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Litter Size Reduction Induces Metabolic and Histological Adjustments in Dams throughout Lactation with Early Effects on Offspring

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Abstract: In the present study we analyzed morphological and metabolic alterations in dams nursing small litters and their consequences to offspring throughout lactation. Offspring sizes were adjusted to Small Litter (SL, 3 pups/ dam) and Normal Litter (NL, 9 pups/ dam). Body weight, food intake, white adipose tissue (WAT) content, histological analysis of the pancreas, mammary gland (MG) and brown adipose tissue (BAT) as well as, plasma parameters and milk composition were measured in dams and pups on the 7th, 14th and 21st days of lactation. In general, SL-dams presented higher body weight and retroperitoneal fat content, elevated fat infiltration in BAT, reduced islets size and hyperglycemia throughout lactation in relation to NL-dams ($p<0.05$). Moreover, MG from SL-dams had reduced alveoli development and high adipocytes content, resulting in milk with elevated energetic value and fat content in relation to NL-dams ($p<0.05$). Maternal states influenced offspring anthropometric conditions during lactation, offspring-SL displayed higher body weight and growth, hyperglycemia, augmented lipid deposition in BAT and elevated islet. Thus, maternal histological and metabolic changes are due to modifications to nursing small litters and reinforce the importance of preserving maternal health during lactation avoiding early programming effects on offspring preventing metabolic consequences later in life.

Key words: dams, metabolic programming, milk, offspring.

INTRODUCTION

Lactation is a critical period during female reproductive life, characterized by a higher energetic demand that imposes substantial changes in the maternal metabolism, necessary for sustaining milk synthesis (Zhao and Cao 2009). Consequently, lactating dams display considerable changes in body composition, food intake and hormonal actions compared to non-lactating rats (Zhao et al. 2010). Since maternal and fetal metabolism, after birth, are linked exclusively via milk, the early life nutritional experience has important consequences on the growth, development and health of

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offspring (López et al. 2006). As such, lactation is considered a critical window of development, during which nutritional and metabolic maternal conditions may program the energetic homeostasis system of offspring, and alter their function later in life, predisposing to obesity and co-related comorbidities (Srinivasan et al. 2006, Cerf et al. 2012).

Obese and protein-restricted lactating dams affect body weight and hormonal states of neonates, via milk, inducing metabolic programming to offspring. Thus, some kind of metabolic changes early in life results in high adipose tissue accumulation, hyperinsulinemia, glucose intolerance, and dyslipidemia in adulthood (McMillen and Robinson 2005, Page et al. 2009, Mozeš et al. 2014). Lactation is defined as the combined processes of milk synthesis, secretion and removal which demand changes in the structure and function of the mammary gland (MG) (McClellan et al. 2008, Laporta et al. 2013). In addition, physiological and morphological modifications in other major sites, such as white adipose tissue (WAT), brown adipose tissue (BAT) and liver are also necessary to sustain milk synthesis in MG (Moffett et al. 2013).

The availability of nutrients for milk production is supplied by other tissues, particularly, WAT and liver (Velazquez-Villegas et al. 2013). As a consequence, despite a high maternal food intake during lactation, increased hepatic gluconeogenesis, decreased glucose utilization by peripheral tissues, as well as a reduction in lipid deposition in WAT is frequently observed during this phase (Williamson 1980, Vernon and Flint 1983, Scow and Chernick 1987). In contrast, lipogenesis and glucose inputs are increased in MG to ensure the preferential uptake of precursors for milk fat and carbohydrates production (Komatsu et al. 2005). These changes in the metabolism of energetic substrates are mediated by alterations in insulin secretion and action (Lellis-Santos et al. 2012). Thus, glucose homeostasis and insulin sensitivity are modified during lactation, resulting in morphological alterations in the pancreatic islets. These changes increase whole body insulin resistance and reduce insulin secretion during lactation (McDowell et al. 1987).

In rodents, the thermogenic function of BAT decreases during lactation, as shown by tissue hypotrophy, a decrease in mitochondrial biogenesis, and an impaired expression of the gene encoding UCP1 (Król et al. 2011). As a result, the capacity for thermogenesis in BAT of lactating dams is greatly reduced (Trayhurn 1983, Isler et al. 1994). Interestingly, milk production correlates well with the expression of some the BAT gene; for example, downregulation of UCP1 gene expression in BAT results in greater milk production (Król et al. 2011, Pedraza et al. 2000).

Finally, the intensity of the morphological and metabolic alterations during lactation depends on the phase of lactation, as well as litter size and nursing intensity (Laporta et al. 2013). For this reason, reductions in litter size to 3 - 4 pups/ dam, after birth, promote overfeeding during the suckling period resulting in obesity and related hormonal and metabolic disorders in offspring over the long term. Thus, offspring reared in small litter (SL) frequently present insulin resistance, glucose intolerance, dislipidemia and cardiovascular diseases in adult life (Mozeš et al. 2014, Xiao et al. 2007); an event attributed to changes in the composition or volume of milk ingested (Purcell et al. 2011). In fact, milk from dams nursing SL display higher calorie contents and fat concentrations and SL-offspring consume a greater milk volume (Cunha et al 2009, Fiorotto et al. 1991). In the present study we characterized the morphological and metabolic changes in dams nursing SL and evaluated the consequences of these alterations on offspring at different stages of the lactation.

MATERIALS AND METHODS

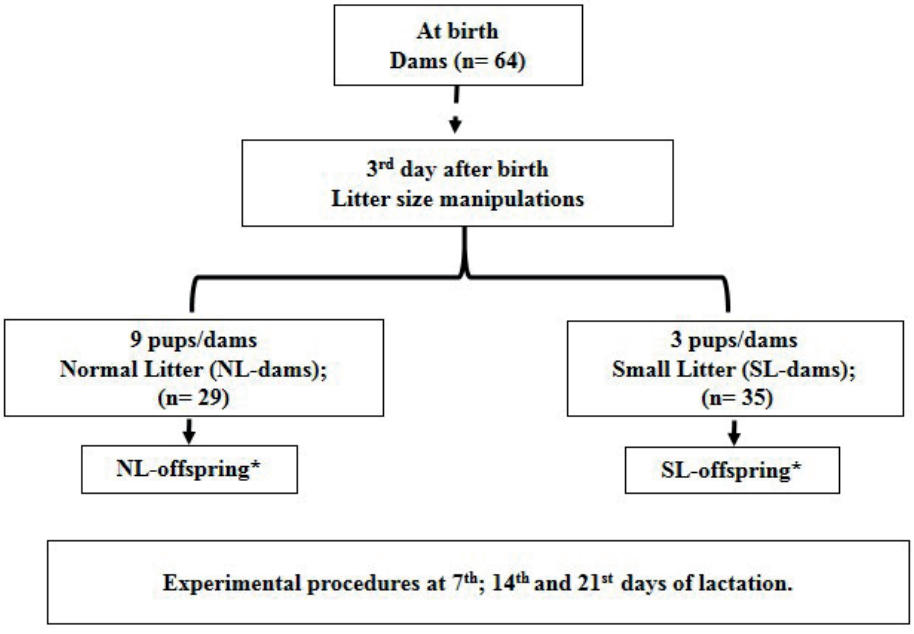
EXPERIMENTAL ANIMALS AND ETHICAL APPROVALS

Rats of the *Rattus norvegicus* species of the Wistar lineage were used in this experimental study. The study was previously evaluated and approved by the Ethics Committee on Animals Use (CEUA) of the Universidade Estadual de Ponta Grossa (UEPG), process number 033/2014 and protocol 9895/2014. After approval by CEUA, the animals were provided by the bioterio of the UEPG. The procedures and methods performed with the animals were followed by the ARRIVE Guidelines and the procedures for the euthanasia of animals followed the norms American Veterinary Medical Association's Guidelines on Euthanasia. The experimental models for the induction of metabolic programming by litter reduction mainly use rats, as established by Kennedy (1957), as these animals have fast reproductive cycles and numerous litters.

