Gestational PFOA Exposure of Mice is Associated with Altered Mammary Gland Development in Dams and Female Offspring

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Perfluorooctanoic acid (PFOA), with diverse and widespread commercial and industrial applications, has been detected in human and wildlife sera. Previous mouse studies linked prenatal PFOA exposure to decreased neonatal body weights (BW) and survival in a dose-dependent manner. To determine whether effects were linked to gestational time of exposure or to subsequent lactational stages, timed-pregnant CD-1 mice were orally dosed with 5 mg PFOA/kg on gestation days (GD) 1–17, 8–17, 12–17, or vehicle on GD 1–17. PFOA exposure had no effect on maternal weight gain or number of live pups born. Mean pup BWs on postnatal day (PND) 1 in all PFOA-exposed groups were significantly reduced and decrements persisted until weaning. Mammary glands from lactating dams and female pups on PND 10 and 20 were scored based on differentiation or developmental stages. A significant reduction in mammary differentiation among dams exposed GD 1–17 or 8–17 was evident on PND 10. On PND 20, delays in normal epithelial involution and alterations in milk protein gene expression were observed. All exposed female pups displayed stunted mammary epithelial branching and growth at PND 10 and 20. While control litters at PND 10 and 20 had average scores of 3.1 and 3.3, respectively, all treated litters had scores of 1.7 or less, with no progression of duct epithelial growth evident over time. BW was an insignificant covariate for these effects. These findings suggest that in addition to gestational exposure, abnormal lactational development of dams may play a role in early growth retardation of developmentally exposed offspring.

Key Words: mammary gland; PFOA; lactation; development; pregnancy.

Perfluorooctanoic acid (PFOA), a synthetic perfluorinated eight-carbon organic chemical has broad industrial applications, and is also a final, persistent degradation product of other perfluorinated materials (Prevedouros et al., 2006). The ammonium salt of PFOA has widespread use, particularly in the production of fluoropolymers. These fluoropolymers are highly resistant to degradation, and since the 1940’s have been employed in the production of consumer and industrial goods, including weather- and stain-resistant materials, as well as electrical, aeronautic, communications, and other industrial applications.

Humans may be exposed to PFOA in occupational settings, through environmental exposures, or through contact with consumer goods. Confirmation of widespread exposure in the general population, and the biological persistence of PFOA, has come by way of survey studies of sera from humans (Emmett et al., 2006; Olsen et al., 2003a, 2005) and wildlife (Giesy and Kannan, 2002). The chemical persistence of PFOA, its widespread presence in humans, and the potential for increased health risk in the occupationally exposed have raised regulatory concern, and spurred the addition of PFOA to the list of contaminants to be addressed in the 2003–2004 National Health and Nutrition Examination Survey (National Center for Health Statistics, 2006), and the proposed National Children’s Study (Needham et al., 2005).

Previously (Olson and Andersen, 1983), evidence of the toxicity of PFOA appeared in the scientific literature, and shortly thereafter PFOA was shown to induce hepatic...
peroxisome proliferation in rats (Ikeda et al., 1985). Today, PFOA is characterized as a peroxisome proliferator–activated receptor alpha (PPAR-α) agonist (Intrasuksri et al., 1998; Maloney and Waxman, 1999). This mode of action is expected to lead to hepatotoxicity in rodents, however, a PPAR-α-mediated mechanism for this health outcome is not considered to be relevant in humans (U.S. Environmental Protection Agency [EPA], 2006). Nonetheless, a PPAR-α mode of action may be relevant for other responses (e.g., during fetal and neonatal development), and it is important to consider that other non–PPAR-α-mediated modes of action are possible and may lead to health effects in animals and humans.

Other differences exist between rats and humans with respect to the toxicity of PFOA. One primary example is a gender difference in the elimination of PFOA by rats, whereby female rats excrete PFOA more rapidly than males (Kudo et al., 2002). This preferential excretion does not occur in humans but in rats is mediated by higher renal expression of organic anion transporter 2 (oat2) in the postpubertal female rat compared to the male rat (or either gender of other species), and results in a significantly reduced PFOA half-life in the adult female to about 2 h, one seventieth of the 5.7-day half-life exhibited in an adult male rat (Kudo et al., 2002). Due to this rapid elimination rate, daily dosing of adult female rats leads to episodic burden of PFOA. Thus, the female rat may not be an appropriate animal model for studies addressing the potential reproductive and developmental toxicity in humans posed by PFOA. In contrast, expression of oat2 in the mouse is more consistent with that in humans, and the mouse displays no sex-dependent elimination difference (Lau et al., 2006). Therefore, these data suggest that the mouse may represent a more suitable model than the rat for human health effects, with respect to the reproductive and developmental toxicity of PFOA.

