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## Milk lipid composition is modified by perinatal exposure to bisphenol A



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## ABSTRACT

To evaluate whether bisphenol A (BPA) modifies the synthesis, composition and/or profile of fatty acids (FAs) in the mammary glands of perinatally exposed animals, pregnant rats were orally exposed to 0, 0.6 or 52 µg BPA/kg/day from gestation day (GD) 9 until weaning. F1 females were bred, and on GD21, lactation day 2 (LD2) and LD10, mammary glands were obtained. On LD10, milk samples were collected, and FA profiles and lipid compositions were established. On GD21 and LD2, BPA exposure delayed mammary alveolar maturation and modified the synthesis of milk fat globules. On LD10, mammary gland histo-architecture was restored; however, the milk of BPA-exposed F1 dams had a FA profile and lipid concentration different from those of the control milk. Furthermore, the body weight gain of BPA52 F2 pups was increased compared with control animals. Thus, perinatal exposure to BPA modifies milk quality, compromising the normal growth of offspring.

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## 1. Introduction

For decades, one environmental factor that has been receiving attention is the ubiquitous presence of bisphenol A (BPA), an endocrine-disrupting chemical (EDC) (Vandenberg et al., 2009). BPA is a monomer commonly used in the manufacture of polycarbonate plastic and epoxy resins, and it is present in routinely used products (Rubin, 2011). Several studies done in rodents have identified adverse effects of BPA at levels equal to or below the acceptable human intake dose (50 µg BPA/kg/day) established by the U.S. Environmental Protection Agency (EPA) (Richter et al., 2007), including alterations in mammary gland development and differentiation in perinatally exposed animals [reviewed in Soto et al., 2013]. It has

also been shown that prenatal exposure to low doses of BPA alters the mammary gland development of female primates and rodents and promotes the development of intraductal hyperplasia and pre-cancerous mammary lesions in adult rodents (Acevedo et al., 2013; Durando et al., 2007; Moral et al., 2008; Muñoz-de-Toro et al., 2005; Paulose et al., 2014; Rochester, 2013; Tharp et al., 2012). Recent epidemiological studies have also indicated that exposure to EDCs is associated with overweight and obesity later in life (Lubrano et al., 2013; Newbold, 2010). Several studies in mice and rats have shown increasing body weight and metabolic complications in animals pre- and perinatally exposed to BPA (Rubin, 2011; Vom Saal et al., 2012).

The milk lipid composition shows marked changes during lactation, especially during the early neonatal period (Guilloteau et al., 2009; Hsieh et al., 2009), and milk is a remarkable source of energy. Milk fat provides a large percentage of the calories, essential fatty acids (FAs) and bioactive lipids required for the development of offspring (German and Dillard, 2006; McManaman, 2009). Therefore, changes in the lipid fraction of milk may establish a set-point for energy intake or the metabolic rate throughout the lives of the offspring (Passos et al., 2000). Lipid secretion by the mammary gland is a specialized apocrine process, during which elements of the apical plasma membrane surround a cytoplasmic lipid droplet (CLD) to produce membrane bound-secreted products referred to as milk fat globules (MFGs) (Barber et al., 1997; Dewettinck et al., 2007; McManaman, 2014; Russell et al., 2007). The pattern of CLD accumulation suggests that milk lipid formation involves sequentially regulated lipogenic, transport and secretion patterns that are functionally linked to the maturation of alveoli into milk-secreting

**Abbreviations:** ACCα, acetyl CoA carboxylase; ADPH, adipophilin; AA, arachidonic acid; BPA, bisphenol A; BTN, butyrophilin; CLD, cytoplasmic lipid droplet; EDCs, endocrine-disrupting chemicals; F0, dams directly exposed to xenoestrogens; F1, F0 offspring; F2, F1 offspring; FA, fatty acid; FAMES, fatty acid methyl esters; FAS, fatty acid synthase; GD, gestation day; LD, lactation day; LA, linoleic acid; LPL, lipoprotein lipase; MFG, milk fat globule; MUFA, monounsaturated fatty acid; ORO, Oil Red O; PLIN, perilipin; PPARγ, peroxisome proliferator-activated receptor gamma; PUFA, polyunsaturated fatty acid; SCD2, stearoyl-CoA desaturase; SFA, saturated fatty acid; SREBP1, sterol regulatory element-binding protein 1; TG, triglyceride; XOR, xanthine oxidase reductase.

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structures (McManaman, 2009; Russell et al., 2007). Additionally, the formation, accumulation and secretion of MFGs have been proposed to depend on intracellular FA synthesis. FAs are either derived from the *de novo* synthesis of medium-chain FAs from glucose in epithelial cells or are transported from the plasma. These functions are regulated both by secretory activation and by the availability of exogenous lipids (Anderson et al., 2007). Another regulator of lipid metabolism in the mammary gland is the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) (Rodríguez-Cruz et al., 2011; Shi et al., 2013), which plays a role in controlling milk fat synthesis, either directly or via the activation of the transcription regulators sterol regulatory-element binding protein 1 (SREBP1) and liver X receptor alpha (Shi et al., 2013).

Recently, we have shown that perinatal exposure to low doses of BPA delays mammary gland differentiation, modifies milk yield and alters milk protein composition in F1 lactating rats (Kass et al., 2012). However, the milk lipid fraction has not yet been analyzed. Therefore, in the present study, we examined whether perinatal exposure to low doses of BPA alters the maturation of alveoli and consequently the regulation of mammary gland lipogenesis and milk lipid secretion and the synthesis, composition and/or profile of FAs in F1 dams during late pregnancy and lactation.

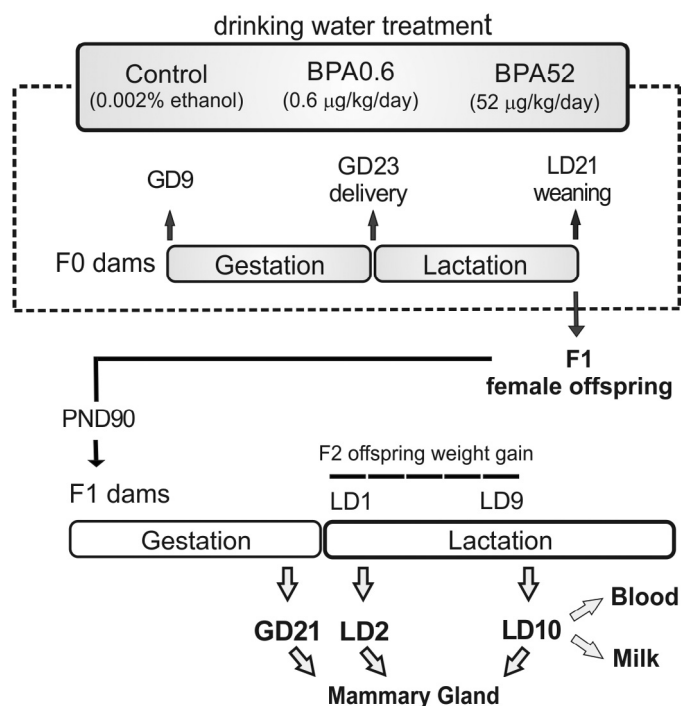
## 2. Materials and methods

### 2.1. Animals

All the experimental protocols were approved by the Ethical Committee of the Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina. Sexually mature female rats (90 days old) of a Wistar-derived strain bred at the Department of Human Physiology (Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral) were used. The animals were maintained in a controlled environment ( $22 \pm 2$  °C; 14 h of light from 06:00 h to 20:00 h) and had free access to pellet laboratory chow (16–014007 Rat-Mouse Diet, Nutrición Animal, Santa Fe, Argentina). For more information regarding the food composition, see Kass et al. (2012), Andreoli et al. (2015) and Supplementary Table S1. To minimize additional exposure to EDCs, the rats were housed in stainless steel cages with sterile pine wood shavings as bedding, and glass bottles with rubber stoppers were used to supply drinking water and oral treatments.