During mating, gestation and lactation periods, the animals were kept in the animal house of the UEPG under standard conditions inside polypropylene boxes with a bed of wood, in a quiet environment with a controlled temperature of 21 ± 2ºC and 12 hour luminosity cycles. The animals had free access to rodent chow (NUVILAB), according to AIN-93 recommendations and drinking water. All animals were submitted to identical conditions.

EXPERIMENTAL DESIGN

Male rats (n = 28) were mated with nulliparous rats (n = 84) in the polygynous mating system (3 females for each male), both aged 70 to 90 days. The female rats were separated into individual boxes after the visual confirmation of the pregnancies. Thus, 64 litters were obtained, which were distributed into 2 experimental groups (29 NL and 35 SL), defined by the number of offspring during the lactational period. The Normal Litter group (NL) was maintained with 9 pups/ dam and the Small Litter (SL) group was maintained



Flowchart - Experimental design. The letter "n" represents the number of litters, i.e., the number of dams in each group. (*) In all lactation stage (7th; 14th and 21st) of 10 -12 male pups rats were analyzed. The pups came from a least 10 different litters.

with 3 pups/ dam. The litter reduction model was carries out according to the model established by Kennedy (1957); the number of offspring per litter was adjusted on the third day of lactation in order to avoid changes in the volume of milk production, as recommended by Plagemann et al. (1999). Preferentially, male offspring were maintained in litters to avoid interferences in the reproductive cycle. Both groups of dams and offspring were evaluated at three stages of lactation; on the 7th, 14th and 21st days, as shown in Flowchart.

BODY WEIGHT AND FOOD INTAKE

The body weight of dams and offspring and the dam's food intake were monitored during the lactation period, on alternate days until the day of euthanasia for each group.

MILK ANALYSIS

Dams were fasted for 8 hours without water restriction before milk collection. Pups were separated from their dams 4 hours before milking in order to accumulate milk in the MG. The pups were kept in boxes, these boxes were close to the dam's boxes. Thereafter, dams were anesthetized by intraperitoneal injections of a ketamine (100mg/kg) and xylazine (2mg/kg) mixture (v/v). Immediately after anesthetic induction, all female rats received a dose of oxytocin (20 IU/kg) via intraperitoneal to stimulate milk ejection and then the manual milking of the rat's breasts was initiated in accordance with the protocol adapted by Leite et al. (2007). The collected milk was stored in sterile micro tubes and immediately frozen at -20°C. Afterwards, the milk samples were used for biochemical measurements of glucose, triglycerides and total proteins by spectrophotometry in an automated analyzer MINDRAY model BS-120 (Shenzhen Mindray Bio-Medical Electronics Co., Nanshan, Shenzhen, China), using reagents for quantitative determination ANALISA (Gold Analisa Diagnóstica Ltda., Belo Horizonte, Minas Gerais, Brazil), following the manufacturer's instructions. In addition, 75μL of milk were also submitted to the analytical technique of creamatocrit, in which the fat content (%) and energetic value (Kcal/L) of the milk were calculated according to Lucas et al. (1978).

SERUM ANALYSIS

The euthanasia method used was guillotine decapitation, the use of anesthesic changes the glycemic profile (De Oliveira et al. 2013) and to perform this work the glycemic data are relevant. After euthanasia, blood from dams and offspring was collected in sterile tubes and immediately centrifuged. Serum samples obtained from centrifugation were used for biochemical measurements of glucose, triglycerides and total proteins by the same method and using the same reagents as the biochemical milk dosage described above.

BIOMETRIC PARAMETERS

The measurement of the naso-anal length (cm) and tissues and organs removal were performed immediately after euthanasia. The naso-anal lengths and body weights were used to calculate the Lee Index (body weight [g]$^{1/3}$/ naso-anal length [cm] x 1000), which is an indicator of obesity in rodents (4). For dams, the liver, pancreas, BAT from interscapular region, ovarian and retroperitoneal WAT depots were removed and weighed, while the pancreas and BAT from interscapular region of pups were also removed and weighed.

HISTOLOGICAL PARAMETERS

A histological technique was performed for qualitative and quantitative analysis of the liver, pancreas, right anterior MG and BAT of the dams, and of the pancreas and BAT of the pups. Two microscope slides were made for each piece of tissue or organ, each one with 3 semi-serial sections in a thickness of 5 μm per section. After

preparation, a digital camera (OLYMPUS C-7070) under an optical microscope (OLYMPUS CX41) photographed the slides. IMAGE J software (version 1.44) was employed for image analysis. In the pancreas, histological analyzes were performed and the number and area of the pancreatic islets were calculated by field (area 103.3 mm^2). In the liver, hepatocyte nuclei were counted and qualitative analysis of lipid infiltration in the hepatic tissue was performed. In the IBAT, the adipocyte nuclei were quantified and the qualitative analysis of the profile of the fat vesicles dispersed in the cytosol was performed. Lastly, the adipocyte count and qualitative analysis of the alveoli in MG were performed.

STATISTICAL ANALYSIS

Data are assessed for normality by Kolmogov-Smimov test and expressed as mean ± standard error of the mean (SEM). Statistical differences between groups of means were evaluated using two-way analysis of variance (ANOVA), with Fisher's LSD pos test. The criterion of significance used was p value <0.05 in analyzes. The GRAPHPAD PRISM 7.00 software was used to perform statistical analysis and graph design.

RESULTS

Food intake was significantly lower in SL-dams, compared to NL-dams between the 7th to 21st days of lactation (Figure 1a); an effect influenced by lactation phase [$F_{(2,52)}$=5.28 p<0.0082], offspring size [$F_{(1,52)}$=14.42 p<0.0082].

