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Effects of maternal protein restriction during pregnancy and lactation on milk composition and offspring development

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Abstract

Before weaning, breast milk is the physiological form of neonatal nutrition, providing pups with all nutrient requirements. Maternal low-protein diet (LPD) during pregnancy and lactation induces adverse changes in key maternal organs, which have negative effects on pup development. We studied the effects of maternal LPD on liver weight, mammary gland (MG) cell differentiation, milk composition and production and pup development throughout lactation. We fed rats with control (C) or LPD (R) during pregnancy and lactation. At 7 d early, 14 d mid and 21 d late lactation stages, maternal biochemical parameters, body, liver and MG weights were analysed. MG cell differentiation was analysed by haematoxylin and eosin staining; milk nutrient composition and production were studied; pup body, liver and brain weights, hippocampal arachidonic acid (AA) and DHA were quantified. Results showed lower body and liver weights, minor MG cell differentiation and lower serum insulin and TAG in R compared with C. R milk contained less protein and higher AA at early and mid stages compared with C. R pup milk and fat intake were lower at all stages. R protein intake at early and mid stages and DHA intake at mid and late stages were lower compared with C. In R pups, lower body, liver and brain weights were associated with decreased hippocampal AA and DHA. We conclude that maternal LPD impairs liver and MG function and induces significant changes in maternal milk composition, pup milk intake and organ development.

Key words: **Maternal undernutrition: Mammary glands: Milk production: Brain: Hippocampus: Neonatal growth**

Maternal milk is widely considered the best feeding source for newborns compared with the alternatives[1–3], even in the setting of maternal undernutrition during pregnancy and lactation, which not only affects maternal health but negatively programmes neonatal growth and organ development and maturation[4–8]. Reduced maternal nutrition is the most extensively studied programming challenge of offspring phenotypic plasticity[9]. Developmental programming is defined as the response to a specific challenge to the mammalian organism during a critical developmental time window that alters the trajectory of development with resulting effects on health that persist throughout life[10,11]. The implications of maternal undernutrition on milk quality and their consequences on maternal neonatal growth and development, to date, have been poorly studied.

During the early stages of lactation (0–7 days of lactation, dL) pups cannot synthesise many key metabolites. These must be provided in the maternal milk[12]. Among the best known examples are the long-chain (LC)-PUFA, especially arachidonic acid (AA) and DHA, which are essential for offspring metabolic functions, for example, in liver and brain maturation[13,14]. This need for LC-PUFA is particularly marked in the hippocampus, a major centre of behavioural control and cognitive function[15–17]. By mid lactation (8–14 dL), maximal milk production is associated with changes in maternal metabolism, hormone synthesis and physiological adaptations[18]. During this stage, pup growth increases dramatically and organ maturation accelerates[7]. If nutrition is adequate, then the neonate has been prepared for an independent existence by the end of lactation (21 dL)[19–21].

Abbreviations: AA, arachidonic acid; C, control group; dL, days of lactation; HOMA, homeostatic model assessment; LC, long-chain; LPD, low-protein diet; MG, mammary gland; R, restricted group.

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We have shown that maternal low-protein diet (LPD) during pregnancy impairs maternal liver fat composition[22] and mammary gland (MG) development and function at the end of pregnancy[4], accompanied by decreased maternal and pup LC-PUFA in both organs. Previous publications have reported nutrient content[23], milk intake[24,25] or pup body weight gain[19,26] using the LPD model in rats. In the present study we evaluated the effects of maternal LPD on maternal liver metabolism, MG development and milk nutrient composition and production, and their consequences on pup milk nutrient intake and development at different lactation stages throughout lactation in rats. We hypothesised that maternal LPD during pregnancy and lactation results in maternal metabolic adaptations that lead to early programming of dysfuction in offspring organ development.

Methods

Care and maintenance of animals

All procedures were approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ, BRE-105). Female albino Wistar rats approximately 120 d of age and weighing 200–240 g were assigned either to a control group (C: 20 % protein; *n* 21; seven animals per each stage of lactation) in which mothers ate control diet during pregnancy and lactation; or a restricted group (R: 10 % protein; *n* 18; six animals at each of the three stages studied) in which mothers ate a protein-restricted diet during pregnancy and lactation[7]. Water and food were provided *ad libitum* to all animals. Food was provided in the form of large flat biscuits retained behind a grill through which the food could be nibbled. Complete details of the maternal diet (Table 1), breeding and management have been published[7]. The day of delivery was considered as postnatal day 0 of life. Maternal and pup weights (g) were recorded throughout lactation: at 7 dL (early lactation), 14 dL (mid lactation) and 21 dL (late lactation)[7]. To ensure homogeneity of study subjects, litters >14 pups or <12 pups were not included in the study. Litters of 12–14 pups were adjusted to 12 pups for each mother while maintaining a sex ratio as close as possible to 1:1.

Maternal parameters

Blood collection. C (*n* 7) and R (*n* 6) rats were anaesthesised with isoflourane, followed by rapid decapitation, by personnel experienced in using a rodent guillotine (Thomas Scientific) on 7, 14 and 21 dL. Trunk blood was collected in polyethylene tubes, allowed to clot at 4°C for 1 h, centrifuged at 1500 ***g*** for 15 min at 4°C and serum stored at −20°C until assayed. Serum glucose (mg/dl) and TAG (mg/dl) concentrations were determined enzymatically using a Synchron CX® autoanalyser (Beckman Coulter Co.). Serum insulin concentration (ng/ml) was determined by RIA using commercial rat kits from Linco Research®. Homeostatic model assessment (HOMA) was calculated from baseline values using the formula: HOMA = glucose (mmol/l) × insulin (μU/ml)/22·5[27]. Each serum sample was determined in duplicate. The intra- and interassay coefficients of variation were <4 and <6 %, respectively[7].

Liver and mammary gland analyses. Maternal liver[22] and complete MG chain were excised and weighed[4]. MG beneath the 6th right nipple (counted from the cephalad end) was longitudinally sectioned into two equal parts; one was immediately frozen at −20°C and the other immediately immersion-fixed in 4 % paraformaldehyde in neutral PBS. After 24 h of fixation, tissue sections were dehydrated with ethanol at increasing concentrations from 75 to 95 % and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin.