### 2.2. Experimental procedures

Females in proestrus were caged overnight with males of proven fertility. The day coinciding with the detection of sperm in the vagina was designated as day 1 of gestation (GD1). On GD9, corresponding to the beginning of organogenesis in the fetus, pregnant rats (F0) were weighed and randomly divided into three groups (8–10 dams/group), as follows: (a) control (0.002% ethanol), (b) BPA0.6 (0.6  $\mu$ g BPA/kg/day) and (c) BPA52 (52  $\mu$ g BPA/kg/day). All treatments were administered in the drinking water of the F0 dams from GD9 to weaning (Fig. 1). The BPA values of all the tap water samples were below the assay limit of detection [LOD = 38 ng/l (Fontana et al., 2011)]. The glass bottles were rinsed and refilled twice a week with freshly prepared solutions. BPA solutions (99% purity, Sigma-Aldrich, Buenos Aires, Argentina) were prepared according to that described in Kass et al. (2012). The individual body weight and water consumption of the F0 dams were recorded twice a week throughout the treatment period, and the doses were calculated on the basis of their average body weight and water consumption during pregnancy and lactation (Table 1). The higher BPA (BPA52) dose used in this experiment was equivalent to the safe dose (50  $\mu$ g/kg/day) established by the EPA (Richter et al., 2007), whereas the other dose (BPA0.6) was 100-fold lower. Taking into consideration that BPA



**Fig. 1.** Schematic representation of the experimental protocol used to study the effects of perinatal (gestation + lactation) exposure to low doses of BPA on the mammary glands of F1 females during late pregnancy and lactation. GD: gestation day; LD: lactation day; and PND: postnatal day.

pharmacokinetics in adult humans, mice and rats is very similar (Doerge et al., 2011; Taylor et al., 2011), and based on prior findings (Angle et al., 2013; Doerge et al., 2011; Taylor et al., 2011), both doses of BPA are predicted to lead to an average serum concentration of unconjugated BPA within human levels (Vandenberg et al., 2010).

After parturition [lactation day 0 (LD0)], F1 pups were weighed and sexed according to the anogenital distance, and litters of eight pups (preferably four males and four females) were left with F0 lactating mothers until weaning on LD21. At weaning, to minimize the exposure to the ubiquitous presence of BPA in the environment, the female F1 offspring (exposed to BPA transplacentally and through the milk) were housed in new stainless steel cages and glass drinking water bottles until the end of the experiment. To evaluate the effects of BPA perinatal exposure on mammary gland differentiation and lactational performance, randomly chosen 90-day-old F1 females were bred to unexposed males of proven fertility on the night of proestrus (as determined by vaginal smears). After pregnancy confirmation, one F1 female per litter from each treatment group was assigned to each of the time-point groups (GD21, LD2 and LD10) or was used in the analysis of the body weight gain of the F2 pups during the first half of lactation (from LD1 to LD9). The remaining females and all males were used for other experiments.

**Table 1**  
F0 daily BPA intake.

	Control	BPA0.6	BPA52
Concentration in drinking water ( $\mu$ g/l)	0	2.5	250
Water consumption (ml/day)	51.56 $\pm$ 5.23	55.46 $\pm$ 3.87	52.04 $\pm$ 3.23
Body weight (g)	255.78 $\pm$ 4.13	253.30 $\pm$ 3.61	250.14 $\pm$ 3.19
Average dose ( $\mu$ g/kg bw/day)	0	0.55 $\pm$ 0.03	52.20 $\pm$ 3.58

Body weight and water consumption were recorded twice a week throughout treatment. Values are expressed as mean  $\pm$  SE of 8–10 F0 dams/group.

### 2.3. Tissue, blood and milk sample collections

Blood and mammary gland samples from F1 dams were obtained after euthanasia on GD21, LD2 and LD10 ( $n = 8$ –10 dams/group for each time point). Blood serum samples were stored at  $-80^{\circ}\text{C}$  until seric glucose, cholesterol and triglyceride (TG) concentrations were determined using enzymatic reagent kits (Wiener Laboratorios S.A.I.C., Rosario, Argentina). The left fourth abdominal mammary gland was fixed in 10% (v/v) buffered formalin and embedded in paraffin, and the fifth mammary gland was placed in an aluminum mold filled with Tissue Freezing Medium (Jung, Leica Microsystems, Buenos Aires, Argentina) and stored at  $-80^{\circ}\text{C}$ , and they were both used for histological analysis. Portions of the fourth contralateral gland (right) excluding the lymph nodes were snap-frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  for RT-PCR analysis. As will be detailed later, milk samples were obtained to determine the total lipid content and FA profile on LD10.

Milk samples were collected from F1 nursing dams as previously described in Kass et al. (2012). Briefly, dams were separated from their pups on LD10 and kept in a chamber in a controlled environment for 2 h. Then, 10 IU of oxytocin (Novartis, Buenos Aires, Argentina) was administered into the peritoneal cavity (i.p.). Ten minutes after oxytocin injection, the nipples were warmed with water-soaked cotton, and the ejected milk was collected by gentle aspiration. The milk samples were frozen for further analysis ( $n = 8$ –10 per treatment).

### 2.4. Lactational performance

Maternal behavior was assessed by observing lactating females in their home cages during a period of 30 min every other day from LD0 to LD10. Nursing (arched back position of the F1 dam over the F2 litter) and suckling (attachment of F2 pups to the nipple when placed with their nursing mother) behaviors were evaluated during this period.

To analyze whether the quality of the milk produced by the BPA-exposed F1 dams modified the growth of the offspring, the body weight gain of the F2 pups was quantified during the first half of lactation. Every other day from LD1 to LD9, the F2 pups were separated from the F1 nursing dams for 1 h and were weighed at the end of this period to obtain a standardized measurement. Body weight gain was defined as the difference in pup weight on each LD compared with the weight on LD1. All animals were kept in a chamber in a controlled environment to avoid body temperature fluctuations.