The maternal retroperitoneal fat depot was affected by lactation phase [$F_{(2,53)}$=4.743 p<0.0127]. Thus, in NL-dams the retroperitoneal content was significantly reduced from 7th at 21st days of lactation (p<0.001). This effect was not observe in SL-dams. However, litter size also influenced maternal retroperitoneal fat content [$F_{(1,53)}$=13.7 p<0.0006]; thus, at the 14th (p<0.0001) and the 21st (p<0.0001) days of lactation the SL-dams displayed greater retroperitoneal content in relation to NL-dams (Figure 1b). Lactation days also influenced maternal ovarian fat depot [$F_{(2,53)}$=13.7 p<0.0001]. Thus, ovarian fat depot was higher at 7th day in relation to 21st day of lactation in both experimental maternal groups (p<0.0001 and p<0.0162, respectively). In NL-dams ovarian fat depot also was significantly smaller at 14th day in relation at 7th day of lactation (p<0.0039). The litter size no influence ovarian fat depot content [$F_{(1,53)}$=0.02031 p<0.8872]. The maternal glycemia was influenced by litter size [$F_{(1,42)}$=9.199 p<0.0041] and by interaction, litter size and lactation days [$F_{(2,42)}$=3.908 p<0.0278] . Thus, in SL-dams groups was found higher glucose plasmatic levels at 14th and 21st days of lactation in relation to 7th day of lactation (p<0.0100 and p<0.0042, respectively). In addition, SL-dams presented higher glucose plasmatic levels compared to NL-dams at 14th (p<0.0033) and 21st (p<0.0100) of lactation. In contrast, neither litter size [$F_{(2,54)}$=0.1834 p=0.8329] no lactation days [$F_{(2,40)}$=0.1989 p=0.8204] influenced maternal plasmatic levels of triglycerides and protein (Figure 1e).

The impact of litter size manipulation on the morphology of MG is shown in Figure 2. The MG from NL-dams possesses alveoli that are significantly dilated by milk, with scarce interlobular connective tissue dispersed in the stroma (Figure 2a, b and c). In contrast, in MG from SL-dams was observed apparent reduction in alveolar area, greater adipocytes content and alveolus with irregular aspects (Figure 2d, e and f). The quantitative analysis of adipose tissue content in MG reveled that number of adipocytes was affected only by litter size [$F_{(1, 53)}$=158.2 p<0.0001]. Thus, in all lactation stages, the number of adipocyte was approximately five times greater in SL-dams than in NL-dams (Figure 2g; p< 0.05). Table I shows the composition and calories of the milk from dams at different lactation stages. The energetic value,

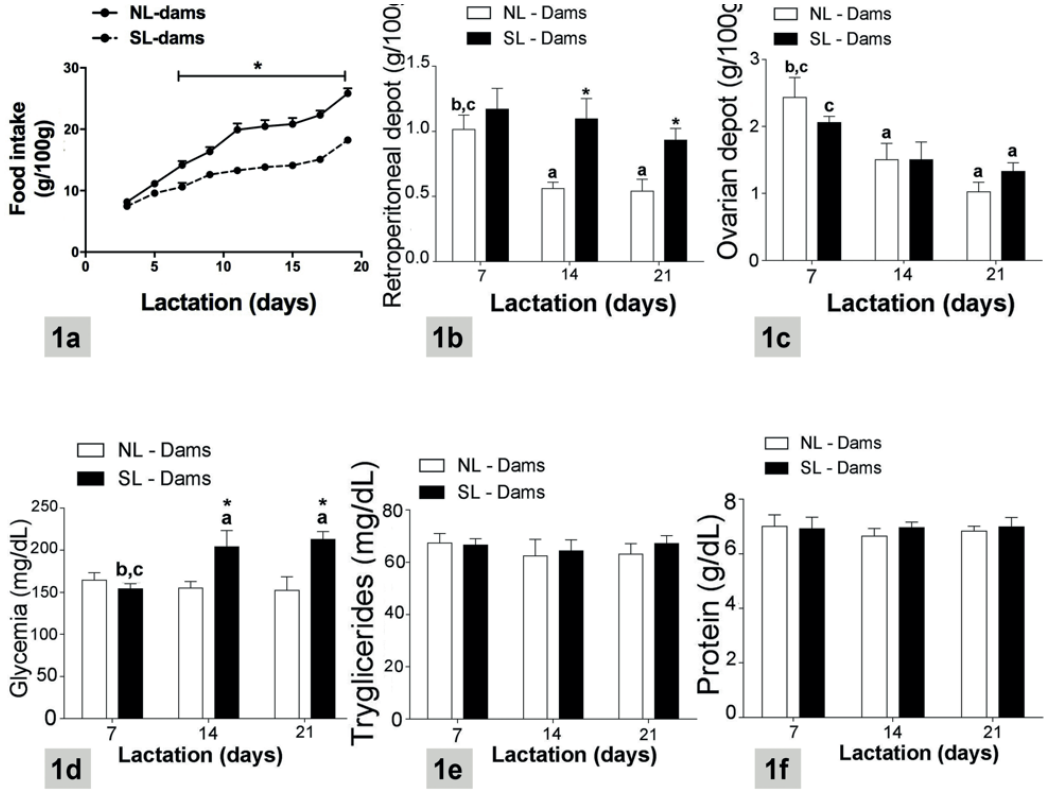


Figure 1 - Biometric, serum parameters and WAT content in lactating SL-dams and NL-dams during different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 9-12 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: *SL versus NL; a 7th day of lactation; b 14th day of lactation and c 21st day of lactation).

fat content and triglycerides content in milk were influenced only by litter size [$F_{(1,42)}$=34.9 $p<0.0001$; $F_{(1,42)}$=35.16 $p<0.0001$ and $F_{(1,37)}$=4.575 $p=0.0391$], respectively. Thus, higher energetic value and fat content were found in milk from SL-dams from 7th at 21st day of lactation in relation to milk from NL-dams. Moreover, at 7th day of lactation the milk from SL-dams presented high triglycerides content in relation to milk from NL-dams in same phase ($p<0.0046$). In contrast, the content of glucose and proteins in the milk were no affected, neither by litter size nor lactation phases, in both experimental groups.

The histological aspects of maternal BAT are shown in Figure 3. Irrespective to litter size, lipid accumulation in BAT of dams progressively increased from the 4th to 21st days of lactation (Figure 3a-c and d-f). However, this effect appears be more evident in BAT from NL-dams. Thus, adipocytes in the BAT from NL-dams have a greater lipid content, characterized by larger fat droplets in the cytosol, compared to SL-dams, at all stages of lactation. The weight of BAT was influenced by lactation phases [$F_{(2,52)}$=4.761 $p<0.0126$]; thus, in SL-dams was observed significantly reduction in weight of BAT on the 14th and 21st day, in relation to 7th day of lactation (Figure 3g; $p< 0.05$). However, neither lactation phases [$F_{(2, 46)}$=0.01763 $p=0.9825$] nor litter size [$F_{(1,46)}$=1.188 $p=0.2814$] significantly affected nucleus number in both dams groups (Figure 3g and h).