Mammary gland cell differentiation morphometric analysis. Morphometric analysis was performed on 10 pictures from 3 to 5 sections from each MG, and at least 150 lobules were quantified at 10× magnification. Adipose and parenchymal tissue (%; acinar and ductal epithelium) areas were determined. Fifty acini per animal were measured at a higher magnification (100×), and results expressed as cytoplasm area (μm^2) and nuclei size (μm^2) for cells in each acinus (around 7–15 cells per acinus) with AxioVision® software. All histological measurements were performed by two independent observers without knowledge of the source of the tissues, and results were averaged for each animal[14].

Milk parameters

Nutrient content. Milk samples were collected on 7, 14 and 21 dL. Pups were removed from their mothers for 4 h after which mothers received 0·8 U oxytocin (intraperitoneally), and milk was expressed 15 min later. Milk samples were vortexed and divided into three aliquots of 1·5 ml and immediately frozen at −20°C until analysed. For analysis, milk samples were thawed at 37°C and shaken vigorously before removing assay aliquots to ensure sample uniformity as previously reported[7]. Water content (% of total milk) was analysed by gravimetric analysis[28]. Protein content (%) was measured by Bradford assay (Biorad®). Total fat content (%) was measured by the Folch method[22]. AA and DHA content from milk fat were measured by gas chromatography[4].

Production and pups' milk component intake. Milk production was estimated as described by Russell and our published data[7,29,30]. At 07.00 hours on 6, 13 and 20 dL, pups were removed from their mothers for 4 h during which time mothers had free access to water and ate *ad libitum* (to produce milk), whereas the pups did not eat. Mothers were weighed at the beginning and end of the 4-h period. Pups were weighed individually immediately before they were returned to the mothers and again 1 h later. Pups' milk component intakes (water,

Table 1. Maternal diet composition during pregnancy and lactation.

	Control diet (%)	Restricted diet (%)
Casein	20	10
Cystine	0·3	0·15
Choline	0·165	0·165
Vitamin mix	1	1
Mineral mix	5	5
Cellulose	5	5
Maize oil	5	5
Carbohydrates		
Maize starch	31·76	37·34
Dextrose	31·76	37·34
kcal/g of diet	4	4
kJ/g of diet	16·7	16·7

protein, fat, AA and DHA) were estimated by milk intake (g/h) × milk component (%)/ 100.

Pup parameters

Body, liver and brain weight. Pup body weight (*n* 12 pups per litter per age for both C and R) was registered and liver and brain excised, cleaned and weighed (g) at 7 (early), 14 (middle) and 21 (late) dL. The hippocampus was dissected and immediately frozen and stored at –70°C until use.

Fatty acid analysis. Maternal milk and pup hippocampal lipids were extracted by a modified Folch technique[22]. Briefly, samples were homogenised with 2 ml of 0·9 % NaCl and 5 ml chloroform–methanol (2:1) as previously described[22]. Fatty acid extraction was performed by the addition of chloroform (3 × 2 ml). The organic phase was pooled and 120–150 µl methanol was added until the organic phase turned transparent, then 1 g Na_2SO_4 was added and vortexed to provide the residue for analysis. The organic phase was evaporated under a stream of N_2.

Preparation of fatty acid methyl esters. Samples of fatty acid methyl esters (FAME) were prepared as previously described[22]. Briefly, 2 ml methanol, 100 µl toluene and 40 µl of 2 % methanolic sulfuric acid were added to the above residue and heated at 90°C for 2 h. Tubes were then placed on ice, and 1 ml of 5 % NaCl was added. FAME were extracted with hexane (3 × 2 ml), and the mixture was centrifuged at 1500 ***g*** for 1 min. The organic phase was pooled and evaporated under a stream of N_2. Hexane (200 µl) was added to the residue, which was then centrifuged at 1500 ***g*** for 5 min. The clear solution was injected in an Agilent® model 6850 gas chromatography system equipped with a flame ionisation detector. Automatic split injection was carried out using an Agilent® 6850 auto-sampler. We used an HP-INNO® wax capillary column (30 m, 0·25 mm, 0·25 m; J & W Scientific). Heptadecanoic acid (125 µg) was added to 100 mg of the sample as internal standard. A 1-µl sample was injected in split mode (50:1) at 250°C. The carrier gas was He with a constant linear velocity of 24 cm/s, and the interface temperature was maintained at 280°C. The oven temperature was raised from 50 to 230°C. The identification of FAME was based upon retention times obtained for methyl ester standards from Poly Science®, and each one was expressed as a percentage of total fatty acids in the sample[22].

Statistical analysis

To assess the differences within lactation periods in the same experimental group (C or R) we performed a one-way multiple ANOVA followed by a Tukey test. To compare C and R groups, we performed unpaired Student's *t* tests at the same lactation period. Preliminary analysis revealed no sex-dependent statistical significance by lactational stage, so pup data from the same litter were averaged for analysis. Data are presented as means with their standard errors. Significance was set at $P \leq 0{\cdot}05$.

Results

Maternal parameters

Body weight. Body weight of C mothers increased at 14 dL compared with 7 dL and then remained stable. Body weight of R mothers decreased during lactation. R mothers weighed lower than C at 14 and 21 dL (Fig. 1(a), $P < 0{\cdot}05$).

Glucose, insulin and homeostatic model assessment. Glucose serum levels were constant throughout lactation in C. However, R glucose increased at 21 dL compared with 7 and 14 dL. Glucose serum levels were higher in R compared with C at 21 dL (Fig. 1(b), $P < 0{\cdot}05$). Insulin serum levels were lower at 21 dL compared with 14 and 7 dL in C. In the R group, insulin serum levels decreased during lactation. R serum levels were lower than in C at all stages (Fig. 1(c), $P < 0{\cdot}05$). HOMA was constant throughout lactation in C and was lower at 21 dL compared with 7 dL in R. However, R HOMA was lower compared with C at all stages (Fig. 1(d), $P < 0{\cdot}05$).

TAG. TAG remained constant throughout lactation in C. However, in R, TAG was similar at 7 and 14 dL and increased between 14 and 21 dL. TAG was lower throughout lactation in R compared with C (Fig. 1(e), $P < 0{\cdot}05$).

Liver weight. Liver weight in C was higher at 14 dL compared with 7 and 21 dL. In R, liver weight increased at 14 dL compared with 7 dL and remained constant until 21 dL (Fig. 1(f), $P < 0{\cdot}05$). Lower liver weights were found throughout lactation in R compared with C. Liver weight relative to body weight remained constant throughout lactation in both C and R but was lower in R compared with C (Fig. 1(g), $P < 0{\cdot}05$).