### 2.5. RT and real-time quantitative PCR analysis

Mammary glands from eight animals from each experimental group (control, BPA0.6 and BPA52) obtained on GD21, LD2 and LD10 were individually homogenized in TRIzol (Invitrogen, Buenos Aires, Argentina), and RNA was prepared according to the manufacturer's protocol. The concentration of total RNA was assessed by measuring the  $A_{260}$  value, and RNA was stored at  $-80^{\circ}\text{C}$  until needed. Equal quantities of total RNA were reverse-transcribed into cDNA according to Ramos et al. (2003). Samples were analyzed in triplicate, and a sample without reverse transcriptase was included to detect contamination by genomic DNA. The primer sequences used for the amplification of adipophilin (ADPH), butyrophilin (BTN), xanthine oxidase reductase (XOR), acetyl CoA carboxylase ( $\text{ACC}\alpha$ ), FA synthase (FAS), lipoprotein lipase (LPL), stearoyl-CoA desaturase 2 (SCD2), perilipin (PLIN), SREBP1, PPAR $\gamma$  and ribosomal protein L19 (housekeeping gene) cDNAs are shown in [Supplementary Table S2](#). cDNA levels were detected using RT-PCR with a Rotor-Gene Q cyclor (Qiagen Instruments AG, Hombrechtikon, Switzerland). A total of 5  $\mu\text{l}$  of cDNA was combined with HOT FIRE Pol EvaGreen qPCR Mix

Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen) at a final volume of 20  $\mu\text{l}$ . After initial denaturation at  $95^{\circ}\text{C}$  for 15 min, the reaction mixture was subjected to successive cycles of denaturation at  $95^{\circ}\text{C}$  for 15 s, annealing at  $52^{\circ}\text{C}$  (BTN),  $54^{\circ}\text{C}$  (XOR, ADPH, and PLIN),  $55^{\circ}\text{C}$  ( $\text{ACC}\alpha$  and LPL),  $56^{\circ}\text{C}$  (SCD2, PPAR $\gamma$  and SREBP1),  $57^{\circ}\text{C}$  (FAS) and  $60^{\circ}\text{C}$  (L19) for 15 s, and extension at  $72^{\circ}\text{C}$  for 15 s. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. All PCR products were cloned using a TA cloning kit (Invitrogen) and specificity was confirmed by DNA sequencing (data not shown). The  $C_T$  for each sample was calculated using Rotor-Gene Q software (Qiagen) with an automatic fluorescence threshold ( $R_n$ ) setting. No significant differences in  $C_T$  values were observed on L19 between the different experimental groups. The relative expression level of each target was calculated using the standard curve method (Cikos et al., 2007), and L19 and the control group were used to normalize the  $C_T$  values.

### 2.6. Histological analysis

Paraffin sections (5  $\mu\text{m}$ ) of mammary gland samples collected on LD2 and LD10 were cut and stained with hematoxylin–eosin for histological examination. The alveolar luminal area in F1 pregnant dams was quantified in 60–80 randomly chosen alveoli at  $200\times$  magnification from five sections per animal. In addition, cryostat sections (10  $\mu\text{m}$ ) were cut and stained with Oil Red O (ORO) (Invitrogen) and counterstained with hematoxylin to identify neutral lipids within the alveolar lumen (Mehlem et al., 2013). The average area of the individual MFGs ( $\geq 1.5 \mu\text{m}$ ) was quantified in 60–80 randomly chosen alveoli at  $600\times$  magnification from five sections per animal. For both histological analyses, images were recorded using a digital camera (C-5000Zoom, Olympus Co., Japan), and measurements were performed using the Image Pro-Plus 4.1.0.1<sup>®</sup> image system analyzer (Media Cybernetics, Silver Spring, MA, USA).

### 2.7. Lipid extraction and assessment of the FA profile

Total lipids were extracted from the mammary glands and milk obtained on LD10 according to the methodology of Folch et al. (1957). The lipid extracts were fractionated into phospholipids and neutral lipid fractions by the micro-column chromatography method (Hurtado de Catalfo et al., 2009) and quantified gravimetrically (Marra and de Alaniz, 1990). After the saponification of all milk samples at  $80^{\circ}\text{C}$  for 45 min under a nitrogen atmosphere in 10% KOH in ethanol, the free FAs were extracted with hexane and evaporated to dryness. Then, they were esterified (de Alaniz and Marra, 1992), and the FA methyl esters (FAMES) obtained were extracted twice with hexane. FAMES were quantified by gas chromatography using a capillary-GLC-Hewlett Packard HP 6890 GC Series System Plus (Avondale, PA, USA) and were identified by comparisons of their relative retention times with authentic standards. The oven temperature was programmed to increase from  $185$  to  $230^{\circ}\text{C}$  in increments of  $3^{\circ}\text{C}/\text{min}$ . Ultra-dry helium was used as a carrier gas at an operating pressure of  $3.0 \text{ kg}/\text{cm}^2$ . FAMES were detected with a flame ionization detector operating at  $280^{\circ}\text{C}$ . In some cases, the identities of the FAs were confirmed by mass spectrometry analysis. Each FA was expressed as  $\mu\text{mol}$  per mg of lipids in the sample.

### 2.8. Statistical analysis

All data are expressed as the mean  $\pm$  SE. ANOVA was performed to obtain the overall significance, and Dunnett's ( $p < 0.05$ ) or Tukey's ( $p < 0.01$ ) post-hoc test was used to compare the experimental and control groups. The Mann–Whitney U test was used for real-time RT-PCR analysis. Values with a  $p < 0.05$  were accepted as significant.

### 3. Results

As previously described in Kass et al. (2012), the treatment with BPA administered in the drinking water did not produce signs of embryo toxicity [i.e. all F0 pregnant dams successfully delivered their pups, and the numbers of live-born pups (F1) per litter were similar among the groups], abnormal maternal or nursing behaviors, or changes in the body weight gain or water consumption of the F0 dams. The length of gestation was unaltered, and no gross malformations were observed in the F1 pups at delivery or weaning. The litter sex ratio showed no alterations and was within the normal range (50% females and 50% males).

In addition, the rate of pregnancy of the F1 BPA-exposed females did not differ from that of the F0 or F1 unexposed animals, and the average numbers of live F2 pups per litter (BPA0.6:  $11.13 \pm 0.22$  and BPA52:  $11.00 \pm 0.44$ ) were not significantly different from that of the control group ( $11.36 \pm 0.44$ ). Moreover, at birth, the F2 pup body weight was similar between the BPA groups (BPA0.6:  $5.84 \pm 0.07$  g and BPA52:  $5.75 \pm 0.08$  g) and control animals ( $5.89 \pm 0.08$  g).

#### 3.1. BPA perinatal exposure impaired milk lipid formation and/or secretion during late pregnancy and early lactation

Previously, we have demonstrated that perinatal exposure to BPA induces a delay in mammary gland differentiation at the end of pregnancy in F1 dams (Kass et al., 2012). To address whether perinatal exposure to BPA affects the differentiation of mammary glands during lactation, mammary gland histology and the area of the alveolar lumen were assessed. On LD2, in the control animals, the alveolar luminal space was expanded, the epithelial cell layer was more prominent than that of the adipocytes, and small lipid droplets were visible at the apical surfaces of epithelial cells. By LD10, the mammary gland contained prominent luminal structures and ducts, and few adipocytes per microscopic field were observed (Fig. 2A). On LD2, although the mammary sections from the BPA-exposed F1 dams exhibited cellular features similar to those of the control dams, the alveolar luminal area was reduced ( $p < 0.05$ ). In contrast, no differences between the groups were observed on LD10 (Fig. 2B).

To elucidate whether the altered mammary gland differentiation observed in the F1 dams during the peri-parturition period is associated with the modified assembly and/or secretion of MFGs, the mRNA expression levels of the MFG membrane proteins ADPH, BTN and XOR were evaluated on GD21, LD2 and LD10. On GD21 and LD2, BTN and XOR mRNA expression levels were lower in all BPA-exposed groups (Fig. 3A and 3B;  $p < 0.05$ ). In addition, ADPH mRNA level was reduced in the BPA0.6 animals on LD2, and it was increased in the BPA52-exposed dams on LD10 compared with the control rats (Fig. 3C;  $p < 0.05$ ).