The liver weight was influenced only by litter size [$F_{(1,52)}$=23.67 $p<0.0001$); thus liver weight was significantly lower in SL-dams, compared

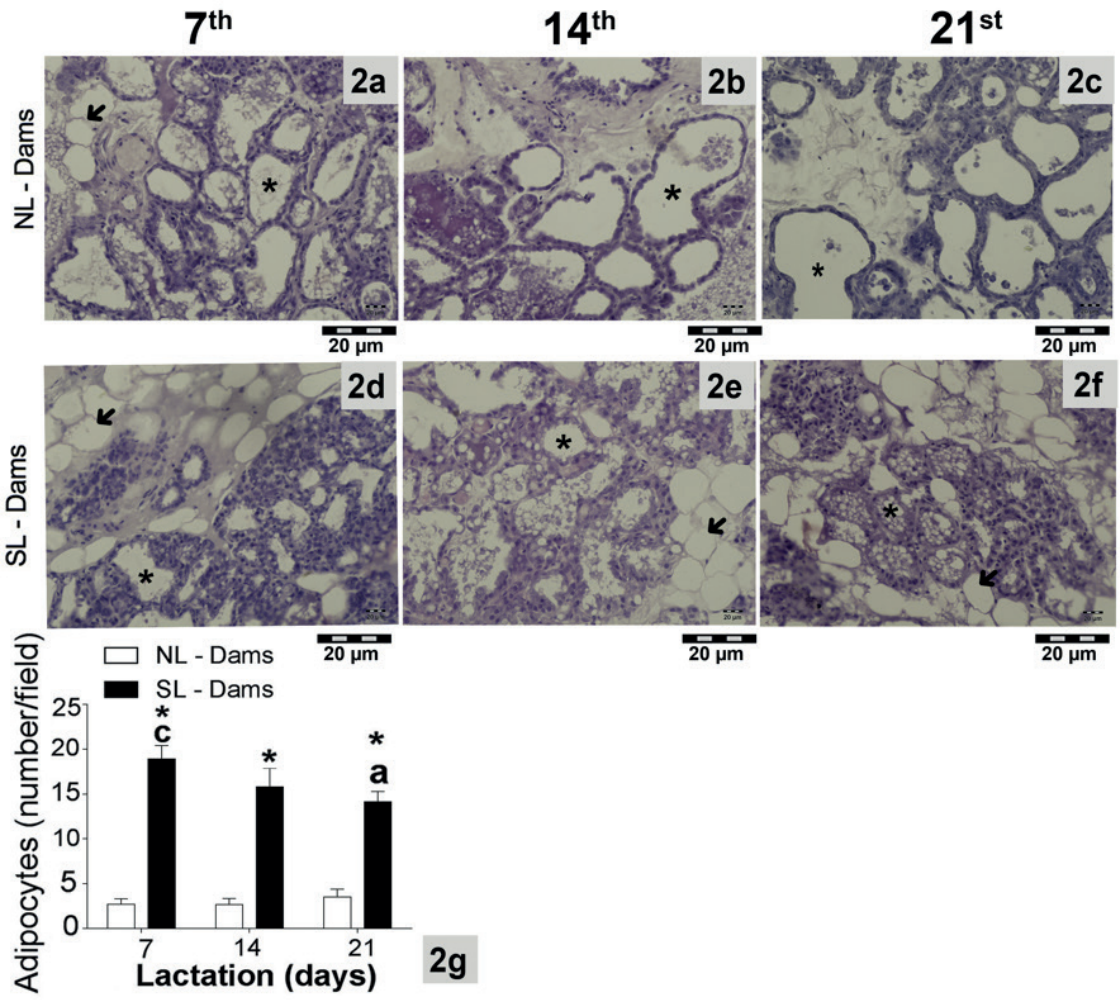


Figure 2 - Histological analysis of the mammary gland from lactating SL-dams and NL-dams during different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 9-11 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: *SL versus NL; a 7th day of lactation and c 21st day of lactation).

to NL-dams, in all lactation phases (Figure 4g; $p< 0.05$). Moreover, hepatocytes nucleus number was influenced by litter size [$F_{(1,53)}=70.75$ $p<0.0001$] and lactation phases [$F_{(2,53)}=10.91$ $p<0.0001$]; thus, in SL and NL-dams the hepatocytes nucleus number were higher at 7th days in relation to 14th ($p<0.0001$) and 21st ($p<0.0047$) days of lactation (Figure 4h). Qualitatively we no observed difference in the hepatic lipid content between dams groups (Figure 4).

The litter size have effect on pancreas number and islets area (Figure 5b and c); thus at 7th and 14th days of lactation SL-dams presented reduction in islets area ($p=0.007$) and number ($p=0.007$) in relation to pancreas from NL-dams in same lactation phases.

The biometric and metabolic profile of offspring during lactation is shown in Figure 6. At day 14 of lactation, SL-offspring displayed increased body weight, compared to NL-offspring; this difference increased throughout lactation (Figure 6a; $p< 0.05$). The naso-anal length was significantly influenced by litter size [$F_{(1,156)}=34.96$ $p<0.0001$], lactation phases [$F_{(2,156)}=1282$ $p<0.0001$]. Thus, naso-anal length progressively increased throughout of lactation

TABLE I
Milk composition during different lactation phases.

Lactation (days)	7th		14th		21st		p-value lactation days	p-value litter size	p-value interaction
	NL-dams	SL-dams	NL-dams	SL-dams	NL-dams	SL-dams			
Glucose (mg/dL)	209.89 ± 15.77	232.00 ± 21.90	234.67 ± 23.20	194.33 ± 22.30	247.25 ± 31.21	202.00 ± 17.71	0.9826	0.3774	0.3170
Triglycerides (mg/dL)	1802.63 ± 48.37	2258.00* ± 110.08	1957.71 ± 135.96	2033.14 ± 92.80	2046.29 ± 112.73	2086.57 ± 140.67	0.8134	0.0391	0.1167
Total Protein (g/dL)	17.70 ± 2.26	18.48 ± 2.58	21.80 ± 1.81	19.59 ± 1.12	24.00 ± 1.27	20.39* ± 0.80	0.2429	0.0765	0.4436
Fat Content (%)	10.22 ± 0.71	13.29* ± 0.97	10.25 ± 0.42	12.99* ± 0.61	10.23 ± 0.70	14.70* ± 0.69	0.4673	<0.0001	0.4568
Energetic Value (Kcal/L)	1326.00 ± 69.11	1624.00* ± 95.03	1329.00 ± 40.13	1594.00* ± 59.08	1328.00 ± 68.17	1761.00* ± 67.45	0.4633	<0.0001	0.4578

The values are expressed as mean ± SEM. The sign (*) indicates a significant difference ($p<0.05$) between the groups of the same lactation stage, by ANOVA two way with Fisher's LSD pos test (NL = Normal Litter x SL = Small Litter); n = 6 to 12 per group.

(7th at 21st day) in Offspring-SL and NL-offspring (Figure 6b). Moreover, Offspring-SL presented higher naso-anal length in relation to Offspring-NL in all stages of lactation ($p<0.0001$). The Lee index was influenced by litter size [$F_{(2,158)}=9.915$] and lactation phases [$F_{(1,158)}=15.26$ $p<0.0001$). Thus, in both offspring groups was observed higher Lee Index at 7th in relation to 21st day of lactation. In addition, in Offspring-NL group the Lee Index was significantly smaller at 21st day compared with 14th day of lactation ($p<0.0004$). Offspring-SL showed higher Lee Index in relation to Offspring-NL only at 21st days of lactation ($p<0.0001$).