Mammary gland weight. In C, MG weight was higher at 14 dL compared with 7 and 21 dL. In R, MG weight was similar at 7 and 14 dL; at 21 dL, MG weight was lower than at 14 dL. Lower MG weights were found in R throughout lactation compared with C (Fig. 1(h), $P < 0{\cdot}05$). MG weight relative to body weight was lower in both C and R at 21 dL compared with earlier time points. Lower values of MG weights relative to body weights were found throughout lactation in R compared with C (Fig. 1(i), $P < 0{\cdot}05$).

Mammary gland morphometric analysis

Parenchymal tissue. In C, the percentage of parenchymal tissue was similar at 7 and 14 dL but higher than 21 dL. In contrast, R percentage of parenchymal tissue increased during lactation. Lower percentage of parenchymal tissue was present in R than C at 7 and 14 dL. However, at 21 dL, R contained a higher percentage of parenchymal tissue compared with C (Fig. 2(a) and (c), $P < 0{\cdot}05$).

Adipose tissue. The percentage of adipose tissue was similar in 7 and 14 dL and increased between 14 and 21 dL in C. In R, the percentage of adipose tissue decreased during lactation. In R, the percentage of adipose tissue was higher at 7 and 14 dL and lower at 21 dL compared with C (Fig. 2(b) and (c), $P < 0{\cdot}05$).

Cell cytoplasm area. In C, cell cytoplasm area was similar at 7 and 14 dL and higher than 21 dL. In R, cytoplasm area was higher at 21 dL compared with 14 dL. But it was lower at 7 and 14 dL compared with C (Fig. 3(a) and (c) , $P < 0{\cdot}05$).

Nuclear size. Nuclear size decreased at 21 dL compared with 14 dL in C, but remained constant throughout lactation in R. Lower values were found in 7 and 14 dL in R compared with C (Fig. 3(b) and (c), $P < 0{\cdot}05$).

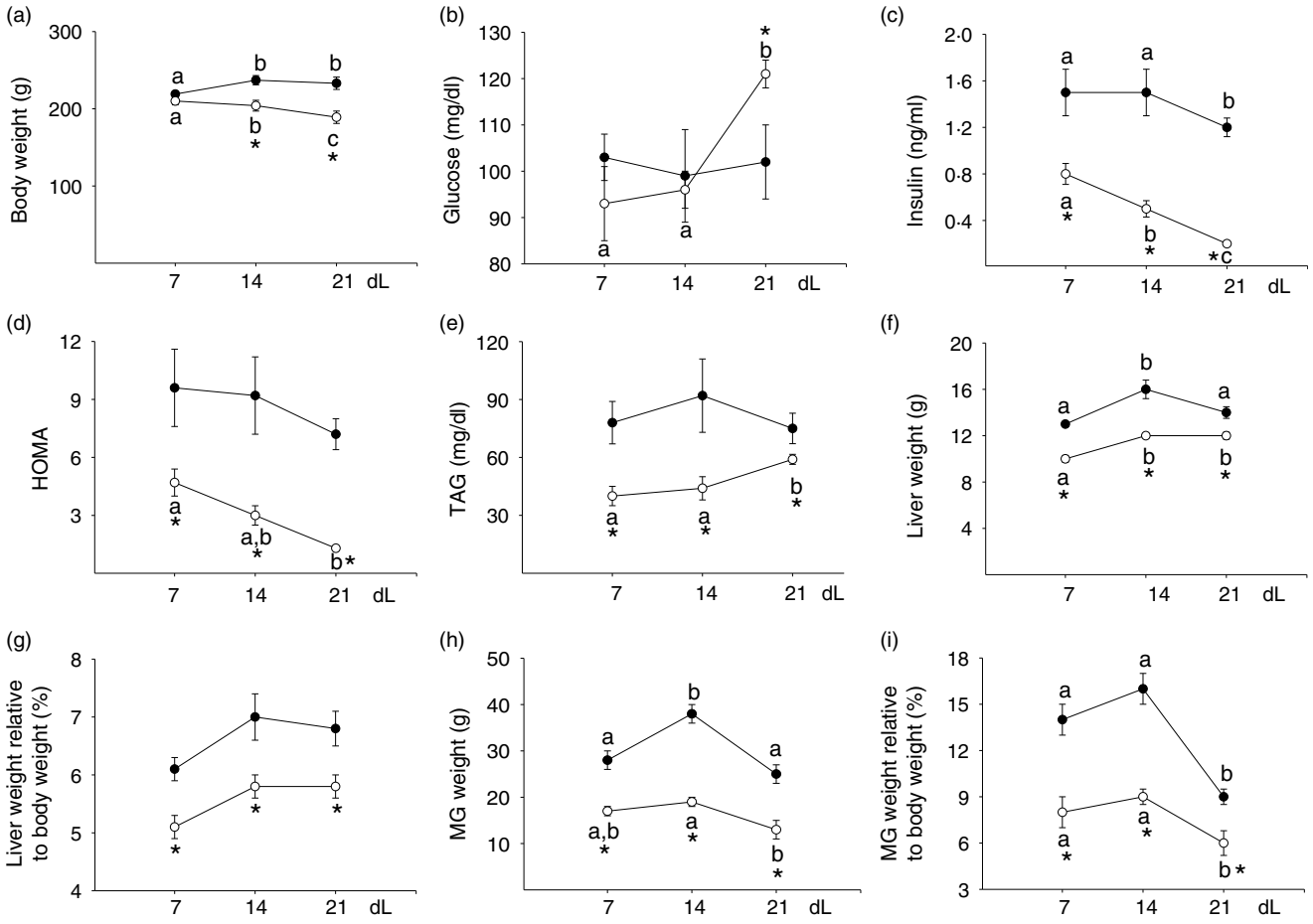

Fig. 1. Maternal parameters. (a) Body weight (g), (b) glucose (mg/dl), (c) insulin (ng/ml), (d) homeostatic model assessment (HOMA), (e) TAG (mg/dl), (f) liver weight (g), (g) liver weight relative to body weight (%), (h) mammary gland (MG) weight (g), (i) MG weight relative to body weight (%) at 7 (early stage), 14 (middle stage) and 21 (late stage) days of lactation (dL). Rats were fed control (C: –20 % protein; *n* 7 per each stage) or restricted (R: –10 % protein; *n* 6 per each stage) diet during pregnancy and lactation. Values are means, with their standard errors represented by vertical bars. **P* < 0·05 *v.* C. Data not sharing a letter are different between ages in the same group (*P* < 0·05). ●, C group; ○, R group. To convert glucose in mg/dl to mmol/l, multiply by 0·0555. To convert TAG in mg/dl to mmol/l, multiply by 0·0113.