Considering that perinatal exposure to BPA altered the normal assembly of MFGs during early lactation, MFG secretion and/or its accumulation in the alveolar lumen were analyzed through the identification of neutral lipids by ORO staining (Fig. 4). On LD2, MFGs in the samples from the F1 BPA-exposed dams showed a decreased size. In addition, the total area occupied by the MFGs in these animals was lower than that in the control dams (Fig. 4A and 4B,  $p < 0.05$ ); however, the growth of the BPA F2 pups at this time point was not affected. At mid-lactation (LD10), the MFG area remained lower in the BPA0.6 samples, and it increased significantly in the BPA52 samples compared with the control animals (Fig. 4C,  $p < 0.05$ ), suggesting that the mammary gland synthesis of TGs could be modified by perinatal BPA exposure.

#### 3.2. Effects of perinatal exposure to BPA on milk lipid synthesis at the end of pregnancy and during lactation

TG production by the mammary gland depends on the expression of several key enzymes involved in FA synthesis (Anderson et al.,

2007). One of the changes associated with the secretory activation of the mammary gland during lactation is the increased expression of these enzymes (Anderson et al., 2007). Thus, to examine whether perinatal exposure to low doses of BPA modifies their transcriptional levels later in life, the mRNA expression levels of LPL, FAS, ACC $\alpha$  and SCD2 were examined on GD21, LD2 and LD10. During late pregnancy (GD21), the mRNA expression levels of ACC $\alpha$ , LPL and SCD2 were down-regulated in both BPA-exposed groups (Fig. 5A, 5C and 5D;  $p < 0.05$ ). These findings suggest that the perinatal exposure to BPA modified the transcriptional levels of these enzymes, limiting FA synthesis in the mammary gland at the end of pregnancy. During lactation, no alterations were observed in the mRNA expression of the evaluated enzymes on LD2, and only SCD2 mRNA expression was up-regulated in the F1 BPA52-exposed dams at mid-lactation (Fig. 5D;  $p < 0.05$ ). The mRNA expression of PLIN, an adipose cell marker (Blanchette-Mackie et al., 1995), was also evaluated on GD21, LD2 and LD10, and no differences were found between the groups (Fig. 5E).

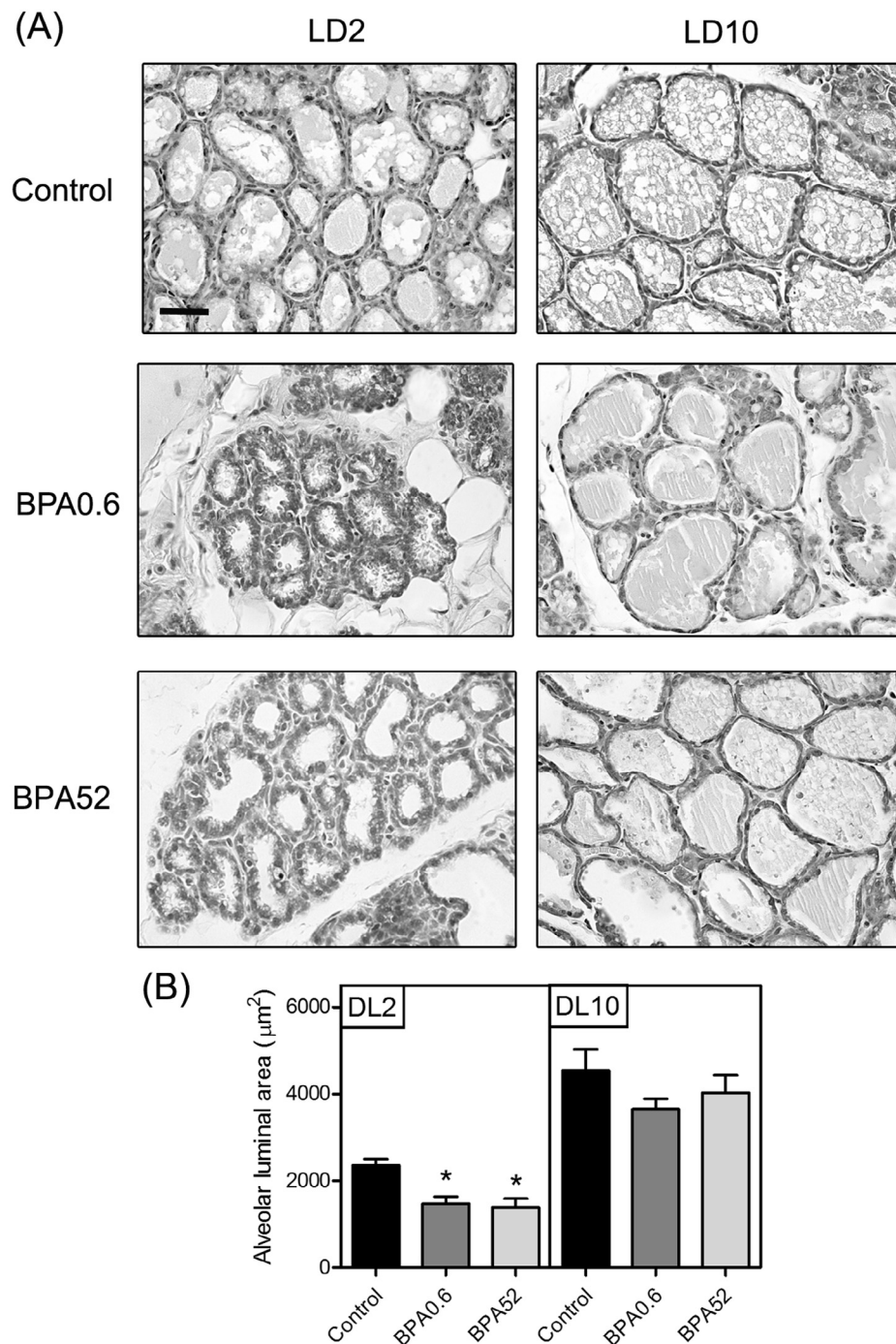
#### 3.3. The milk FA profile was altered by BPA perinatal exposure at mid-lactation

To elucidate whether the alterations in milk lipid synthesis and secretion observed in the F1 BPA-exposed dams during lactation modified the lipid fraction of the milk, the lipid content and FA profile were analyzed on LD10.

First, the milk concentrations of total lipids, TGs and phospholipids were determined gravimetrically at mid-lactation. The F1 BPA0.6 dams showed lower milk levels of total lipids, TGs and phospholipids compared with the control animals (Table 2). In contrast, the milk of the F1 BPA52 lactating dams had increased concentrations of total lipids, TGs and phospholipids compared with the control animals (Table 2).

To understand the lipid metabolism underlying the differentiation of the mammary glands, FAs were grouped according to their chemical features into saturated (SFA), monosaturated (MUFA) and polyunsaturated FAs (PUFA) (Table 3). The SFA level was higher in the BPA52-exposed group, mainly because of the higher levels of C8:0, C10:0, C12:0, C14:0, C16:0, C18:0 and C20:0 SFAs. In the BPA0.6 group, the proportions of C8:0 and C20:0 SFAs were reduced in the milk. The most strongly represented FA, C18:1n-9 MUFA, was lower in the BPA0.6 group and higher in the BPA52 group compared with the controls. Additionally, the levels of C18:2n-6 and C20:3n-6 PUFAs were reduced in the BPA0.6 group and increased in the BPA52 group. By contrast, the level of arachidonic acid (AA, C20:4n-6) was higher in the BPA0.6 group and lower in the BPA52 group than in the control group. In addition, the C22:4n-6, C22:5n-3 and C22:6n-3 PUFA levels were increased in the BPA0.6 F1 dams. The complete milk FA profiles for all experimental groups are shown in Supplementary Table S3.

