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Neonatal Overfeeding Induced by Reducing the Litter Size Leads to an Obese Phenotype and Increases Preference for Sweet Food in Adult Male Rats

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Authors' contributions

Author CN: Data analysis, interpretation and writing; authors SC and VB: Data acquisition, analysis and interpretation; authors AKP, CD, MZG and PPS: Project idea, data acquisition, analysis and interpretation. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: the aim of this study was to induce obesity in rats using the neonatal overfeeding protocol and evaluate in adult male animals standard chow intake, sweet food intake, the preference between sweet food and standard chow, locomotor activity and anxiety-like behavior.
Methodology: The neonatal overfeeding protocol consisted of reducing the litter size to 4 animals (small litters = SL) compared to 8 animals in normal litters (NL). In these experiments we used 55 offspring from 18 litters.
Results: obesity was successfully induced as observed by increased body weight and

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depots of abdominal fat in SL animals compared to NL; [F(1, 53)=15.018; *P*<.001] for body weight and [t(48.06)=2.186 *P*=.03] for abdominal fat.No difference between groups was found in standard chow [t (16)=1.843 *P*=.08] and sweet food intake [t(53)=0.453 *P*=.65], however in the test that evaluated the preference between both foods SL animals consumed more sweet food than NL [t(48) =2.481 *P*=.02]. Additionally, there was no difference between groups regarding locomotor activity [t(52)=0.073 *P*=.94] but SL animals showed reduced anxiety-like behavior compared to NL [t(39.36)=2.205 *P*=.03].
Conclusion: this study supports the use of neonatal overfeeding protocol as a model of early obesity and showed for the first time the increased preference for sweet food in adult neonatal overfed animals.

Keywords: Anxiety; obesity; overfeeding; rats; sweet.

1. INTRODUCTION

Obesity has become one of the major threats for public health in industrialized world among adults, but also among adolescents and children [1]. Many obesity-related health conditions once thought applicable only to adults are now being seen in children and with increasing frequency [2]. Examples include high blood pressure [3], type 2 diabetes [4] and nonalcoholic fatty liver disease [5]. Obesity is influenced by the interaction of genes, nutrition, environment, and lifestyle [6]. Additionally, the way an individual grows and develops early in life directly impacts upon their cardio-metabolic health in later life [7, 8]. Weight gain in the first years of life is important in programming body mass index (BMI) in young adults [9]. It was shown that weight at 1 year of age was associated with adult fat mass, suggesting that postnatal environmental factors, such as infant feeding, could be more important than prenatal factors for the development of adult adiposity [10].

Studies in animals [11,12] and humans [13] have shown that early postnatal overfeeding represents a risk factor for obesity and associated metabolic disturbance in adulthood. In the experimental field a protocol used to study short and long-term consequences of childhood obesity consists in raising rats in small litters [14,12]. It was shown that this protocol of neonatal overfeeding led to rapid early weight gain, resulting in a metabolic syndrome phenotype, i.e., hyperleptinemia, hyperglycemia, hyperinsulinemia, and an increased insulin/glucose ratio [14]. Hyperphagia and hypertension have also been observed in adult rats submitted to early overnutrition [11].

Human desire for sweet taste spans all ages, races and cultures. Given that sweetness has a powerful hedonic appeal, sweet foods and beverages have come under scrutiny as potential contributors to the obesity epidemic worldwide [15]. Craving for carbohydrates has been reported to affect obese patients [16]. On the other hand, the consumption of foods rich in fat and carbohydrates, also known as comfort food have been used for consolation when feeling down and out [17]. In the short term, or in societies where there is not immediate and continual access to comfort foods, occasional relief of anxiety with sweet or fatty foods is probably useful, however easy and continuous access to this type of food can lead to a consequent epidemic of obesity.

Therefore, given the importance and increasing prevalence of obesity in children, the aim of this study was to induce obesity in rats using the neonatal overfeeding protocol (by raising them in small litters) and evaluate in adult male animals behavior parameters as: standard chow intake, sweet food intake, preference between sweet food and standard chow,

locomotor activity and anxiety-like behavior. Our hypothesis was that neonatal overfeeding would lead to an obese phenotype and an increased preference for sweet food that could be associated with an increased anxiety-like behavior.