Milk parameters

Water. No difference in the percentage of water content was observed at any stage of lactation within or between groups (Fig. 4(a), $P < 0{\cdot}05$).

Protein. Milk protein content in C was higher at 14 dL compared with 7 and 21 dL, and higher at 21 than 7 dL. In R, the protein content was higher at 21 dL compared with other stages. Protein content was lower at 7 and 14 dL in R compared with C (Fig. 4(b), $P < 0{\cdot}05$).

Total fat. Total fat content was lower at 14 dL in comparsion with 7 and 21 dL, and lower at 21 dL compared with 7 dL in both C and R. No differences were found between C and R throughout lactation (Fig. 4(c), $P < 0{\cdot}05$).

Arachidonic acid. AA increased at 21 dL in C compared with 7 and 14 dL, while AA in R was higher at 21 dL compared with 14 dL. Higher AA content was observed at 7 and 14 dL in R compared with C (Fig. 4(d), $P < 0{\cdot}05$).

DHA. DHA decreased at 21 dL compared with 14 and 7 dL in both C and R. No differences were found between C and R throughout lactation (Fig. 4(e), $P < 0{\cdot}05$).

Milk production and pup milk intake

Total intake. Total pup milk intake increased between 7 and 14 dL and remained constant until 21 dL in both C and R. Lower total milk intake throughout lactation was found in R compared with C (Fig. 5(a), $P < 0{\cdot}05$).

Protein intake. Pup protein intake increased between 7 and 14 dL and remained constant until 21 dL in C. In R, protein intake was higher at 21 dL compared with 7 dL. Lower protein intake was found at 7 and 14 dL in R compared with C (Fig. 5(b), $P < 0{\cdot}05$).

Total fat intake. Pup total fat intake increased at 21 dL compared with 7 dL in both C and R. Fat intake was lower throughout lactation in R compared with C (Fig. 5(c), $P < 0{\cdot}05$).

Arachidonic acid intake. Pup AA intake increased by 21 dL in C compared with 7 and 14 dL and in R compared with 7 dL. No differences were found between the groups (Fig. 5(d), $P < 0{\cdot}05$).

DHA intake. Pup DHA intake remained constant throughout lactation in both C and R (Fig. 5(e)). Lower DHA intake was found at 14 and 21 dL in R compared with C (Fig. 5(e), $P < 0{\cdot}05$).

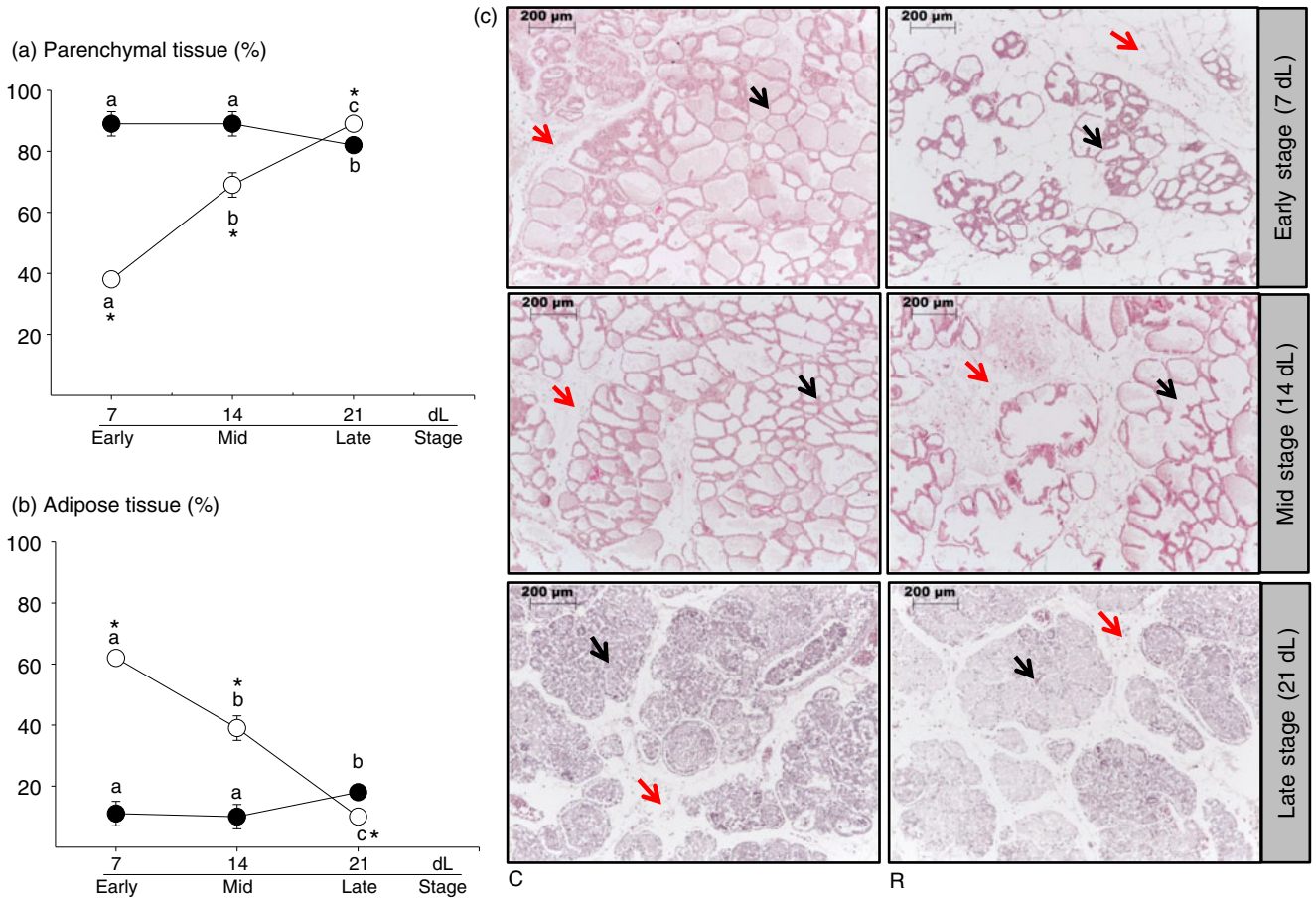

Fig. 2. Mammary gland. (a) Parenchymal tissue (%), (b) adipose tissue (%), (c) microphotography with haematoxylin and eosin; 40×. Black arrows point to parenchymal and red adipose tissues, respectively, at 7 (early stage), 14 (middle stage) and 21 (late stage) days of lactation (dL). Rats were fed control (C: –20 % protein; *n* 7 per each stage) or restricted (R: –10 % protein; *n* 6 per each stage) diet during pregnancy and lactation. Values are means, with their standard errors represented by vertical bars. *$P < 0{\cdot}05$ *v.* C. Data not sharing a letter are different between ages in the same group ($P < 0{\cdot}05$). ●, C group; ○, R group.

