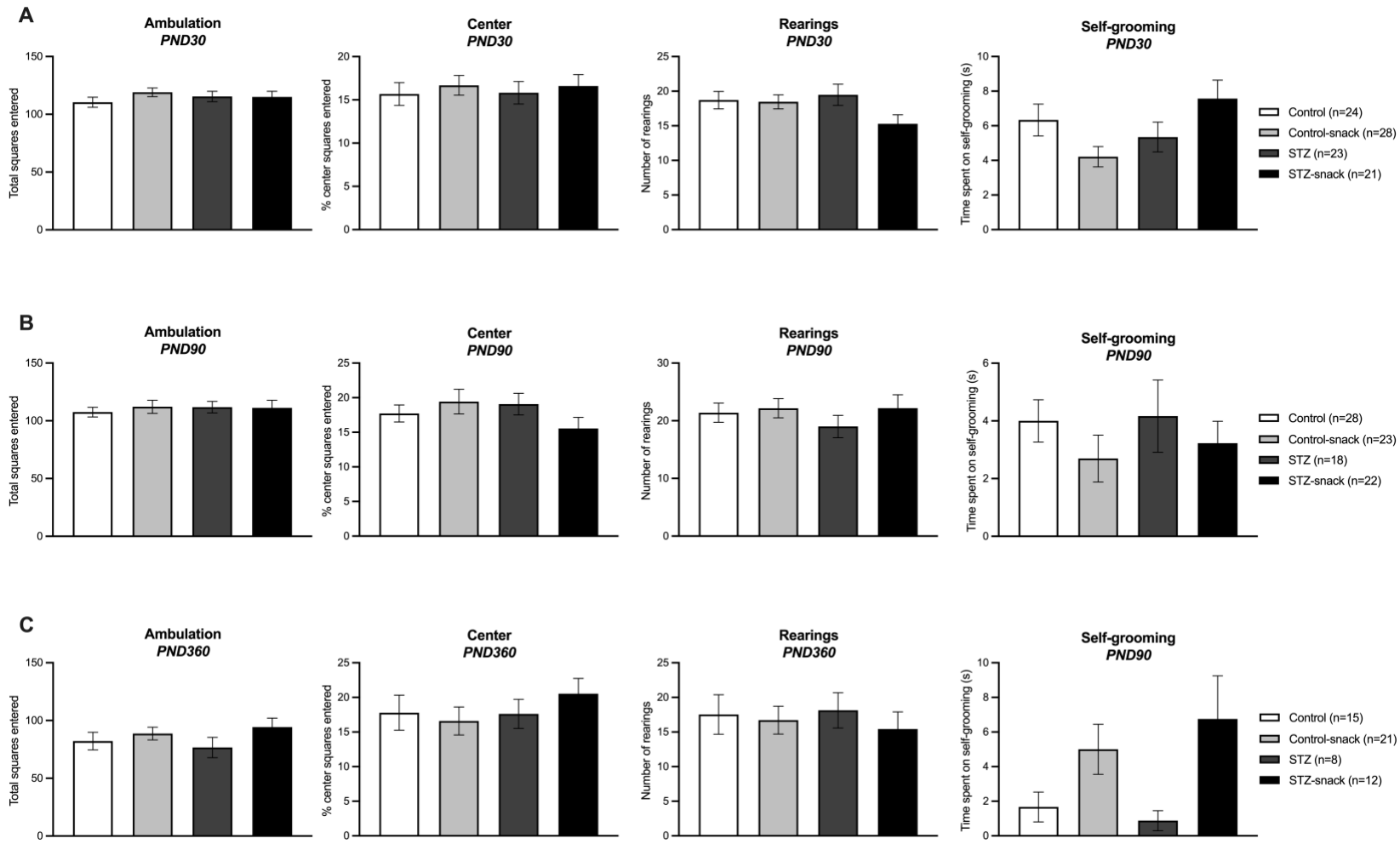
**Figure 4**


A
PND30
B
PND90
C
PND360
Glycemia (mg/dL)
Minutes
0
15
30
60
120
Males (n=34)
Females chow-fed (n=18)
Females snack-fed (n=17)
D
E
F
Control
Control-snack
STZ
STZ-snack
0 min
120 min
*
**
***

## Supplemental material – Legends

**SM Figure 1.** Exploratory behavior on PND 30, 90, and 360. Values expressed as mean ± standard error of the mean. Total ambulation, center squares entered, rearing and self-grooming on PND 30 (A), 90 (B), and 360 (C) (Three-way ANOVA, $p>.05$).