2. MATERIALS AND METHODS

2.1 Subjects

Wistar rat matrices (60 days, 180-200g) were obtained from Universidade Federal de Pelotas and acclimatized to our rat facility for 2 weeks prior to mating. All rats were housed in Plexiglas cages in groups of 2-4 rats, in a controlled environment: lights on between 07:00 and 19:00h, temperature of 25+/-2 ºC and humidity of 35%. Cage floor was covered with wood chips, and cleaning was done 2-3 times per week. Food and water were left *ad libitum*. After mating, the females were left in single cages until giving birth. The day of birth was considered as day 0. At day 1 after birth, litters were culled to 4 animals (3 males and 1 female per litter) in the small litter group (SL), and to 8 pups (5-6 males and 3-2 females) in the normal litter group (NL) and were maintained undisturbed until weaning at day 21, when rats were separated by gender. Only males were used in this study and were grouped in 3-4 per cage. In these experiments we used 55 offspring from 18 litters. All the experiments were performed after 60 days of life.

2.2 Body Weight

Body weight was measured weekly from day 21 until day 86 of life. A digital scale with a precision of 0.01g was used for the measurements.

2.3 Standard Chow Intake

After 60 days of life, rat's standard chow intake was measured by leaving a previously known amount of food in the cage cover and measuring the remaining after the desired period. Care was taken to start the measurement in a clean cage and to search the cage for food spills afterwards.The consumption was measured in two consecutive periods of 24h, with the rats in their usual groups of 3-4 littermates, and the amount consumed was presented as an average per cage per rat per 24h.

2.4 Sweet Food Intake

To evaluate the consumption of sweet food, after 70 days of life animals were individually placed in a box similar to their home cage, containing 10 pellets of sweet food (Froot Loops®, Kellogs, 3.78 kcal/g), for 5 minutes. The number of ingested pellets was counted and a protocol was established, so that when the animals ate part of the Froot Loops® (e.g., 1/3 or 1/4), this fraction was considered. During five consecutive days prior to testing, the animals were habituated to the new food under the same experimental conditions of the test and 3 Froot Loops® pellets were given per rat in their home cages after habituation. The habituation to the Froot Loops® is important because animals may avoid the consumption of unfamiliar food.

2.5 Food Preference Test

Preference test was done by placing 50g of sweet pellets (Froot Loops®, Kellogs) and 50g of regular habitual chow, in the cage cover of the animals' home cages, and measuring the remaining after 24h. Animals were evaluated individually in this test after 80 days of life.

2.6 Open Field Test

After 86 days of life, animals were tested for locomotor activity and anxiety using the open field paradigm in an automated apparatus (EP-149 IR, Insight Equipamentos Científicos LTDA, RibeirãoPreto, SP). The evaluation was performed in two days, being the first day considered as training for novelty exposure with duration of 5 minutes and the following day as the testing with duration of 30 minutes. Total locomotion measured in distance traveled (locomotor activity) was verified considering the test day and percentage of time spent exploring the central area of the open field (anxiety parameter) was evaluated considering the training day. The open field was thoroughly cleaned with 30% ethanol between different animals.

2.7 Light/dark Exploration Test

After 90 days of life, animals were submitted to the light/dark apparatus as described by [18]. It consisted of an open-topped wooden arena (70cm x 10cm), half painted black and half white. Twenty-seven centimeter high walls bordered the field, and the two compartments were freely communicated at the center. The white compartment was illuminated by bright, direct white light, and the dark compartment received no light at all. The experiment was conducted with room lights off. Crossing from one side to another was considered when the rat left only the distal third of the body in the original compartment. Time spent in the lit compartment and entries made to the lit compartment were recorded during 15min.

2.8 Abdominal Fat Deposition and Glycemia

Between postnatal day 110 to 120, animals were sacrificed by decapitation and the two major portions of abdominal fat (gonadal and retroperitoneal adipose tissue depots) were dissected and weighted using a scale with a precision of 0.01g. Results were expressed as % of body weight. Plasma glucose was measured by the glucose oxidase method using a commercial kit (Wiener lab).

2.9 Statistical Analysis

Continuous data in a one-factor model was analyzed by Student's t test with litter sizeas the between factor (standard chow and sweet food intake, preference test, open field test, light/dark exploration test and abdominal fat deposition). Repeated measures ANOVA was used for analysis of body weight measures (group and time as factors) and Greenhouse-Greisser correction was applied considering violation of the sphericity assumption as shown by the Mauchly test. Data was presented as mean and standard error of the mean (SEM). Statistical significance was set at $P \leq .05$.