Pup parameters

Body weight. Pup body weight increased throughout the lactation period in C. However, in R, pup body weight increased at 14 dL and remained constant thereafter. Body weight was lower in R compared with C at 14 and 21 dL (Fig. 6(a), $P < 0{\cdot}05$).

Liver weight. Pup liver weight increased throughout lactation in C. However, in R, pup liver weight increased by 14 dL and remained constant through 21 dL. Liver weight was lower throughout lactation in R compared with C (Fig. 6(b), $P < 0{\cdot}05$).

Brain weight. Pup brain weight increased throughout lactation in both C and R. Brain weight was lower at 14 and 21 dL in R compared with C (Fig. 6(c), $P < 0{\cdot}05$).

Arachidonic acid in pup hippocampus. Pup hippocampal AA content decreased by 21 dL compared with 7 dL in C (AA %: 7 dL = $11{\cdot}2 \pm 0{\cdot}3^{a}$; 14 dL = $10{\cdot}2 \pm 0{\cdot}2^{ab}$; 21 dL = $8{\cdot}4 \pm 0{\cdot}01^{b}$). Hippocampal AA levels in R were constant throughout lactation (AA %: 7 dL = $6{\cdot}5 \pm 0{\cdot}02$*; 14 dL = $5{\cdot}6 \pm 0{\cdot}01$*; 21 dL = $5{\cdot}8 \pm 0{\cdot}01$*) and lower than in C at all stages (Fig. 6(d), $P < 0{\cdot}05$).

DHA in the pup hippocampus. Pup hippocampal DHA content in C decreased by 14 dL compared with 7 dL and remained constant until 21 dL (DHA %: 7 dL = $7{\cdot}2 \pm 0{\cdot}02^{a}$; 14 dL = $5{\cdot}5 \pm 0{\cdot}01^{b}$; 21 dL = $6{\cdot}0 \pm 0{\cdot}02^{b}$). In R, pup hippocampal DHA decreased throughout lactation (DHA %: 7 dL = $5{\cdot}8 \pm 0{\cdot}02^{a}$*; 14 dL = $4{\cdot}1 \pm 0{\cdot}08^{b}$*; 21 dL = $3{\cdot}2 \pm 0{\cdot}01^{c}$*). In R, hippocampal DHA was lower than in C at all stages of lactation (Fig. 6(e), $P < 0{\cdot}05$).

Discussion

Under optimal conditions, breastfeeding provides all the necessary nutrients for neonatal growth and maturation[31,32]. Delay in offspring growth velocity during the lactation period may predispose the offspring to metabolic syndrome during adulthood, suggesting that milk composition and intake are important mediators of offspring metabolic programming[24]. We studied the effects of maternal LPD on milk quality and production. Previous studies in rats have documented that maternal LPD negatively impacted maternal milk nutrient content[23,24,33], pup milk intake[24,25] and pup body weight[19,26], with negative effects in offspring development[34].

The present study is the first to attempt to analyse the effects of LPD on the delivery of milk nutrients to offspring during