FAs were also categorized according to their origins. The proportion of medium-chain FAs derived from the *de novo* synthesis ( $<C16$ ) was decreased in the milk samples from the BPA0.6 group compared with the controls. Moreover, in the milk samples from the BPA0.6-exposed F1 dams, the proportion of long-chain FAs derived from circulation ( $>C16$ ) and FAs originating from both sources ( $C16$ ) were lower than in the controls (Fig. 6). These results were consistent with the lower milk TG concentration found previously. The opposite results were observed in the BPA52-exposed group, in which the proportions of milk FAs were higher regardless of the FA source, and the same result was observed for the TG concentration. Taken together, our results suggest that perinatal exposure to low doses of BPA modifies the composition and proportions of FAs in milk during lactation. This finding could indicate that milk lipid synthesis in these animals is altered, although their serum concentrations of glucose, cholesterol and TGs were similar to those of the control animals (data not shown).



**Fig. 2.** Alveolar maturation during lactation. (A) Representative hematoxylin–eosin photomicrographs that illustrate the histological features of the mammary glands on LD2 and LD10. On both days studied, the lactating mammary glands showed organized epithelia, a reduced area of adipose tissue, a thin layer of connective tissue surrounding the alveolus, very small or indiscernible lipid droplets within the epithelial cells, and fully expanded alveoli with lipid droplets and milk in the luminal space. Whereas during early lactation, the luminal area of the alveoli was reduced by BPA exposure, on LD10, no differences were found between the groups. Scale bar: 50  $\mu\text{m}$ . (B) Quantifications of the alveolar luminal area on LD2 (left) and LD10 (right). The bars represent the mean value  $\pm$  SEM of 8 animals/group (\* $p < 0.05$ ; ANOVA followed by Dunnett's post test).

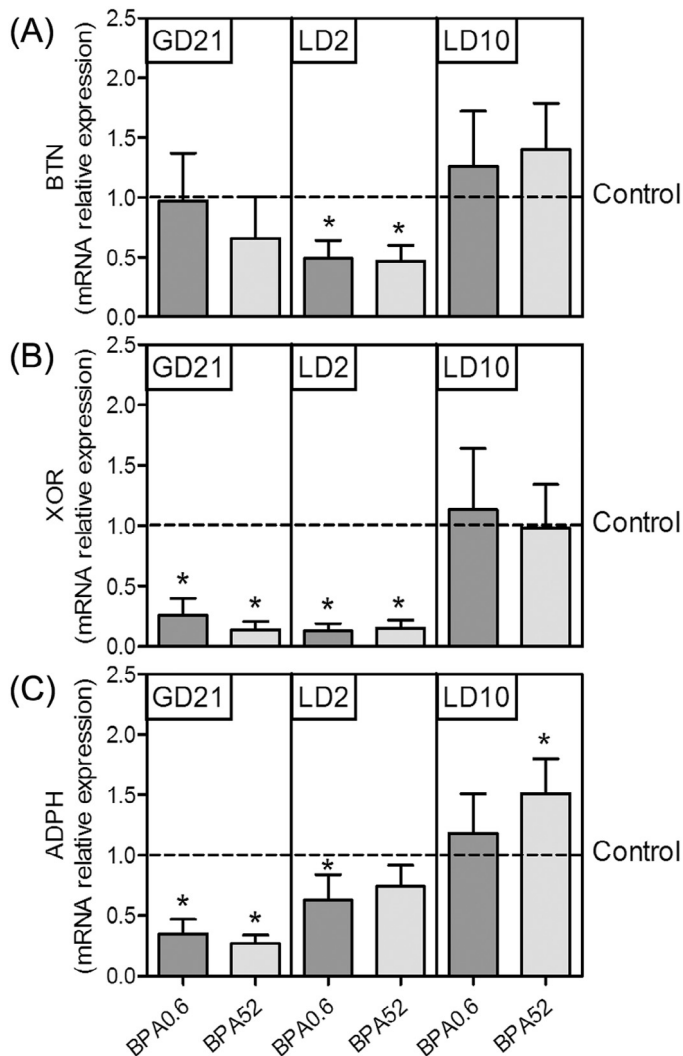
#### 3.4. *PPAR $\gamma$* and *SREBP1* mRNA expression was modified by perinatal exposure to BPA

*PPAR $\gamma$*  is known to regulate both the expression of several genes involved in *de novo* FA synthesis, such as *SREBP1*, and lipid droplet storage in mammary glands during lactation (Kang et al., 2015; Shi et al., 2013). Therefore, to examine whether BPA-induced changes in the milk lipid fraction are associated with alterations in lipid metabolism regulation, the mRNA expression levels of *PPAR $\gamma$*  and *SREBP1* were evaluated in F1 BPA perinatally exposed dams at mid-lactation.

On LD10, the mRNA expression levels of *PPAR $\gamma$*  and *SREBP1* were up-regulated in the BPA52 F1 dams (Fig. 7;  $p < 0.05$ ), whereas no significant difference was observed between the BPA0.6 and control dams.

#### 3.5. The weight gain of the F2 offspring was affected by perinatal exposure of F1 dams to BPA

Milk fat, a major source of lipids, provides a large percentage of the calories, essential FAs and bioactive lipids required for neonatal growth and development (McManaman, 2009). To evaluate



**Fig. 3.** Effects of perinatal exposure to BPA on mRNA expression of MFG membrane proteins. On GD21, LD2 and LD10, the mRNA expression levels of BTN (A), XOR (B) and ADPH (C) were quantified by real-time RT-PCR in the mammary glands of F1 dams. The bars represent the mean value  $\pm$  SE of 8 animals/group. The samples were normalized to L19 expression and to control animals, and a value of 1 was assigned to the control group (\* $p < 0.05$ , Mann–Whitney test).

whether the modified milk FA profile and lipid content induced by BPA alters F2 offspring growth, the body weight gain of F2 pups was assessed during lactation.

The body weight gain of the BPA52 F2 offspring was increased from LD5 to LD9 (Fig. 8). This finding could indicate that perinatal exposure to BPA52 not only modified the FA profile but also perturbed the growth and development of the F2 suckling pups. In contrast, no changes were observed in the body weight gain of the BPA0.6 F2 pups, despite the alterations detected in the milk lipid content and FA profile.