3. RESULTS

3.1 Body Weight

As plotted in Fig.1.all rats gained weight as the time passed by. A Repeated Measures ANOVA showed a main effect of time [F(2.10, 111.27)=3730.634; *P*<.001, correction for Greenhouse-Geisser]. There was also a group effect showing that SL animals were heavier than NL ones [F(1, 53)=15.018; *P*<.001]. This group effect was confirmed individually for every age that the weight was verified by Student's t test (*P*<.001 for ages of 21, 28, 35, 42 and 49 days; *P*=.001 for 56 days; *P*=.004 for 63 days; *P*=.003 for 70 days; *P*=.01 for 78 days and *P*=.03 for 86 days).

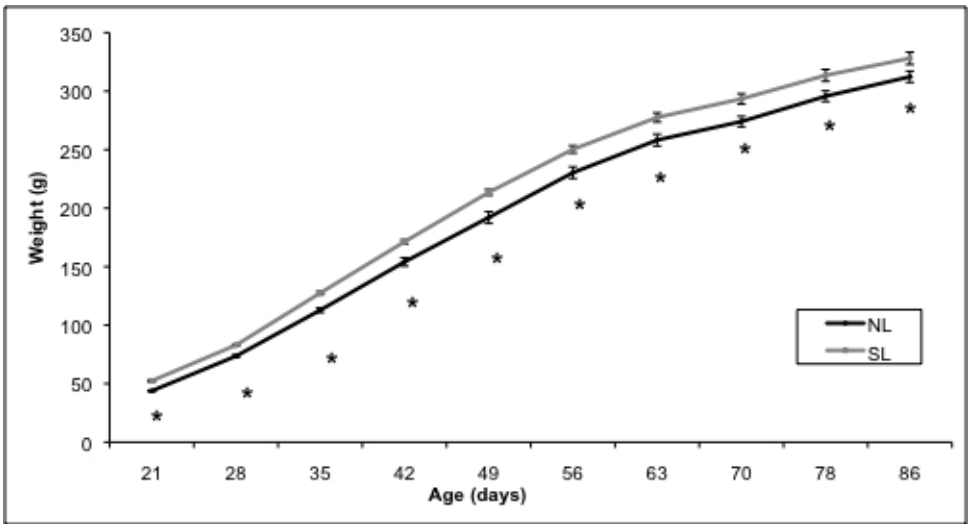

Fig. 1. Body weight of rats raised in normal (NL) and small (SL) litters

Data is expressed as mean ± SEM. NL n=27, SL n=28. Repeated Measures ANOVA showed a main effect of time [F(2.10, 111.27)=3730.634; P<.001, correction for Greenhouse-Geisser] and a group effect [F(1, 53)=15.018; P<.001].

** Different compared to normal litters (Student's t test P<.05).*

3.2 Standard Chow Intake

There was no difference in the consumption of standard chow between the groups (Student's t test, t (16)=1.843 *P*=.08, n=8-10/group,Table 1).

3.3 Sweet Food Intake

There was no difference in the consumption of sweet food between NL and SL animals, after habituation (Student's t test, t(53)=0.453 *P*=.65, n=27-28/group,Table 1).

3.4 Food Preference Test

Fig. 2.illustrates the food preference test. Student's t test showed again that there was no difference in the consumption of standard chow between NL and SL animals [t(48)=0.248

P=.81], however considering sweet pellets SL rats consumed more calories than NL ones when having these two types of food available [t(48) =2.481 *P*=.02]. Therefore, SL animals ate more total calories than NL rats [t(48) =2.842 *P*=.007], due to an increased consumption of sweet food.