Plasma glucose level was affected by litter size [$F_{(1,83)}=14.1$ $p<0.0003$] and lactation phases [$F_{(2,83)}=10.73$ $p<0.0001$]; Figure 6d. Thus, in SL and NL offspring groups, the glucose plasmatic level was significantly higher at 21st day in relation at 7th day of lactation ($p<0.0001$ and $p<0.0341$, respectively). In Offspring-NL glucose plasmatic level at 21st day also was higher in relation than 14th day of lactation ($p<0.0095$). Moreover, Offspring-SL showed increased glucose plasmatic level at 7th and 14th day of lactation compared to Offspring-NL group ($p<0.0031$ and $p<0.0102$; respectively). The plasmatic level of triglycerides was influenced by interaction effect [$F_{(2,78)}=4.333$ $p=0.0164$] and by lactation phases[$F_{(2,78)}=47.97$ $p<0.0001$]. Thus, Offspring-NL and Offspring-SL presented smaller triglycerides plasmatic level at 7th day in relation to 21st day of lactation ($p<0.0001$ and $p<0.0001$). In Offspring-NL the plasmatic level of triglycerides

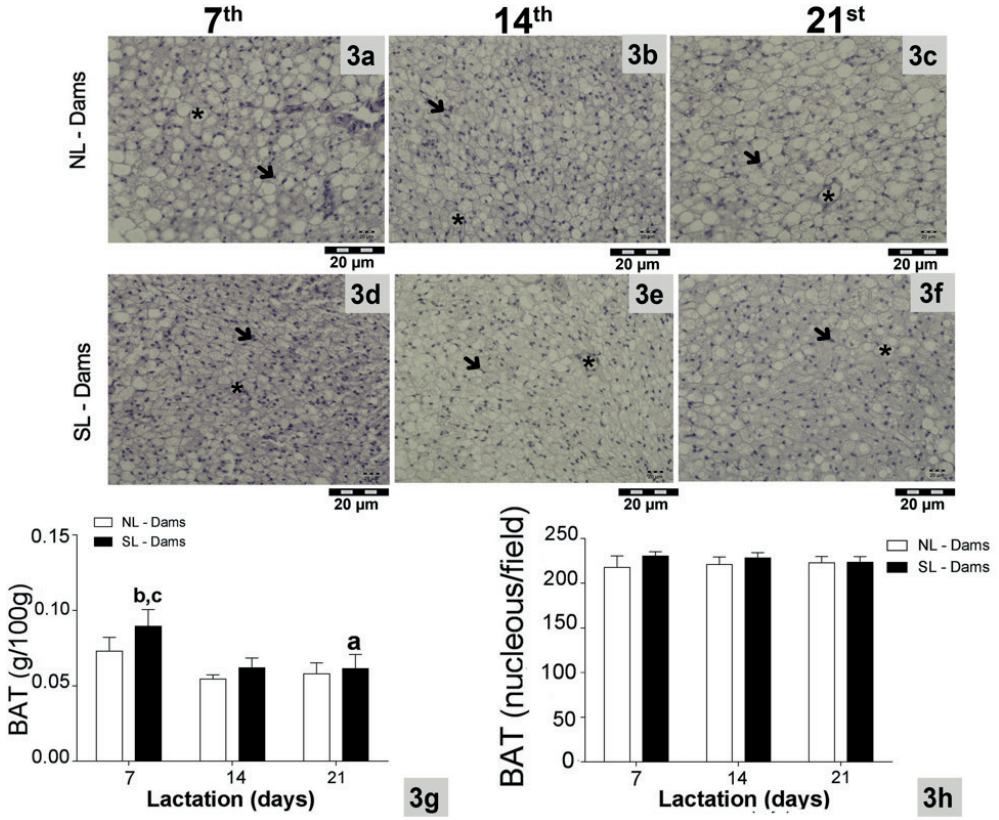


Figure 3 - Histological analysis of the BAT from lactating SL-dams and NL-dams during different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 8-12 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: a 7th day of lactation; b 14th day of lactation and c 21st day of lactation). The arrows in the image indicate the adipocyte nucleoli and the asterisks in the image indicate the adipocytes.

also was reduced at 14th day in relation to 7th day of lactation ($p<0.0028$). Similar reduction in triglycerides plasmatic level was found in Offspring-SL from 14th to 21st day of lactation ($p<0.0001$). Only at 14th day of lactation Offspring-SL presented higher triglycerides plasmatic level compared with Offspring-NL ($p<0.0046$; Figure 6e). The protein plasma level was influenced by lactation days [$F_{(2,65)}=15.65$ $p<0.0001$] and litter size [$F_{(1,65)}=14.96$ $p=0.0003$]. Thus, in both Offspring groups protein plasma level was augmented at 7th day in relation at 14th day of lactation ($p<0.0001$ and $p<0.0012$). In Offspring-NL rats protein plasma level also was higher at 7th day in relation to 21st day of lactation ($p<0.0003$). In addition, at 14th and 21st day of lactation the protein plasma level was significantly higher in Offspring-SL compared to Offspring-NL group ($p<0.0384$ and $p<0.011$; respectively).

The weight and histological aspects of BAT of offspring are represented in Figure 7. The BAT weight was affect only by litter size [$F_{(1,180)}=22.32$ $p<0.0001$]; thus Offspring-SL presented higher BAT weight at 7th and 14th day of lactation in relation to Offspring-NL in same lactation phases ($p<0.0009$ and $p<0.0019$; respectively). In Offspring-SL rats the weight of BAT was significantly higher at 21st day in relation to 7th day of lactation ($p<0.0180$; Figure 7g). The nucleus number in BAT was influenced only by lactation phase [$F_{(2,52)}=14.49$ $p<0.0001$]; thus in both Offspring groups was observed higher nucleus number at 7th days in relation to 21st day of lactation ($p<0.0186$ and $p<0.0001$; respectively). In

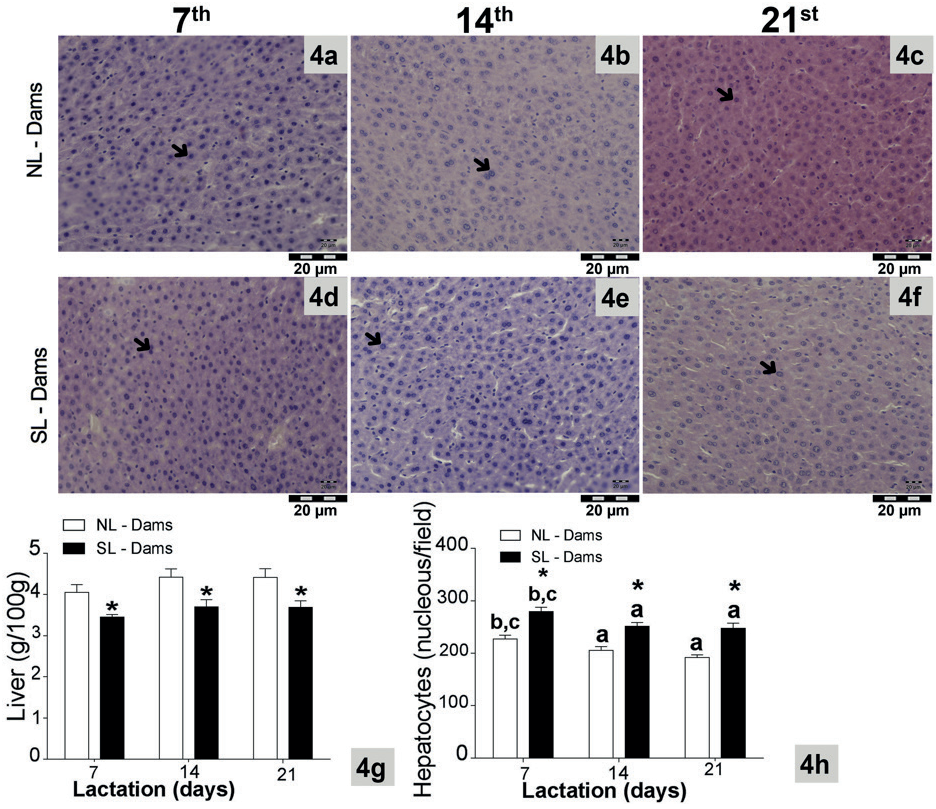


Figure 4 - Histological hepatic analysis from lactating SL-dams and NL-dams during different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 9-12 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: *SL versus NL; a 7th day of lactation; b 14th day of lactation and c 21st day of lactation). The arrows in the image indicate the hepatocytes.