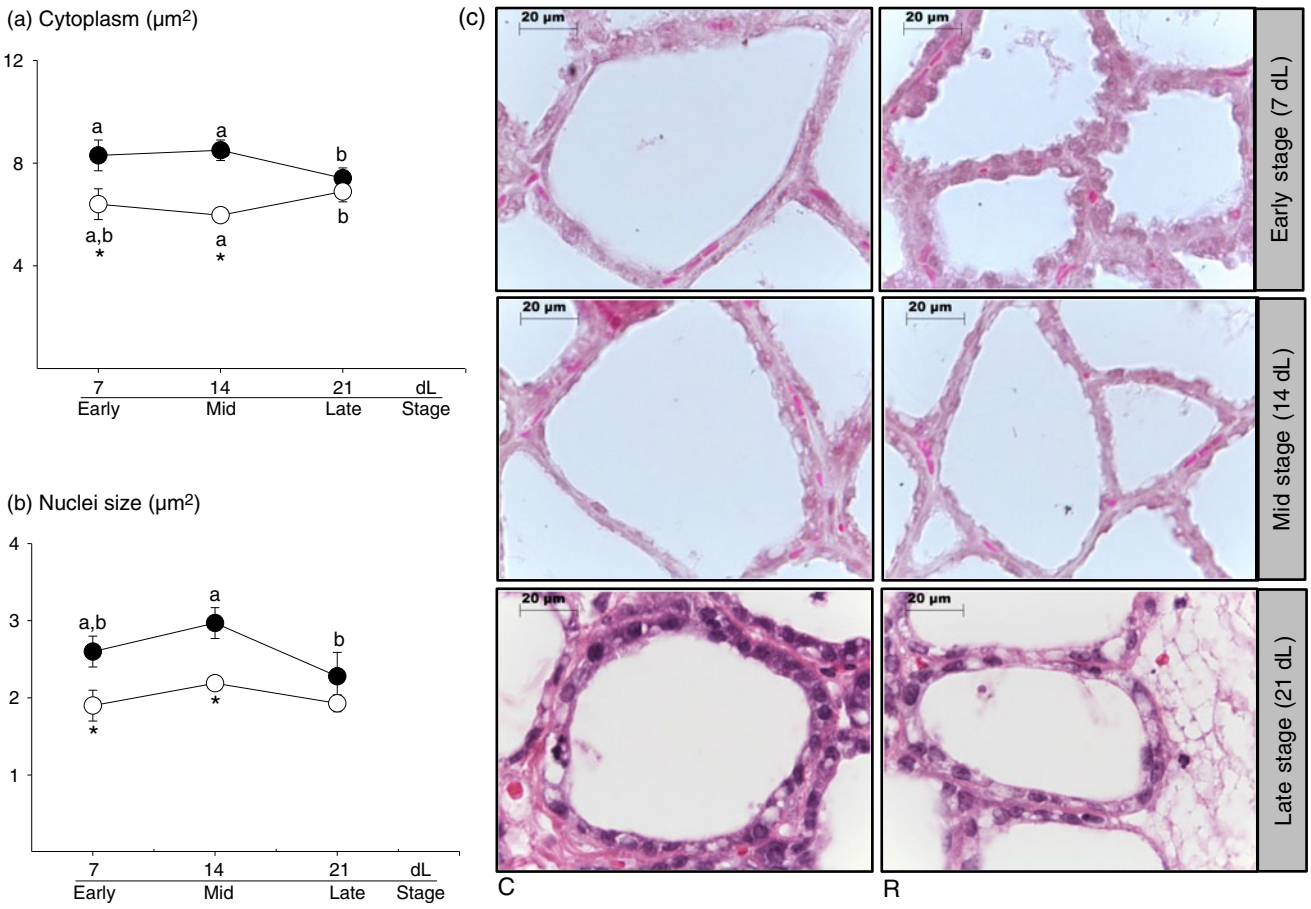

Fig. 3. Mammary gland. (a) Cytoplasm area (μm²), (b) nuclei size (μm²), and (c) microphotography with haematoxylin and eosin; 100× at 7 (early stage), 14 (middle stage) and 21 (late stage) days of lactation (dL). Rats were fed control (C: –20 % protein; *n* 7 per each stage) or restricted (R: –10 % protein; *n* 6 per each stage) diet during pregnancy and lactation. Values are means, with their standard errors represented by vertical bars. **P* < 0·05 *v.* C. Data not sharing a letter are different between ages in the same group (*P* < 0·05). ●, C group; ○, R group.

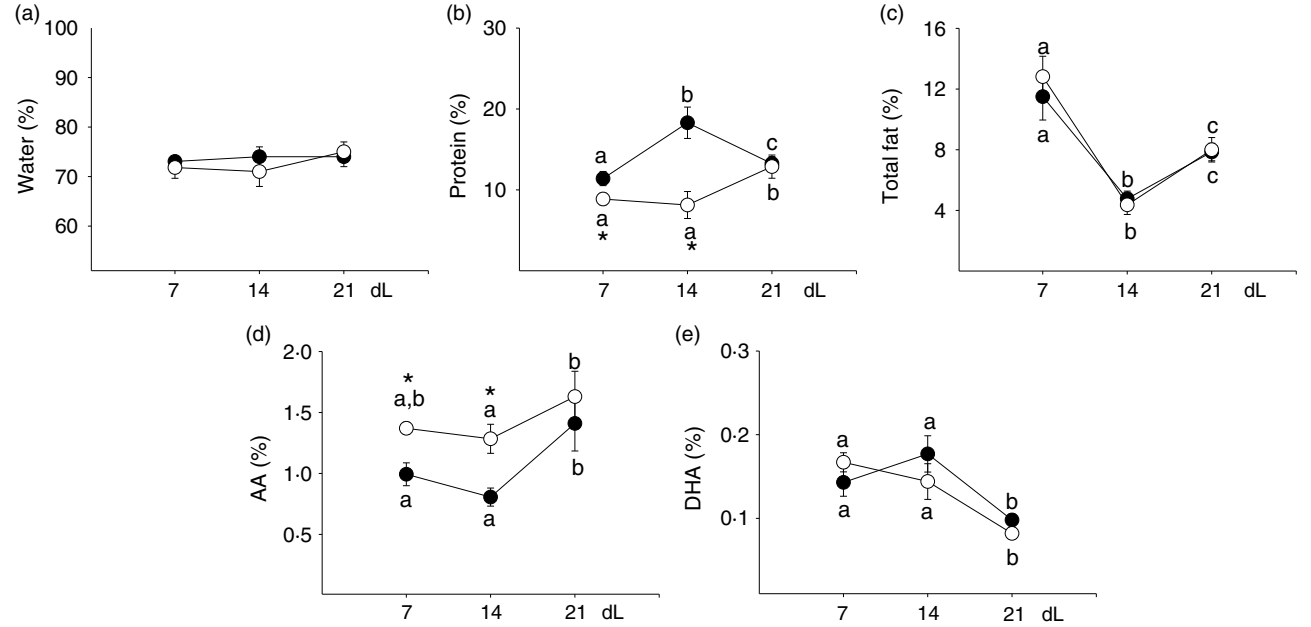

Fig. 4. Milk parameters. (a) Water (%), (b) protein (%), (c) total fat (%), (d) arachidonic acid (AA) (%), and (e) DHA (%) at 7 (early stage), 14 (middle stage) and 21 (late stage) days of lactation (dL). Rats were fed control (C: –20 % protein; *n* 7 per each stage) or restricted (R: –10 % protein; *n* 6 per each stage) diet during pregnancy and lactation. Values are means, with their standard errors represented by vertical bars. **P* < 0·05 *v.* C. Data not sharing a letter are different between ages in the same group (*P* < 0·05). ●, C group; ○, R group.