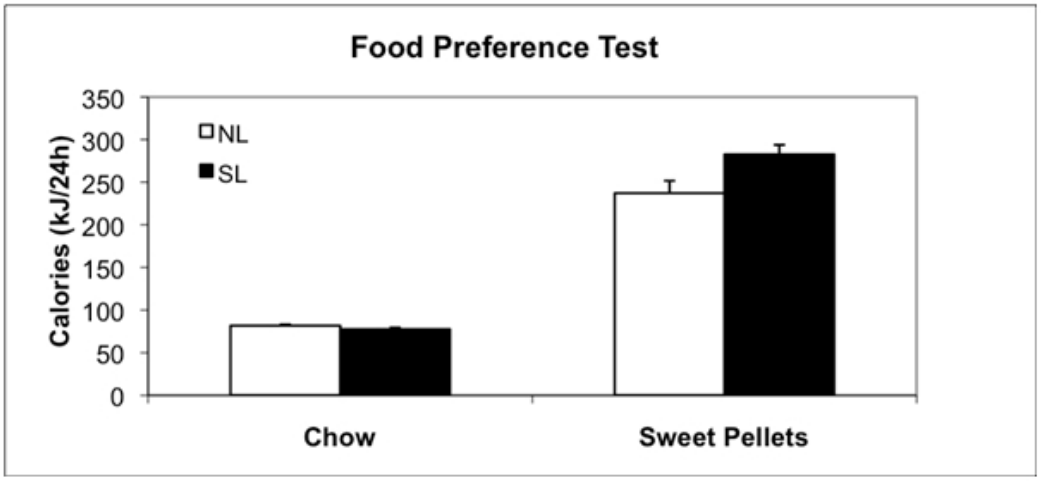

Fig. 2. Food preference test in rats raised in normal (NL) and small (SL) litters
Data is expressed as mean + SEM, (NL n=25, SL n=25). On food preference test, considering sweet pellets SL rats consumed more calories than NL ones when having the two types of food (standard chow and sweet food) available [t(48) = 2.481 P=.02].
** Significantly different compared to NL animals, regarding sweet pellets.*

3.5 Open Field test

The total distance travelled during the 30 minutes of exposure to the Open Field apparatus was not different between the groups [Student's t test, t(52)=0.073 *P*=.94, n=26-28/group, Table 1). However, SL rats spend more time in the centre of the apparatus [Student's t test, t(39.36)=2.205 *P*=.03, n=26-28/group, Table 1], demonstrating a behavior compatible with decreased levels of anxiety as proposed in [33,38].

3.6 Light/dark Exploration Test

There was no difference between groups in the time spent [Student's t test, t(15)=0.540 *P*=.60, n=8-9/group, Table 1] and in the entries made to the lit compartment [Student's t test, t(15)=1.584 *P*=.13, Table 1].

Table 1. Data is expressed as mean + SEM,

Behavior	NL group	SL group	P
Feeding behavior			
Standard chow intake (g/24h)	24.47±0.48	25.75±0.48	0.084
Froot Loops (g/5 min)	0.41±0.10	0.49±0.13	0.625
Open Field			
Distance travelled (meters)	9.88±0.59	9.83±0.36	0.942
Time central area (%total time)	19.5±1.39	26.43±2.81*	0.033
Light-dark test			
Time spent in the lit compartment (seconds)	414.94±18.20	429.37±19.57	0.597
Number of entries in the lit compartment	20±2.49	25.5±2.39	0.134

** Significantly different compared to NL animals.*

3.7 Abdominal Fat Deposition

Adult SL animals had increased depots of abdominal fat when compared to NL rats [Student's t test, t(48.06)=2.186 *P*=.03, Fig. 3].