#### 4. Discussion

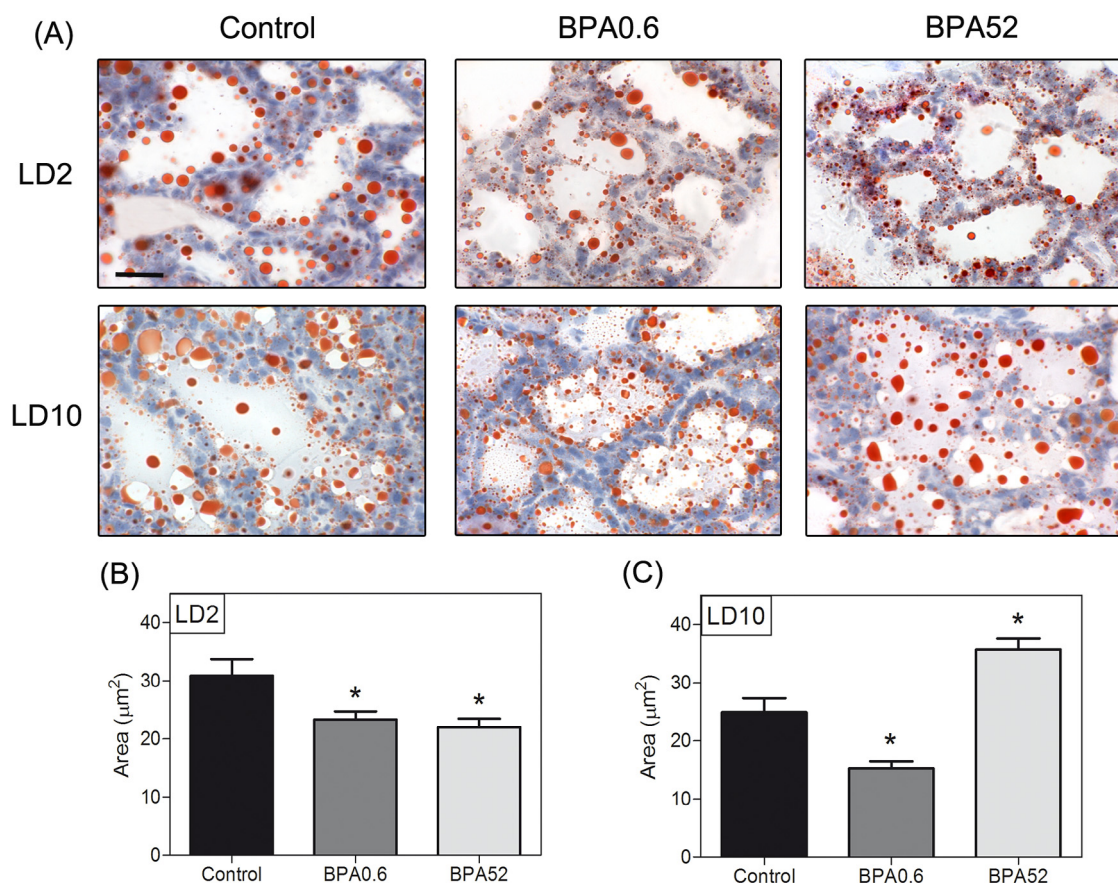
As the mammary gland differentiates into a secretory organ, milk-secreting cells require the coordination of multiple biochemical processes to produce large quantities of milk lipids (Anderson et al., 2007; Russell et al., 2007). In the current study, we observed that perinatal BPA exposure delayed the alveolar maturation of the mammary gland during secretory activation and modified the synthesis and/or secretion of MFGs during late pregnancy and early

lactation in rats. In addition, changes in the milk lipid content and FA composition induced by BPA exposure resulted in the increased body weight gain of F2 suckling rats that were born from dams perinatally exposed to a BPA dose equivalent to that established by the EPA as acceptable for human intake.

Previously, we have established that perinatal BPA exposure impairs the functional differentiation of the rat mammary gland and alters the composition of milk proteins during late pregnancy (Kass et al., 2012). The findings reported here demonstrate that the mammary gland histo-architecture in the BPA-exposed F1 dams remained modified during early lactation (LD2), but not during mid-lactation (LD10). Taken together, the previous and present results suggest that perinatal exposure to low doses of BPA delays alveolar maturation during the secretory activation of the mammary gland. The interaction between epithelial cells and the extracellular matrix, especially the basement membrane, is critical for successful lactogenic differentiation (Morrison and Cutler, 2010). The exposure of the mammary gland to BPA during critical periods of morphogenesis could modify the mechanical properties of the extracellular matrix, resulting in a delay in alveolar maturation during secretory activation. When lactation was established in this study, the mammary glands of the F1 BPA-exposed dams were capable of synthesizing and secreting milk; however, the milk had altered protein and lipid contents and a FA profile different from that of the control animals.

It is well known that defects in milk lipid secretion and/or synthesis are associated with alterations in the alveolar maturation of the mammary gland (McManaman, 2014; Russell et al., 2011). Deficiencies in XOR and/or BTN, or the interference with their interactions with ADPH, have been predicted to perturb the secretion of MFGs (McManaman et al., 2002, 2007). Our data demonstrate that perinatal BPA exposure not only impairs alveolar maturation, but also modifies the mRNA expression of BTN, XOR and ADPH and perturbs the assembly and/or secretion of MFGs during late pregnancy and early lactation. The mRNA expression of BTN and XOR was down-regulated by perinatal BPA exposure on GD21 and LD2, suggesting that MFG secretion could be reduced during the secretory activation of mammary epithelial cells. In both BPA groups, the MFG size was smaller than that in the control rats on LD2. In contrast, on LD10, whereas the MFG area remained smaller in the BPA0.6 F1 dams, it was increased in the BPA52 F1 dams. A similar pattern of expression was observed for ADPH mRNA in both BPA-exposed groups during lactation. It has been shown that ADPH regulates CLD growth by preventing the access of lipases to the TG core of CLD (Listenberger et al., 2007) and through alveolar maturation in the mammary gland (Russell et al., 2011). Therefore, one of the mechanisms by which BPA could modify MFG homeostasis in the mammary gland is through interfering with ADPH expression. In addition, the PLIN mRNA expression levels observed suggest that perinatal exposure to BPA does not modify adipocyte maturation in the lactating mammary gland during this period.

Considering that CLD growth and MFG size were modified during secretory differentiation by perinatal BPA exposure, milk FA synthesis could also be altered during this period. On GD21, perinatal exposure to BPA decreased the transcriptional levels of ACC $\alpha$  and LPL, limiting FA synthesis in the mammary gland during secretory activation. In contrast, the mRNA expression of the evaluated lipogenic enzymes was similar between the groups on LD2, and only SCD2 mRNA expression was increased in the F1 BPA52-exposed dams on LD10. Despite the restoration of the architecture of the mammary gland and the similar mRNA expression levels of the lipogenic enzymes at mid-lactation, our results clearly demonstrate that the milk FA composition and profile were modified by perinatal BPA exposure. The discrepancies between mRNA expression and milk lipid content observed in our experiment could be due to the BPA-induced post-transcriptional regulation of lipogenic enzymes in F1