**SM Figure 1.**


A
Ambulation
PND30
Total squares entered
Center
PND30
% center squares entered
Rearings
PND30
Number of rearings
Self-grooming
PND30
Time spent on self-grooming (s)
Control (n=24)
Control-snack (n=28)
STZ (n=23)
STZ-snack (n=21)
B
Ambulation
PND90
Total squares entered
Center
PND90
% center squares entered
Rearings
PND90
Number of rearings
Self-grooming
PND90
Time spent on self-grooming (s)
Control (n=28)
Control-snack (n=23)
STZ (n=18)
STZ-snack (n=22)
C
Ambulation
PND360
Total squares entered
Center
PND360
% center squares entered
Rearings
PND360
Number of rearings
Self-grooming
PND90
Time spent on self-grooming (s)
Control (n=15)
Control-snack (n=21)
STZ (n=8)
STZ-snack (n=12)

## CONCLUDING REMARKS

The present study evaluated if snack intake during pregnancy and lactation would aggravate previously established maternal hyperglycemia and its consequences to maternal care, as well as offspring development, metabolism, and behavior throughout life. Our hypothesis was that snack intake during pregnancy and lactation would trigger further impairments in maternal glycemia homeostasis, resulting in changes in maternal behavior as well on offspring development, metabolism, and behavior from weaning to senescence, that could be sex specific.

This hypothesis was explored in chapters 1 and 2. Chapter 1 described how snack intake during pregnancy and lactation altered maternal food intake and changed the glucose tolerance of STZ-treated females, as well as its impact on maternal behavior and offspring birth weight. We have shown that snack intake aggravates glucose intolerance in hyperglycemic females. This maternal metabolic impairment led to changes in offspring birth weight and altered maternal motivation during lactation. Birth weight classification was normalized in the offspring from hyperglycemic dams with access to snacks, which showed a patter similar to Control offspring. Corroborating previous studies, offspring from hyperglycemic dams were more frequently classified as large por pregnancy age, while offspring from snack-fed dams were more frequently classified as small. Interestingly, birth weight is a strong predictor of long-term consequences to the offspring, being related to an increased risk of developing obesity and metabolic impairments later in life.

On this way, some findings may not be a direct consequence of maternal manipulations themselves, but they may occur as a reflection of these manipulations on offspring birth weight, which is a strong predictor of future outcomes. Mechanisms related to this growth impairment could also be investigated in future studies. In the study conducted during the internship at Carleton University placentas were collected that could help to elucidate how maternal hyperglycemia and snack intake may change nutrients' flow during pregnancy, which might explain offspring outcomes later in life.

Short and long-term consequences to the offspring were then explored in Chapter 2, which described the effects of the association of an impaired maternal metabolism and altered nutrition to offspring glucose metabolism, behavior, and biometric aspects in adolescence, adulthood, and senescence. While offspring metabolism was not further compromised, changes were observed on offspring reproductive function and general behavior in adulthood and senescence, respectively, showing that consequences may only be evident in the long-term. Although no major

impairments were observed on the behavioral tasks evaluated, it is known that both maternal diabetes and inappropriate nutrition are linked to offspring cognitive impairments [49, 62]. For that reason, object recognition and spatial memory in the Morris water maze were also evaluated in the offspring of hyperglycemic dams with access to snacks, which may help to elucidate these behavioral impairments in all age groups. However, given the complexity of this evaluation, the analysis of the videos from this behavioral task is still under way. Results will be assembled in a manuscript addressing impacts of maternal hyperglycemia and snack intake on offspring learning. Additionally, besides morphometric analysis, offspring from both sexes and from all age groups were perfused, and brains were collected for future evaluation. Brain analysis will be planned in order to correlate with possible learning impairments.

Despite not reproducing all aspects of the diabetic syndrome, the experimental model used in this study is useful to study the consequences of maternal hyperglycemia associated with an inappropriate nutrition, since the glycemic levels observed resemble those found in pregnant women diagnosed with clinical or gestational diabetes. Another positive aspect of this study is the analysis of both male and female offspring outcomes. Studying both sexes is extremely important since sex-specific differences related to maternal metabolism have already been described and could help to delineate future experimental and clinical research. Furthermore, offspring outcomes are rarely followed until senescence. Frequently, experimental studies evaluate offspring up until earl adulthood, while most clinical studies still follow only children and adolescents. Therefore, this study broadens the analysis of offspring outcomes, both related to animals' sex and age.