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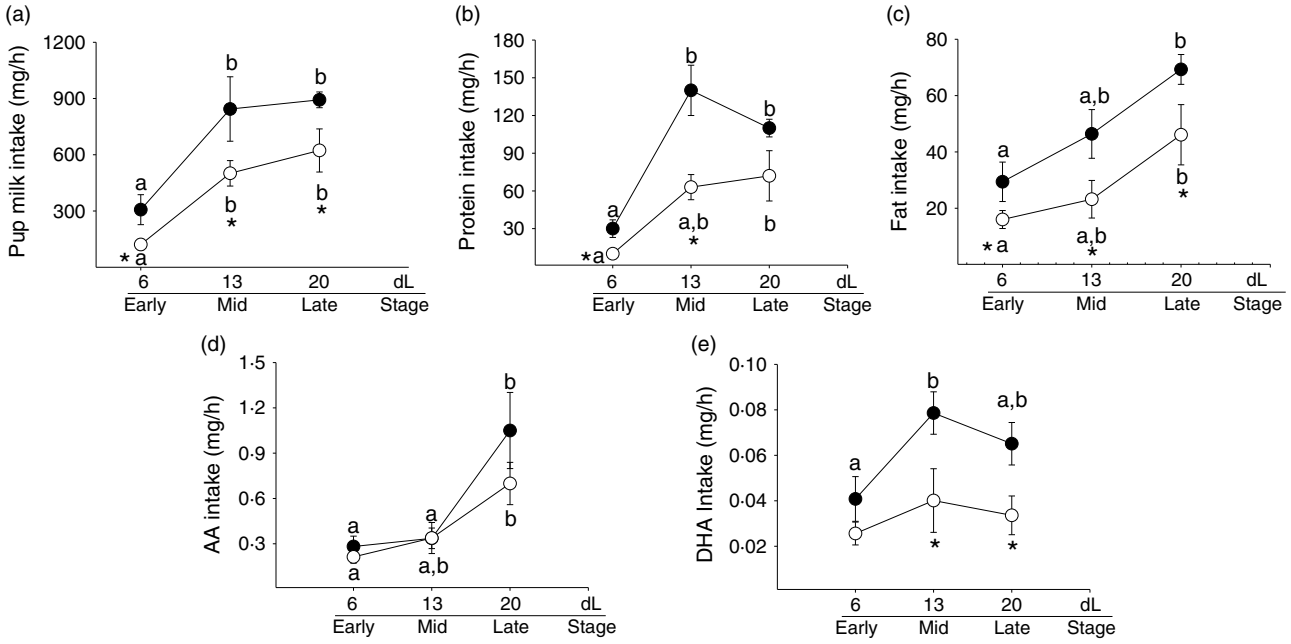

Fig. 5. Pup parameters. (a) Milk intake (g/h), (b) protein intake (g/h), (c) total fat intake (mg/h), (d) arachidonic acid (AA) intake (mg/h), and (e) DHA intake (mg/h) at 7 (early stage), 14 (middle stage) and 21 (late stage) days of lactation (dL). Rats were fed control (C: −20 % protein; *n* 7 per each stage) or restricted (R: −10 % protein; *n* 6 per each stage) diet during pregnancy and lactation. Values are means, with their standard errors represented by vertical bars. **P* < 0·05 *v.* C. Data not sharing a letter are different between ages in the same group (*P* < 0·05). ●, C group; ○, R group.

lactation and its association with pups' liver and brain weight and hippocampal AA and DHA content at weaning.

Carefully controlled studies in precocial and altricial mammalian species provide insights into the mechanisms involved. Humans are monotocous and precocial species, meaning that women generally bear only one fetus at a fairly advanced stage of metabolic development. In contrast, rodents are polytocous and altricial, bearing large litters born at an immature stage after relatively short pregnancies and requiring considerable maternal care in the immediate postnatal period to regulate basic neonatal functions. Thus, even under optimal feeding conditions, the nutritional demands of pregnancy and lactation on the litter-bearing rodent mother are much greater compared with humans. The present study analyses the effects of maternal LPD on mother, milk and offspring outcomes in a rat model, which is a limitation of the study. However, comparative physiology provides the opportunity to observe the differences and similarities between species by understanding the differences in molecular, cellular, biochemical and hormonal mechanisms. Understanding these differences can provide insights that can guide interventions in cases of abnormal human development[10].

It has been reported that maternal food intake relative to the body weight, liver and MG weights is greater in lactating compared with non-lactating rats[26,32]. In line with our findings, an increment in liver weight during lactation is related to increased maternal lipogenesis and β-oxidation processes[18,35–38]. Normal MG proliferation as well as functional differentiation are complex phenomena controlled by many hormones and growth factors[39]. C mothers showed increased MG weight at mid lactation associated with increased milk production. In contrast, maternal LPD impairs MG differentiation, proliferation and development throughout lactation accompanied by negative effects on milk nutrient content, milk production and pup milk intake.

Numerous metabolic adaptations occur during pregnancy[4] and lactation[40] to support milk synthesis without jeopardising maternal substrate homeostasis while optimising the delivery of appropriate nutrition to the offspring[40,41]. We showed low desaturase and elongase gene expression at the end of gestation in LPD-fed mothers in both liver[22] and MG[4]. These lower levels were further associated with a low percentage of LC-PUFA in maternal liver[22] and MG[4], as well as with poor fetal brain maturation, demonstrating negative impacts on both maternal and fetal homeostasis. Adequate maternal protein intake is necessary during both gestation and lactating periods[4]. Protein restriction during pregnancy does not affect maternal nursing behaviour, but the mobilisation of maternal stores may be insufficient for nutrient delivery to the pups[42].

Pup milk intake was higher in C than R at all stages. Maternal LPD was found to decrease the suckling stimulus in newborns due to a low milk volume[19,43]. Milk yield also decreased because of protein reduction in the diet during gestation and lactation[44,45]. As a result, low neonate suckling behaviour negatively impacts total milk production, potentially due to a change in orexigenic drive in the pups' appetitive centre at the hypothalamic level[7]. Non-human primate fetuses whose mothers were undernourished showed increased orexigenic and decreased anorexigenic peptides in the arcuate nucleus appetitive centres[46].

Milk protein concentration was higher at mid lactation in C, the time of maximum milk production in rodents[7,47,48]. In contrast, in R, protein concentration was lower than in C and did not show a peak during lactation. A reduction in maternal protein intake led to lower protein and amino acid availability to the pup, which together impaired pup's health and growth[49,50]. Contrasting