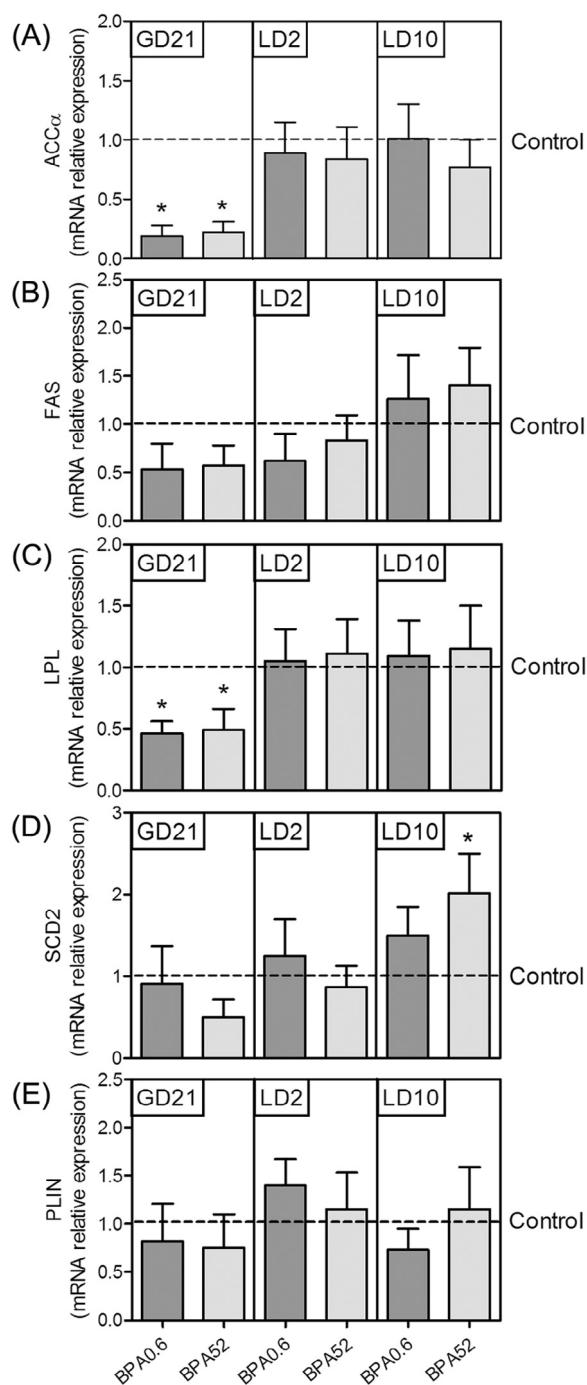
**Fig. 4.** Altered accumulation of MFGs in the mammary glands of BPA-exposed F1 dams. (A) ORO staining of neutral lipids (red) in mammary gland cryosections. On LD2, the BPA-exposed F1 dams showed decreased accumulation of MFGs in the alveolar lumen compared with the control animals. On LD10, the mammary glands of BPA52 F1 dams had an increased accumulation of MFGs, whereas those of BPA0.6 F1 dams showed fewer MFGs than control animals. Scale bar: 40  $\mu\text{m}$ . The area of MFGs within the alveolar lumen was quantified on LD2 (B) and LD10 (C). The bars represent the mean value  $\pm$  SEM of 8 animals/group (\* $p < 0.05$ ; ANOVA followed by Dunnett's post test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lactating rats. In addition, PPAR $\gamma$  and SREBP1 mRNA expression levels were increased in the BPA52 F1 dams at mid-lactation. PPAR $\gamma$  has a central role in regulating the transcriptional levels of several genes involved in lipid metabolism in the mammary gland, including SREBP1 (Bionaz and Looor, 2008; Shi et al., 2013), which in turn regulates *de novo* FA synthesis through ACC $\alpha$  and FAS (Liang et al., 2002; Rudolph et al., 2010). Furthermore, it has been shown that the activation of PPAR $\gamma$  signaling in goat mammary epithelial cells promotes the accumulation of lipids by up-regulating ADPH expression and promoter activity (Kang et al., 2015). Perinatal exposure to BPA could have directly or indirectly activated PPAR $\gamma$  signaling in the mammary gland, thereby increasing both *de novo* FA synthesis and ADPH mRNA expression in the BPA52 F1 lactating rats on LD10.

As previously mentioned, the milk lipid content was modified by perinatal BPA exposure. The concentrations of total lipids, TGs and phospholipids were lower in BPA0.6 F1 dams and higher in BPA52 F1 dams than in control rats. In addition, the FA profile was altered, and a relationship between MFG size and the milk FA profile was established in both BPA groups. The BPA0.6 F1 dams showed smaller MFGs, decreased levels of medium-chain SFAs and increased levels of long-chain PUFAs in the milk. On the other hand, the BPA52 F1 dams had the largest MFGs, with a higher level of medium-chain SFAs and a lower level of long-chain PUFAs at mid-lactation. In this regard, compositional modifications in the polar lipid envelopes of MFGs and the milk FA profile have been associated with MFG size in bovines (Mesilati-Stahy et al., 2011). According

to our results, perinatal exposure to BPA52 appeared to induce the *de novo* synthesis of FAs due to increased levels of myristic acid, palmitic acid and stearic acid in the milk, indicating that FAS activity was augmented although mRNA expression was similar in all groups. In contrast, the levels of these milk FAs were reduced in the lactating BPA0.6 F1 rats. Another important FA in milk is AA, which is required for the growth and neuronal development of offspring (Rodriguez-Cruz et al., 2011). Chen et al. (2012) reported that BPA exposure may increase desaturase activity and enhance the conversion of linoleic acid (LA) to AA in the testis. Our data showed that in BPA0.6 F1 dams, in accordance with the lower level of LA, there was a significant increase in FA desaturase products (AA) compared with control F1 dams. In contrast, BPA52 F1 dams had an increased level of LA and a reduced level of AA in the milk. These findings indicate that whereas BPA0.6 exposure enhanced the conversion of LA to AA, BPA52 exposure decreased this metabolic process. These results suggest that perinatal exposure to low doses of BPA modifies the quality of the lipid fraction of the milk, a fact that may alter the growth and development of F2 pups.

To examine whether changes in milk FA composition induced by perinatal BPA exposure affect the growth of F2 offspring, their body weight gain was analyzed during the suckling period. BPA52 F2 pups had an increased body weight gain between LD5 and LD9 compared with control pups, suggesting that in addition to altering the milk FA composition, BPA52 also perturbed the growth and development of F2 suckling pups. On the other hand, BPA0.6-induced alterations in the milk FA profile were not accompanied



**Fig. 5.** Effects of perinatal exposure to BPA on mRNA expression of enzymes involved in mammary gland lipid synthesis. On GD21, LD2 and LD10, the mRNA expression levels of ACCα (A), FAS (B), LPL (C), and SCD2 (D) as well as the adipocyte marker PLIN (E) were quantified. The bars represent the mean value  $\pm$  SE of 8 animals/group. The samples were normalized to L19 expression and to control animals, and a value of 1 was assigned to the control group (\* $p < 0.05$ , Mann–Whitney test).

by changes in the body weight gain of F2 pups. In addition to the contribution of *de novo* FA synthesis in the mammary glands of BPA52 F1 dams to a higher milk lipid content, an increased synthesis of FAs in the liver and hepatic steatosis induced by BPA have been reported in male mice (Marmugi et al., 2012). In addition, recent studies have shown that prenatal or perinatal exposure to EDCs increases body weight and causes transgenerational effects on adipogenesis (Chamorro-Garcia et al., 2013; Zhang et al., 2014).

**Table 2**

Effect of perinatal exposure to BPA on milk concentrations of total lipids, triglycerides and phospholipids in F1 dams at mid-lactation.

Milk	Treatment groups		
	Control	BPA0.6	BPA52
Total lipids (mg/ml)	214.9 $\pm$ 4.4	182.8 $\pm$ 5.1*	238.7 $\pm$ 4.8*
Triglycerides (mg/ml)	203.3 $\pm$ 6.1	177.5 $\pm$ 4.4*	217.1 $\pm$ 4.2*
Phospholipids (mg/ml)	11.3 $\pm$ 1.49	6.1 $\pm$ 0.9*	22.4 $\pm$ 1.9*

Values are expressed as mean  $\pm$  SE of 8–10 F1 dams/group.

\*  $p < 0.01$  compared with control group (ANOVA followed by Tukey's post-hoc test).