Despite the gender difference in elimination, most PFOA studies have been performed in rats. One 2-year feeding study in rats (Sibinski, 1987) reported a statistically significant increase in mammary fibroadenomas compared to controls but these observations were deemed equivocal, as tumor rates were considered “comparable to some historical background indices” (U.S. EPA, 2006). Additionally, described in this study was an increased occurrence of Leydig cell adenomas, suggesting that PFOA may impact a range of reproductive tissues. Because the mammary fibroadenoma data were regarded as equivocal by the U.S. EPA Science Advisory Board, there have been recommendations to reconsider the possible impact of PFOA on mammary tissue (Kropp and Houlihan, 2005). Recent data from Lau et al. (2006) indicated a decrease in postnatal weight gain in mouse pups following oral exposure on gestation days (GD) 1–17 to 5 mg PFOA/kg body weight (BW), without the dramatic effects on postnatal survival that were seen with higher doses of PFOA (<30% survival among 10 and 20 mg PFOA/kg BW exposures). Because neonatal BW gain is highly dependent upon the quantity and quality of milk received from the dam, these findings may reflect nutritional deficits associated with mammary gland alterations in lactating dams, in utero toxicity, or some combination of the two.

These observations led us to examine the specific effects of PFOA on the mouse mammary gland. In addition to the direct influence of PFOA on developing maternal mammary tissue, the possible role of impaired lactation in PFOA-exposed dams on neonatal BW gain and survival was investigated. Furthermore, the impact of prenatal PFOA exposure on neonatal mammary gland development in female pups was evaluated as a developmental toxicity endpoint. For this purpose, exposure was timed to correspond with known gestational windows of sensitivity for offspring mammary gland development (most lactational mammary gland development in dams occurs during the second half of gestation [GD 9–18], whereas the mammary bud in offspring forms after GD 13 in the mouse; Borellini and Oka, 1989). Hence, the effects of PFOA on differentiation and development of mammary tissue were determined in both the dam and female offspring, in order to describe the as yet unknown effects of PFOA on this important developing tissue, and move toward further characterizing the mode(s) of action of PFOA toxicity in tissues other than the liver.

MATERIALS AND METHODS

Animals

Timed-pregnant CD-1 mice were acquired from Charles River Laboratories (Raleigh, NC). Sperm-positive females were designated GD 0, and delivered on the same day to U.S. EPA’s Association for Assessment and Accreditation of Laboratory Animal Care facilities. Animals were weighed upon arrival and randomly distributed among four treatment groups. Mice were housed individually in polypropylene cages and received food (LabDiet 5001, PMI Nutrition International LLC, Brentwood, MO) and tap water ad libitum. Animal facilities were maintained on a 14:10-h light-dark cycle, at 20–24°C with 40–50% relative humidity. All animals were found to be free of infectious diseases prior to beginning the study. All animal protocols were reviewed and approved by the U.S. EPA’s National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee.

Dosing Solution and Procedures

PFOA as its ammonium salt (>98% pure) was acquired from Fluka Chemical (Steinheim, Switzerland). PFOA dosing solution was prepared fresh daily in deionized water, at a concentration of 0.5 mg/ml. Mice received either water vehicle or PFOA at 5 mg/kg BW by oral gavage, once daily over the dosing periods described below. The dose of 5 mg PFOA/kg BW/day was chosen based on previous work that found this dose to reduce neonatal BW gain (Lau, et al., 2006). This dose was not meant to reproduce average human serum PFOA concentrations in the United States, as the current range of mean serum PFOA in humans is between 5.6 ng/ml (general biomonitoring; Olsen et al., 2005) and 329 ng/ml (no occupational exposure, but potential community exposure; Emnett et al., 2006).