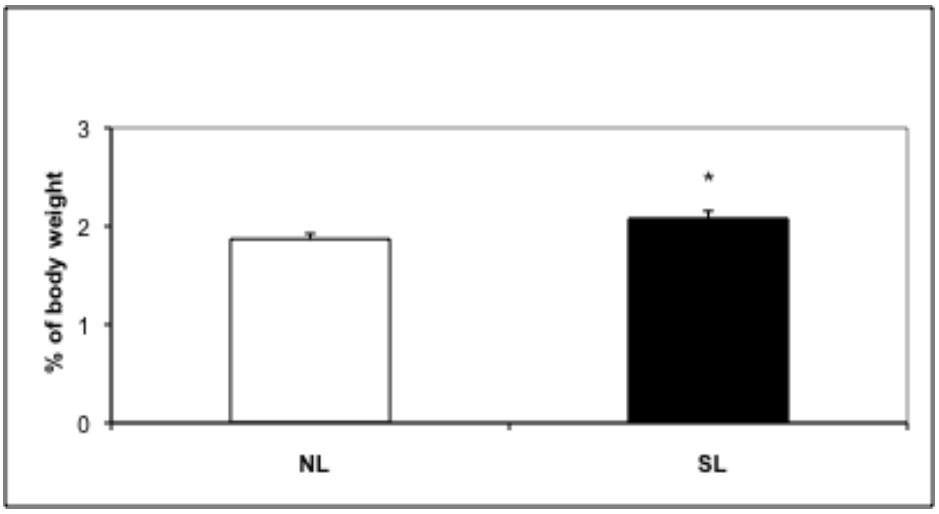

Fig. 3. Abdominal fat deposition in rats raised in normal (NL) and small (SL) litters
Data is expressed as mean + SEM, NL n=26, SL n=28. Student's t test showed increased depots of abdominal fat in SL animals compared to NL ones [t(48.06)=2.186 P=.03].
** Significantly different compared to NL animals.*

3.8 Glycemia

No differences were seen in glycemia between SL and NL rats [NL=8.6±0.4mmol/L; SL=8.1±0.3mmol/L; Student's t test, t(14.623)=1.017 *P*=.32, n=9/group].

4. DISCUSSION

The main findings of this study were that neonatal overfeeding induced by raising the offspring is small litters lead to an obesity phenotype in male rats represented by increased abdominal fat depots and body weight. Additionally, we saw that adult SL animals had an increased preference for sweet food and showed a reduced anxiety-like behavior when compared to NL animals.

Obesity was successfully induced in rats that were raised in small litters. Our results, regarding body weight and abdominal fat depots comparing SL and NL adult animals are in agreement with previous studies mentioned in the introduction section [11,12]. Although we do not have data on percentage of body fat, measuring the two major depots of abdominal fat has been used as a marker of abdominal study in many studies from our group and others [34,35]. They also agree with a study performed in mice where neonatal overfeeding led to accelerated body weight gain with adult SL animals showing higher body weight than NL rats [19]. Considering that, our hypothesis that neonatal overfeeding would lead to an obese phenotype was confirmed. However, no differences were seen in glycemia, as opposed to the description of [37].

With regard to the hypothesis that neonatal overfeeding would lead to an increased preference for sweet food, our results also sustained this point. When animals were exposed to sweet food and standard chow simultaneously for 24h, there was no difference between groups in the consumption of standard chow, but when considering sweet pellets SL rats consumed more calories than NL ones. One possibility to explain this behavior would be a decreased dopaminergic activity in the nucleus accumbens induced by obesity. It was shown in humans that striatal dopamine D2 receptor availability was significantly lower in obese individuals compared to controls [20]. Since dopamine modulates motivation and reward circuits, the deficiency in obese individuals may lead to pathological eating in order to compensate for decreased activation of these circuits [20]. It has also been proposed an association between striatal D2 receptors and prefrontal metabolism in obese subjects suggesting that decreases in striatal D2 receptors could contribute to overeating via their modulation of striatal prefrontal pathway, which participate in inhibitory control and salience attribution [21]. We also verified the sweet food consumption when only this type of food was available for a short period to evaluate craving behavior. The absence of effect may be related to the fact that this test was performed during the light phase of the circadian cycle when rats are less active and eat less [22]. Another possibility is that longer periods of exposure are needed to visualize a significant difference between the groups and the craving behavior might not be an issue in this case.

No effect concerning the consumption of standard chow (when this was the only type of food available) was observed in this study. It was shown that adult rats from small litters consume significantly more rat chow in a one-week period than adult rats from large litters [23]. The different results between our study and theirs may be explained by the fact that we evaluated the consumption of chow during two consecutive periods of 24h while in their study a one-week period was used. Another difference was the size of the litters used, in both studies the small litter had 4 pups however in our study the intervention group was compared against a litter of 8 pups (normal litter) and in theirs against a litter of 16 pups (large litter). The size of the litter might also explain the difference between our results and the increased consumption of standard chow over 24h from weaning until adulthood showed by SL rats when compared to NL ones in another study [11]. In that study the small litter contained 3 pups and the normal litter 10 animals.