Further, perinatal exposure to BPA alters the maternal behavior of F1 dams and induces body weight increases in the F2 generation only (Boudalia et al., 2014). Changes in maternal behavior have also been shown in female CD-1 mice exposed to BPA as fetuses or as adult dams in late pregnancy (Palanza et al., 2002). In contrast, our results showed that neither F0 nor F1 BPA-exposed dams exhibited changes in their nursing behavior, thus suggesting that the differences in F2 body weight gain are related to the milk composition and not to the maternal behavior. Not only increases in FA synthesis in the mammary gland and liver but also changes in adipogenesis in the BPA F1 dams could be responsible for the

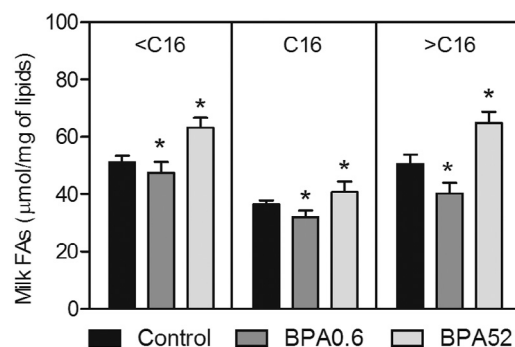
**Table 3**

Milk fatty acid (FA) profile of F1 BPA-exposed dams at mid-lactation.

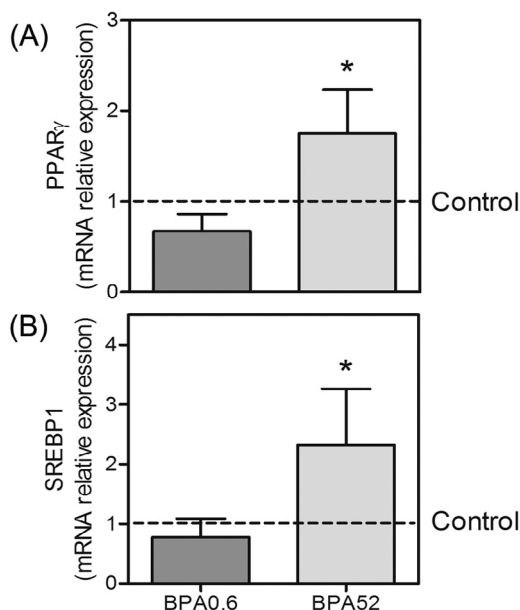
Type of FA ( $\mu$ mol/mg of lipids)	Treatment groups		
	Control	BPA0.6	BPA52
SFA			
C8:0	14.7 $\pm$ 0.2	12.9 $\pm$ 0.3*	18.3 $\pm$ 0.2*
C10:0	12.0 $\pm$ 0.4	11.3 $\pm$ 0.2	16.1 $\pm$ 0.3*
C12:0	10.2 $\pm$ 0.3	9.5 $\pm$ 0.2	12.7 $\pm$ 0.1*
C14:0	13.1 $\pm$ 0.2	12.6 $\pm$ 0.3	15.0 $\pm$ 0.3*
C16:0	32.5 $\pm$ 0.5	28.8 $\pm$ 0.5*	36.7 $\pm$ 0.6*
C18:0	5.5 $\pm$ 0.2	3.2 $\pm$ 0.1*	8.9 $\pm$ 0.2*
C20:0	2.2 $\pm$ 0.1	1.0 $\pm$ 0.1*	3.9 $\pm$ 0.1*
MUFA			
C16:1n-7	3.8 $\pm$ 0.1	3.2 $\pm$ 0.1	3.9 $\pm$ 0.3
C18:1n-9	18.6 $\pm$ 0.3	15.2 $\pm$ 0.4*	22.7 $\pm$ 0.3*
PUFA			
C18:2n-6	15.4 $\pm$ 0.3	11.0 $\pm$ 0.3*	19.1 $\pm$ 0.5*
C20:3n-6	2.5 $\pm$ 0.1	0.9 $\pm$ 0.1*	4.5 $\pm$ 0.1*
C20:4n-6	1.8 $\pm$ 0.1	2.5 $\pm$ 0.1*	1.0 $\pm$ 0.1*
C22:4n-6	0.5 $\pm$ 0.1	1.8 $\pm$ 0.1*	0.4 $\pm$ 0.1
C22:5n-3	0.3 $\pm$ 0.04	0.5 $\pm$ 0.1*	0.4 $\pm$ 0.01
C22:6n-3	0.5 $\pm$ 0.05	0.8 $\pm$ 0.1*	0.5 $\pm$ 0.02*

Values are expressed as mean  $\pm$  SE of 8–10 F1 dams/group.

\*  $p < 0.01$  compared with control group (ANOVA followed by Tukey's post-hoc test).



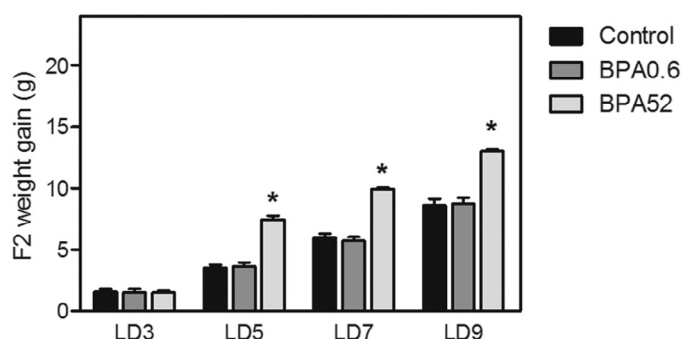
**Fig. 6.** Milk FA profiles at mid-lactation (LD10). FAs were categorized according to their origin as follows: *de novo* FAs (<C16), preformed FA (>C16) and FAs from both sources (C16). The bars represent the mean value  $\pm$  SE of 8–10 animals/group (\* $p < 0.01$ ; ANOVA followed by Tukey's post-hoc test).



**Fig. 7.** Regulation of lipogenesis in the mammary glands of BPA F1 dams at mid-lactation. The mRNA expression levels of PPAR $\gamma$  (A) and SREBP1 (B) were evaluated on LD10. The bars represent the mean value  $\pm$  SE of 8 animals/group. The samples were normalized to L19 expression and to control animals, and a value of 1 was assigned to the control group (\* $p < 0.05$ , Mann–Whitney test).

modifications in the lipid fraction of the milk that ultimately modified the growth of F2 pups. On the other hand, we have reported that the body weights of F2 pups are similar among groups on LD14 (Kass et al., 2012). The lower milk yield and protein content previously described in BPA-exposed F1 dams (Kass et al., 2012) could have counterbalanced the higher milk lipid fraction observed in the present study, explaining why, on LD14, no differences in body weight were observed between the groups despite the increased body weight gain between LD5 and LD9.

In conclusion, our study demonstrates that perinatal exposure to low doses of BPA induces a delay in the functional differentiation of the mammary gland during secretory activation, which is overruled during lactation. Moreover, the quality of the milk produced at mid-lactation is affected by BPA exposure, suggesting that the normal growth and development of the F2 generation could be affected.



**Fig. 8.** Weight gain of the F2 pups during lactation. The F2 offspring of BPA52-exposed F1 dams showed a higher weight gain than the controls from LD5 to LD9. Body weight gain was defined as the difference in the weight of the pup on each LD compared with the weight on LD1. Taking into consideration that no difference in body weight gain was recorded between male and female F2 pups, the bars represent the mean value  $\pm$  SE of 8–10 litters/group (\* $p < 0.01$ ; ANOVA followed by Dunnett's post test).

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## Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.05.007.

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