Experimental Design

Sixty dams, divided equally among two blocks, were treated once daily on GD 1–17 (N = 14 dams), 8–17 (N = 16 dams), or 12–17 (N = 16 dams) with 5 mg PFOA/kg BW, or on GD 1–17 with water vehicle (control, N = 14 dams). Dams were weighed daily throughout gestation. At birth, pups were individually weighed and sexed. Pups were pooled and randomly redistributed among the dams of respective treatment groups, and litters were equalized to...
10 pups (both genders represented). Dams which delivered small litters (N < 4 pups) were excluded from the remainder of the study. On postnatal days (PND) 5, 10, and 20 the litters were weighed, and average neonatal BWs were calculated. Half of the dams and respective litters in each treatment group were randomly chosen and necropsied at PND 10. Remaining dams and litters were necropsied on PND 20. Figure 1 graphically depicts this study design.

In a separate study, timed-pregnant CD-1 mice (N = 5 per group) were dosed GD 1–17 with 0 or 5 mg PFOA/kg BW/day and sacrificed on GD 18 to evaluate, via whole mount, the development of the maternal mammary gland prior to parturition and ensuing lactation.

**Necropsy**

Dams and pups were sacrificed on PND 10 or 20 by decapitation and trunk blood was collected and stored at –80°C in polypropylene tubes for dosimetric studies. At the time this study was initiated, no validated protocol for serum PFOA measurements was available to us. Therefore, pup blood and unperfused liver (quick frozen under dry ice) were collected and maintained at –80°C for use in (at the time undetermined) future studies. Extra pups remaining from individual treatment groups at PND 1 (following equalization of litters) were euthanized and livers removed and frozen as stated for future studies. The fourth and fifth inguinal mammary glands were collected from dams and female pups on PND 10 and 20. One side was prepared as whole mounts, and portions of the contralateral glands were placed either in TRI Reagent (Sigma-Aldrich Corp., St Louis, MO) for RNA isolation, or in 10% neutral buffered formalin for histological preparation. Uteri were dissected from dams to determine the number of implantation sites.

**Uterine Implantation**

Upon removal, uteri were placed in phosphate-buffered saline. The number of uterine implantation sites per dam was visually determined by light microscope (Leica WILD M420 microscope, Leica, Wetzlar, Germany), to determine rates of postimplantation loss.

**Mammary Gland Preparations**

Mammary glands were removed on PND 10 and 20 because these time points represent peak lactational output from the dam, and the time that pups begin to wean themselves from the dam, respectively. The entire fourth and fifth glands were removed from dams and female pups, and mounted flat on glass slides. Whole mounts were fixed in Carnoy’s solution, stained in alum carmine stain, and dehydrated and cleared in xylene, as previously described (Fenton et al., 2002). A portion of the contralateral mammary glands was removed on PND 10 and 20, placed in a histology cassette, fixed in 10% neutral buffered formalin for 48 h, and stored in 70% ethanol. The glands were paraffin-embedded and 5-μm sections were prepared and stained with hematoxylin and eosin (H & E). Whole mounts and histological sections were visualized by light microscope (magnification up to ×70).

Mammary gland whole mounts from female offspring were scored on a 1–4 subjective, age-adjusted developmental scale (as described by Rayner et al., 2004; 1 = poor development/structure; 4 = normal development/structure, given age). The developing tissue was assessed for number of primary ducts, number of large secondary ducts, and lateral side branching, appearance of budding from the ductal tree and longitudinal outgrowth of the epithelia. Slides were separated by score as they were evaluated, compared within a score for consistency, and then recorded. Two independent scorers, blind to treatment, scored glands within the age groups. Mean scores for the two ages, within treatment groups, were calculated and analyzed statistically for treatment and time-related differences.

Maternal mammary gland H&E slides were similarly subjectively scored. The differentiated tissue was assessed for amount of epithelial tissue filling the gland, presence of well formed, productive alveoli (lipid/milk), and in the case of the involuting tissue (PND 20), the normal presence of apoptotic bodies and regressing alveoli. Typical mammary glands, representing the mean score for each treatment group, were photographed using the above described macrocope and mounted camera (Photometrics CoolSNAP, Roper Scientific, Inc., Tucson, AZ).