In our initial hypothesis we thought that neonatal overfeeding would lead to an obese phenotype and to an increased preference for sweet food as confirmed by our results and that this could be related to an increased anxiety-like behavior. Preschool obesity in children has been associated with increased feeling of anxiety [24] and in the general population an association between these two factors has also been suggested [25]. However, we observed that SL rats spent more time in the centre of the open field apparatus when compared to NL animals, showing a behavior compatible with decreased anxiety. Studies in animals have shown controversy results concerning anxiety and obesity that may be related to the use of different protocols. Souza et al. [26] showed that the consumption of highly palatable diet enriched with sucrose led to an obese phenotype and induced some anxiety-like behavior in rats. The consumption of a high-fat diet and resulting obesity has also been associated with increased anxiety observed in mice by decreased time spent in open arms in the elevated plus maze and decreased time in the centre of the open field [27]. On the other hand, neonatal overfeeding, induced by changing the litter size in which the animals were raised, led to a reduced anxiety-like behavior in adult rats in our study, which is in agreement with others [23], even though there are some methodological differences between the studies. On Spencer and Tilbrook [23] manuscript, on the day of birth all offspring were removed from their matrices and randomly reallocated to new dams in litters of four or 16. No dam received any of her own pups and each litter was made of 50% males and 50% females. In our study, at day 1 after birth, litters were culled to 4 animals (3 males and 1 female per litter) or 8 pups (5-6 males and 3-2 females) and were raised by their own mothers. We chose to use normal litters of 8 pups because this allows a suitable milk feeding avoiding the possibility of an under feed effect with higher number of offspring. Others have already attributed as normal litters those containing 8-10 pups [28,29]. It is important to highlight for the number of animals that we used in the evaluation of the open field test in this study (26 for NL and 28 for SL). One possible explanation for the decreased anxiety-like behavior seen in neonatal overfeeding adult rats may be the amount of maternal care received by these animals, as already proposed by Spencer and Tilbrook [23]. Rats raised in smaller litters would have reduced competition for maternal care and it is well known that the higher amount of maternal attention such as licking or grooming can have pronounced long-term effects on the animals as reduced manifestation of fear [30] and reduced anxiety-like behavior [31]. However, SL animals are shown to have enhanced central responses to restraint stress including greater activation of the paraventricular nucleus of the hypothalamus and paraventricular nucleus of the thalamus [23]. However, considering that classically increased maternal care leads to reduced stress responses [30], it is somewhat contradictory to think that the diminished anxiety described here is due to increased maternal care. We also used the light/dark exploration test to evaluate anxiety-like behavior in this study, however no difference between groups was verified. The inconsistence between the results of the open field and the light/dark exploration test can be explained by the fact that different tasks actually measure a mixture of different aspects of anxiety [32] in such a way that different results may be found with different tests [33].

As expected, our study is not free from limitations. A better estimative of obesity would help to characterize if the neonatal overfeeding indeed leads to increased body weight, instead of increased body mass as a whole. However, many studies using this model have described the induction of increased body weight, hyperinsulinemia and hyperleptinemia by the reduction of the litter size [14,19,23,28,29,37]. In addition, separate the effects of overnutrition from increased maternal care is difficult experimentally, considering that the intervention (reduction of the litter size) is applied the pre-weaning period. Despite that, two main observations suggest that is indeed overnutrition and not maternal care the main player in body weight increase in this model. Despite that, it is unlikely that all the maladaptive

responses observed in this model [14,19,23,28,29,37] would be a result of increased maternal care.

5. CONCLUSION

In conclusion, this study supports the use of neonatal overfeeding protocol as a model of early obesity, showing for the first time that adult neonatal overfed animals have increased preference for sweet food in adult life. Our main objective here was to show that neonatal overfeeding leads to sweet food preference in adulthood, and supposedly this could be related to increased anxiety. Surprisingly, we found decreased anxiety in these animals, which may suggest that other factors are involved in the sweet food preference in these animals. Excessive weight gain and, especially, increased fat deposition in early infancy in humans may lead to increased risk of developing metabolic and cardiovascular disturbances later on [36], which is probably preceded by altered food preferences over the life course. The knowledge about how early life events may shape the individual's feeding behavior and food preferences during development may serve as a basis for studies investigating particular interventions for specific populations (e.g. obese infants and children).

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CONSENT

Not applicable.

ETHICAL APPROVAL

Animal procedures followed international standards and the project was approved by the local Ethics Commitee (Grupo de Pesquisa e Pós-Graduação - Hospital de Clínicas de Porto Alegre, project 09-409).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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