As the experimental model of snack intake described in this study was proven effective to further impair the glucose tolerance of hyperglycemic rats, new research questions emerged, aiming to better understand the impact of this maternal manipulation on other offspring outcomes, such as food intake control and preference. This resulted in a research grant submission in which I'm a collaborator that was granted and is currently under way (Impact of maternal diabetes and snack consumption on male and female offspring control of food intake, FAPESP 2019/01306-2). This project provided the basis for two Master's thesis (Effects of chronic central leptin infusion on food intake of offspring of rats with mild hyperglycemia, FAPESP 2019/06974-3; Effects of chronic central leptin infusion on sexual behavior and reproductive tract of offspring of rats with mild hyperglycemia, CNPq) and an undergraduate thesis (Impact of maternal diabetes and snack consumption on offspring food preference, FAPESP 2020/03604-8) that are also under way with my collaboration. Plus, I was co-advisor in an undergraduate thesis

(Maternal hyperglycemia and overnutrition: effects on anxiety-like behavior of Wistar rats during lactation).

The experimental model employed in the present study was adjust to better suit the new projects. A change in rat lineage, from Wistar to Sprague-Dawley rats, was judged more adequate to the new proposed aims. In addition, as we started using Sprague-Dawley rats, we observed that the experimental model of neonatal STZ injection, which worked well on Wistar rats, was not the most adequate for this lineage. Thus, a modified protocol of STZ administration during pregnancy was employed with success. Although the protocol for inducing hyperglycemia and rat lineage was changed, the aggravated glucose tolerance phenotype was similar to the one described in the present study, reinforcing the validity and consistence of the experimental model.

In conclusion, the present study showed that snack intake during pregnancy and lactation further impaired maternal hyperglycemia, leading to disruptions in offspring birth weight and maternal motivation during lactation, as well as to impaired offspring reproductive function in adulthood and changes in anxiety-live behavior in senescence. Future studies will describe effects on offspring learning and memory, and its possible neural substrates. Although snack intake aggravated the glucose intolerance of mild hyperglycemic rats, glycemic levels were still within the mild range (120 – 300 mg/dL), which might explain why this condition did not lead to major impairments for both mother and offspring. However, the experimental model used in this study is useful to study the consequences of maternal diabetes associated with inappropriate nutrition, since the glycemic levels observed resemble those most observed in pregnant women diagnosed with clinical or gestational diabetes. On the other hand, this experimental model may not be the most suitable to evaluate intervention strategies during pregnancy and lactation that might improve the consequences of this maternal metabolic impairment, because maternal and fetal impairments are limited. However, it should be considered that even this mild maternal condition was enough to change maternal and offspring outcomes, reinforcing the importance of women sustaining target glucose levels and a healthy diet during pregnancy and lactation.

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## ETHICAL COMMITTEE CERTIFICATE

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

# *Certificado*

Certificamos que o projeto intitulado "Associação entre hiperglicemia materna e supernutrição: efeitos no cuidado materno e no desenvolvimento e comportamento dos descendentes em diferentes fases da vida", Protocolo nº **919-CEUA**, sob a responsabilidade de **Ana Carolina Inhasz Kiss**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 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 da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

| | | |
|---|---|---|
| Finalidade: | ( ) Ensino | (x) Pesquisa Científica |
| Vigência do Projeto: | Início: 01/09/2016 | Término: 31/08/2020 |
| Espécie/linhagem: | *Rato Wistar* | |
| Nº de animais: | *605* | |
| Peso: | *De 4 a 600 gramas* | Idade: 0 a 360 dias |
| Sexo: | *Macho e fêmea* | |
| Origem | *Centro Multidisciplinar para Investigação Biológica – CEMIB da Universidade Estadual de Campinas/UNICAMP – Campinas/SP – CNPJ: 046.068.425/0001-33* | |

Botucatu, 26 de agosto de 2016.

Prof. Adj. Wellerson Rodrigo Scarano
Presidente da CEUA

Comissão de Ética no Uso de Animais 1982 unesp

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