addition, at 21st day of lactation the nucleus number was significantly smaller in BAT from Offspring-SL compared to Offspring-NL ($p<0.0388$; Figure 7h). By descriptive qualitative analysis was possible observe that BAT from Offspring-SL displayed augmented lipid deposition, characterized by greater fat droplets in the cytoplasm, compared to BAT from Offspring-NL (Figure 7a-f), an event progressively increased throughout lactation.

The pancreas weight was influenced by litter size [$F_{(1,183)}$=8.7,14 $p<0.0036$] and lactation phases [$F_{(2,183)}$=39.14 $p<0.0001$]. Thus, at 21st day of lactation the weight of pancreas was significantly higher in Offspring-SL in relation to Offspring-NL ($p<0.0275$). In addition, in both Offspring groups the weight of pancreas progressively augmented of 7th at 21st day of lactation ($p<0.0001$ and $p<0.0001$; respectively) without alter islets area (Figure 8g). In contrast, the nucleus number in pancreatic islets was affect only by litter size [$F_{(1,58)}$=34.42 $p<0.0001$]; thus at 14th and 21st day of lactation, the number of nucleus in pancreatic islets was significantly higher in Offspring-SL in relation to pancreas from Offspring-NL group ($p<0.0001$ and $p<0.0018$, respectively).

DISCUSSION

To sustain milk synthesis and nourish the offspring, the maternal metabolism of dams adapts to the specific demands of lactation; thus, during this period, food consumption increases two to three fold, compared to that of non-lactating dams

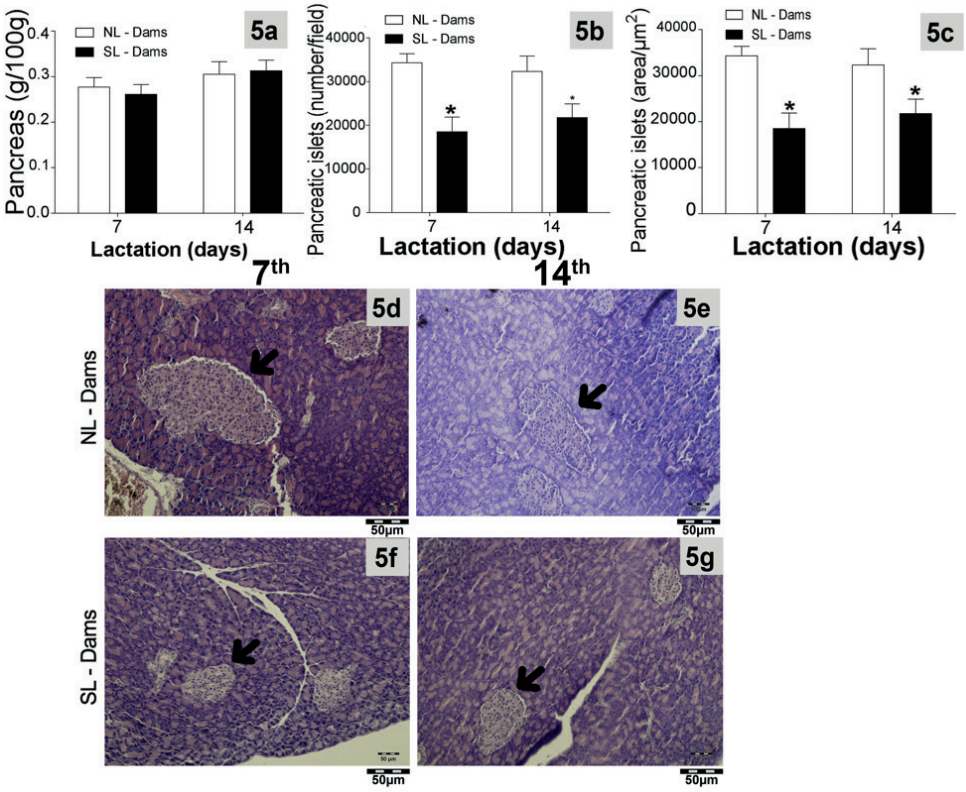


Figure 5 - Histological analysis of the endocrine pancreas from lactating SL-dams and NL-dams during different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 9-12 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: *SL versus NL; a 7th day of lactation; b 14th day of lactation). The arrows in the image indicate the pancreatic islets.

(Abizaid et al. 2008). Metabolic homeostasis depends on mechanisms that control food intake and energy expenditure, and both processes are regulated by hypothalamic neurons that are activated by peripheral adiposity signals, such as leptin (Wang et al. 2011). Hyperphagia in lactating rats is associated with hypoleptinemia (Brogan et al. 1999, Denis et al. 2003). In our study, SL-dams presented a reduced food intake and higher retroperitoneal fat depot during lactation, compared to NL-dams; suggesting altered secretion or action of leptin in dams nursing litter size. Moreover, our data also demonstrated that SL-dams presented hyperglycemia in relation to NL-dams. Maternal obesity and hyperglycemia during lactation are conditions that favors metabolic programming in offspring (Desai et al. 2014). Corroborating this hypothesis, in the present study Offspring-SL showed disruption in glucose homeostasis, higher body weight and obesity during lactation.

An adjustment in lipid metabolism from WAT is necessary to maintain milk synthesis throughout lactation (Barber et al. 1992). Thus, as consequence of the decreased rate of fatty acid synthesis and a concomitant increased lipolysis WAT frequently present reduction during lactation (Vernon and Flint 1983, Williamson 1980). Corroborating these findings, in our study, NL-dams displayed a significant reduction in retroperitoneal and ovarian fat depots at long of the lactation. Moreover, independently of litter size manipulation, the ovarian fat depot was preferentially mobilized at long of lactation; suggesting that this depot is more sensitive to lipolysis in this phase.