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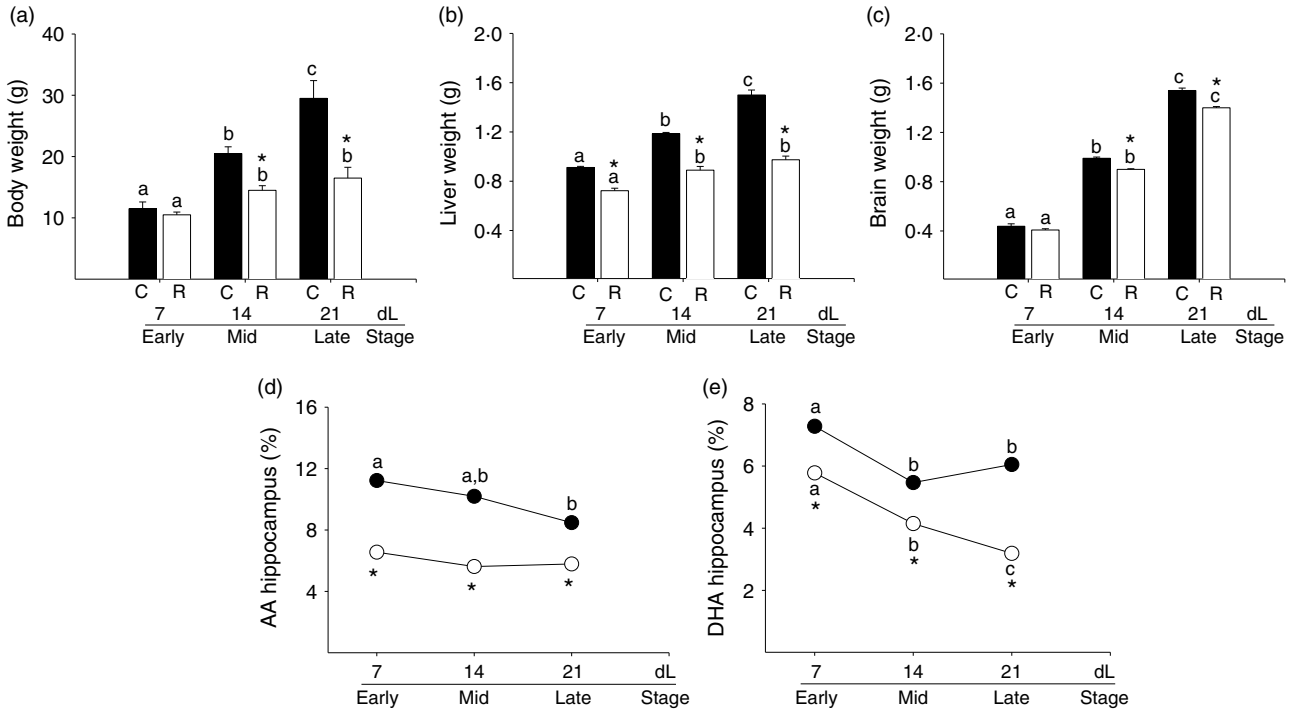

Fig. 6. Pup parameters. (a) Body weight (g), (b) liver weight (g), (c) brain weight (g), (d) arachidonic acid (AA) hippocampus (%) and (e) DHA hippocampus (%) at 7 (early stage), 14 (middle stage) and 21 (late stage) days of lactation (dL). Rats were fed control (C: −20 % protein; *n* 7 per each stage) or restricted (R: −10 % protein; *n* 6 per each stage) diet during pregnancy and lactation. Values are means, with their standard errors represented by vertical bars. **P* < 0·05 *v.* C. Data not sharing a letter are different between ages in the same group (*P* < 0·05). ●, C group; ○, R group.

our results, Grigor *et al.* found no changes in protein and lipid concentrations in the milk of rats fed low-protein or energy-restricted diets at mid lactation[51]. In agreement with our results, other studies showed that protein restriction in the first half of the lactating phase reduced milk protein concentrations[52–55]. Total protein and casein concentrations gradually increased throughout lactation in rats that doubled their pup weight in 5 d out of a weaning period of 21 d. Human babies doubled their weight in 120 d by consuming only their mother's milk[20]. Therefore, from the beginning of lactation, rats need to provide sufficient proteins to support the pups' organ development[56,57]. Human milk contains 1·2 mg/l protein, while rat milk contains 10 times that amount (around 11·8 mg/l), providing enough nutrients to double the infant's birth weight in <5 d[50,56]. Under normal conditions, rats produce around 1·5 ml/h milk for ≥10 pups[14], while women produce 50 ml/h milk for one or two infants[58,59]. Changes in the protein composition of human milk from colostrum to late lactation are associated with environmental variables such as feeding daytime[50]. Differences are also associated with maternal geographical location (temperature, elevation, etc.), cultural, and ethnic factors[60,61]. In contrast, since rats were maintained under controlled experimental conditions, changes in the protein content of rat milk only depended on the lactation stage and were relatively independent of environmental changes[31].

Our study did not modify the quantity or quality of fat in the maternal diet; only protein content was changed. Milk fat concentration was similar in both groups, in contrast to another study that reported a higher milk fat concentration at the beginning of lactation in rats fed a LPD[55]. However, pup fat intake reduced in R mothers at all stages of lactation. Maternal LPD reduced the percentage of milk DHA as well as milk DHA intake at late lactation.

Other studies have shown that maternal LPD negatively programmed pup's brain development[22,62] and behaviour[63] in ways that continued into adulthood. DHA supplementation in rat mothers fed with a LPD restored milk fatty acid composition and brain development[64]. Maternal DHA consumption increased milk and infant plasma DHA levels in both human[65] and baboon studies[66,67]. Importantly, the biochemical form of long-chain polyunsaturates in milk affects incorporation into the neonatal brain[68].

Early programming by maternal LPD affects body, liver and brain development. Lower percentages of hippocampal AA and DHA correlate with neural cell membrane composition and cognitive function in early life[67,69]. These results also are in line with our previous studies showing that maternal LPD programmed offspring liver and brain development, metabolic dysfunction[70,71], adult life appetitive behaviour, lower body weight and leptin serum concentrations[70,71]. LPD was found to impair reproductive functions[72,73], delaying sexual maturation, onset of puberty and decreased sperm quality[74]. LPD also causes offspring anxiety-type behaviour and learning and cognitive problems[9,63,75]. Here we found that offsprings of LPD-fed rats had lower hippocampal AA and DHA (%). The brain is the most complex and interactive organ in the body. The hippocampal region is closely involved in the control of both short- and long-term memory and in memories associated with spatial learning and planning[67,69,76,77].

To our knowledge few studies have been conducted to identify the effects of inadequate maternal protein diet on maternal MG function and offspring nutrient delivery during lactation; studies that have been reported addressed mostly milk composition[44]. This is unfortunate since a LPD has been extensively studied to induce developmental programming in the offspring[54,78]. The present study provides data on the effects of LPD on maternal outcomes in key organs, milk production and composition, and pups' organ development in a rat model. Milk also confers bioactive molecules that are known to protect the mother and offspring from infections and inflammation, contributing to maternal and neonatal immune maturation, organ development and healthy microbial colonisation[50].

In conclusion, maternal LPD delayed MG differentiation and milk production, affecting the pups' milk nutrient intake, with negative consequences on the development of multiple organs in pups by weaning. Adequate maternal nutrition during lactation is a key factor in offspring's life course health.

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