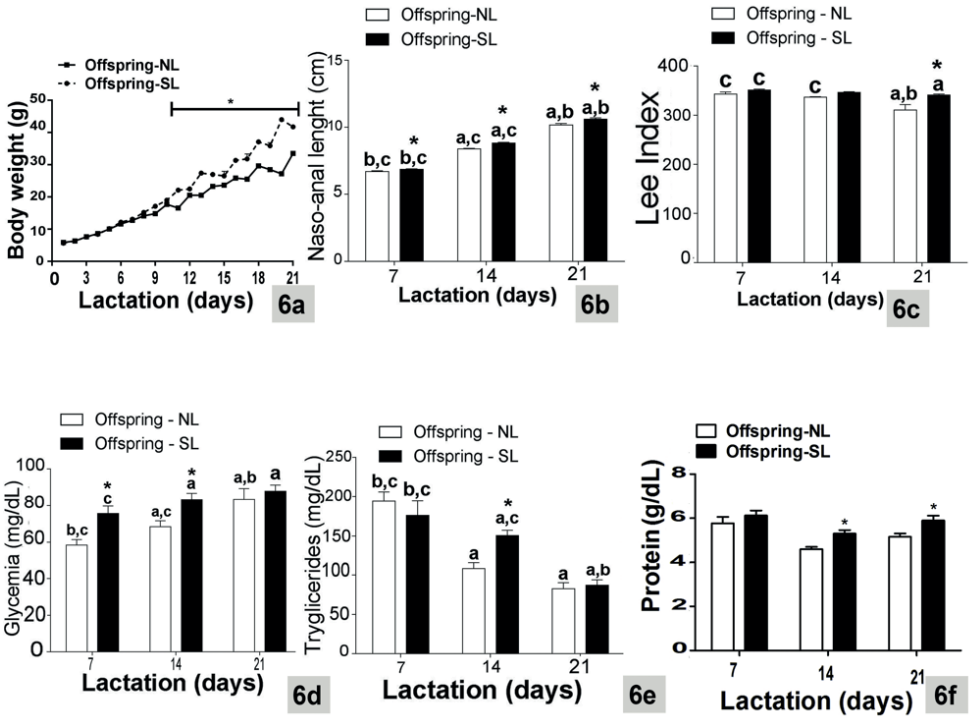


Figure 6 - Biometric parameters and blood biomarkers from SL-offspring and NL-offspring during different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 9-12 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: *SL versus NL; a 7th day of lactation; b 14th day of lactation and c 21st day of lactation).

The WAT of MG is considered as an extra-parenchymal tissue, which is enlarged during the early fetal and postnatal development phases, decreasing drastically during lactation (Denis et al. 2003). The histological analysis in our study showed that, MG from lactating SL-dams had higher WAT content and a concomitant reduction in alveoli structures, suggesting reduced MG activity in this group (Lang et al. 2012). In addition, as mentioned by Fiorotto et al. (1991), a decrease in suckling stimulus reduces milk secretion favoring the involution of the MG. Despite the histological involution of the MG of SL-dams, their milk displayed a higher caloric and greater fat content, compared to NL-dams. Triglycerides make up 98% of the lipid content of milk in many mammal species, including rodents (Neville and Picciano 1997). Triglycerides were found increased in the milk of SL-dams only on the 7th day of lactation, suggesting that other types of lipids could be augmented, along the lactation in this group. The profile of maternal diet, as well as, body fat maternal composition can influence milk lipid content (Neville and Picciano 1997). Taken together, these data indicate that the higher fat mass and hyperglycemia, found in SL-dams, could contribute to the increase in fat content in their milk. Interestingly, at 21st day of lactation, the milk from SL-dams also presented reduced protein levels. Results presented herein are in agreement with earlier research suggesting that litter manipulation modulates milk composition in lactating dams (Mozeš et al. 2014).

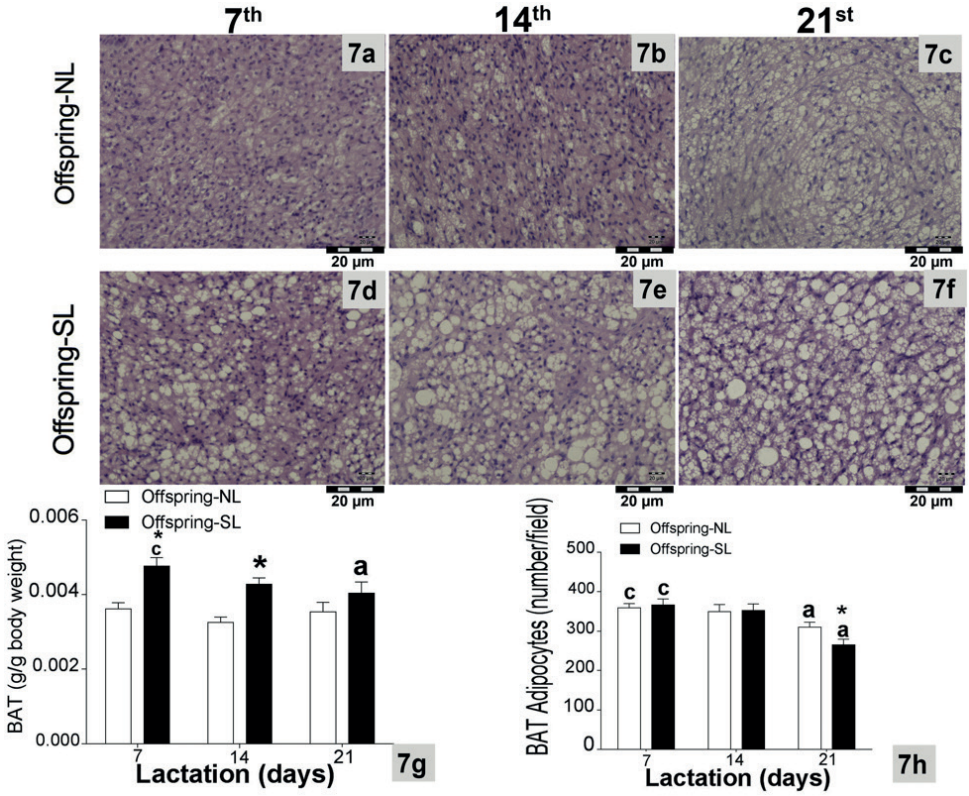


Figure 7 - Histological analysis of the BAT from SL-offspring and NL-offspring in different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 9-12 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: *SL versus NL; a 7th day of lactation; b 14th day of lactation and c 21st day of lactation).

Reduction in thermogenic BAT activity is frequently observed in lactating dams in relation to non-lactating dams; an event necessary to milk synthesis (Król et al. 2011). Study done by Isler et al. (1994) showed that BAT activity is altered by litter size. Corroborating with this findings, our study demonstrated that SL-dams present a lower lipid deposition in BAT, at all lactation stages, suggesting elevated thermogenic activity, compared to the BAT of NL-dams nursing. As demonstrated by our data, lactating SL-dams also presented a lower liver weight, despite of greater nucleus count, in relation to liver from NL-dams. Similarly, maternal high fat diet induces a reduction in weight of liver as well as, affects hepatic cell proliferation; both events are associated with changes in hepatic lipid metabolism (Martín-Hidalgo et al. 2005). It is possible that the greater maternal hyperglycemia and WAT content of SL-dams favors hepatic lipogenic activity, contributing to a higher fat content in the milk, as observed here. Further studies, exploring gluconeogenesis and lipogenic activity in dams nursing SL-offspring, are necessary to clarify the contribution of hepatic metabolism to the milk composition.

Increased glucose and lipid requirements, necessary to sustain the demand of lactation, are hormonally driven (Martín-Hidalgo et al. 2005). In this regard, lactating dams present lower insulin levels, an event associated with catabolic processes, such as lipolysis in WAT (Neville and Picciano 1997). Insulin modulates secretory differentiation,

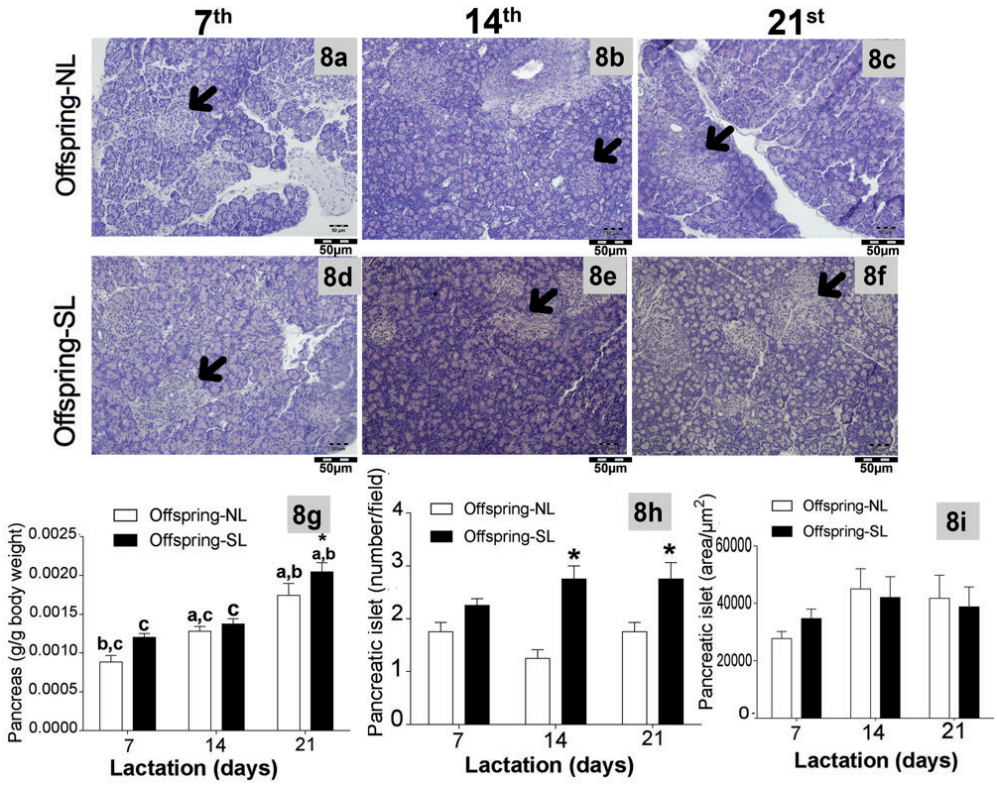


Figure 8 - Histological analysis of the endocrine pancreas from SL-offspring and NL-offspring in different lactational phases. The values are expressed as mean ± Standard Error of the Mean (n= 9-12 animals/group). The symbol above bars indicates a significant difference ($p<0.05$) between the groups (Two-way ANOVA with uncorrected Fisher´s LDS: *SL versus NL; a 7th day of lactation; b 14th day of lactation and c 21st day of lactation). The arrows in the image indicate the pancreatic islets.

secretory activation, and mature milk production in the MG (Nommsen-Rivers 2016). Thus, during lactation, pancreatic islets undergo important structural and secretory adjustments; in particular, the pancreas of lactating dams lose β-cell mass and display a reduced glucose-induced insulin secretion, compared to non-lactating dams (Lellis-Santos et al. 2012). Our results demonstrated that SL-dams presents reduction in islet size on the 7th and 14th days of lactation, compared to the NL-dam. This event persistent at 21st days of lactation, as recently confirmed by our research group (Cancian et al. 2016). Reduction in islets area suggests a decrease in pancreatic function. Prolactin is essential for lactogenesis, having hypertrophic effect on the endocrine pancreas (Grattan et al. 2008). In this regard, the magnitude of prolactin released by lactating dams is regulated by litter size, suckling frequency and intensity (Grattan et al. 2008). Thus, it is probable that in SL-dams occurs a reduction in suckling stimuli, resulting in a decrease in prolactin secretion culminating with lower proliferative effects on the endocrine pancreas.

In our study offspring reared in SL displayed augmented body weight, growth, obesity, hyperglycemia, and hyperproteinemia during lactation. Furthermore, the increase in body fat stores, observed in SL rats prior to weaning, might be mainly attributed to changes in the composition of the milk macronutrients ingested up to 10th day postpartum (Howie et al. 2009). Higher energy and milk fat concentrations were documented in dams nursing small litter, and their consumption was substantially higher in their pups (Cunha et al.

2009, Fiorotto et al. 1991). Interestingly, in both offspring we observed increases in glycaemia and reductions in triglyceridemia, suggesting changes in the metabolism of pups at long of lactation. These events are probably due to the suckling–weaning transition, which is associated with a drastic nutritional shift in which fat-enriched maternal milk is replaced by a carbohydrate-rich diet (Neville and Picciano 1997).

Maternal obesity can modify BAT thermogenic activity in offspring (Zhao and Cao 2009). We observed that pups reared in SL have greater lipid droplets in their BAT adipocytes, suggesting a premature reduction in BAT thermogenesis, during suckling. In contrast, offspring reared in SL have augmented BAT thermogenesis at pre-weaning (21 days of lactation) but reduced BAT thermogenesis during adult life (Zhao 2011).

Finally, the suckling period consists of a critical postnatal window for structural and physiologic maturation of the endocrine pancreas, where lactational programming could be more marked. Indeed, in newborn infants an immature beta cell phenotype, characterized by a reduced glucose-induced insulin secretion associated with higher proliferation rates of islet cells (Cerf 2015). We observed that offspring, reared in SL, presented increased islet numbers, indicating that lactational hypernutrition, can stimulates the proliferation of pancreatic islets altering glucose-induce insulin secretion. Interestingly, islets from Offspring-SL secreted less insulin in response to glucose at 26 days of life, compared to islets from Offspring-NL (Waterland and Garza 2002).

CONCLUSIONS

The reductions in litter size during lactation stages promote precocious maternal metabolic and histological adjusts, characterized by high body fat mass accumulation in WAT and BAT, reduced MG development, and higher fat content in milk. These maternal metabolic conditions have impact in the growth and metabolism of offspring, as shown by high body weight, greater lipid accumulation in BAT, hyperglycemia and augmented islet proliferation, supporting early programming effects. These findings reinforce the importance of preserving maternal metabolic state during lactation, avoiding early disruption in energy homeostasis in offspring, which may predispose to disease in adulthood.

AUTHOR CONTRIBUTIONS

JL: manipulation of the animals, obtaining the samples, conducting the analyzes and preparation of the manuscript. D: obtaining the samples and preparation of the manuscript. C: manipulation of the animals and obtaining the samples. P: manipulation of the animals, conducting the analyzes and obtaining the samples. M, J, F, Makcine: manipulation of the animals and obtaining the samples. F: graphic development of images. G: preparation of the manuscript. S: performance of statistical tests and preparation of the manuscript.

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