**Real-Time Polymerase Chain Reaction**

Total RNA was extracted from lactating glands using TRI reagent according to the manufacturer’s suggestions, employing two chloroform extractions, and dissolving the RNA pellet in RNase-free water. Samples were digested with DNaseI (Promega M6101; Madison, WI) and quantitated using RiboGreen reagent (Invitrogen R11490; Carlsbad, CA) according to the manufacturer’s instructions. RNA was then reverse transcribed (ABI complementary DNA [cDNA] Archive kit 4322171; Foster City, CA) and 10 ng (for β-casein and α-lactalbumin [α-Lac]) or 50 ng (for epidermal growth factor [EGF] and lactotransferrin [Lactof]) of the corresponding cDNA was amplified in a reaction containing 0.4 mM deoxy-nucleotide triphosphate, 8 mM MgCl₂, 1 × Platinum Taq buffer (Invitrogen 10966-034), 1 U Platinum Taq enzyme, 0.24 μM forward primer, 0.24 μM reverse primer, and 0.12 μM dual-labeled fluorescent probe. Dual-labeled (fluorescein, BHQ) hydrolysis probes were synthesized by IDT (Coralville, IA) according to the sequences shown in Table 1. PCR cycling conditions were an initial 95°C for 3 min, then 40 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 10 s. Quantitative real-time PCR was performed in a BioRad iCycler (LaJolla, CA). All samples were run in duplicate. A cDNA standard was synthesized for each gene and quantitated using PicoGreen (Invitrogen PT589). Known quantities of this cDNA were diluted and amplified in each plate to generate a standard curve for each particular gene. Each RNA sample was compared to the gene-specific standard cDNA curve to determine relative copy number.

**Dosimetry**

**Blood.** After collection, blood specimens were stored at –80°C until shipped on dry ice to the Centers for Disease Control and Prevention’s (CDC) National Center for Environmental Health, and frozen at –40°C until analysis. PFOA in blood was semiquantitatively measured through a multiple reaction monitoring experiment using online solid-phase extraction coupled to reversed-phase high-performance liquid chromatography-tandem mass spectrometry.

![Study Timeline](https://example.com/study_timeline.png)

**FIG. 1.** Study design. Time-pregnant CD-1 mice received 5 mg PFOA/kg BW/day for different periods of gestation, depicted by bar lengths, ending on GD 17. Stars represent BW data collection (for both dams and pups after PND 1). Other data collection time points are also shown.
(HPLC/MS), as described (Kuklenyik et al., 2005). The necessary dilution of blood samples was performed in two steps. First, at least 10 µl of blood was diluted to 1 ml with water in a 2-ml polypropylene tube, then a second dilution was performed by aliquoting the appropriate amount of the diluted sample into an autosampler vial and adding blank calf serum (calibration standards also contained blank calf serum). The diluted blood sample was further diluted with 0.1M formic acid and injected into a commercial column switching system allowing for concentration of PFOA on a C18 solid-phase extraction column. The column was automatically positioned in front of a C8 analytical HPLC for chromatographic identification of PFOA.

Detection and quantification utilized negative-ion TurboIonSpray ionization, a variant of electrospray ionization, tandem MS. The isotope-labeled internal standard used for quantification was 1,2-13C2-PFOA. Quality control (QC) materials, prepared in calf serum, were analyzed with the samples to ensure the accuracy and reliability of the data (Kuklenyik et al., 2005). The analytical batch consisted of nine calibration standards and 61 samples (including two high-concentration QCs [QCH], two low-concentration QCs [QCL], two reagent blanks, and one serum blank). The concentrations of the two QCH and the two QCL were evaluated and averaged to obtain one measurement of QCH and of QCL per batch, using standard statistical probability rules. Because of the high dilution factors and the fact that blood instead of serum was used for the measurements (the analytical method is validated for serum), only estimated values of PFOA concentration ranges are provided.

Ranges of PFOA concentration (ng/ml) were reported and were rounded up to create five categories, (<100 = 100, 100–499 = 500, 501–1000 = 1000, 1001–10,000 = 10,000, 10,001–20,000 = 20,000, >20,000 = 50,000) in order to calculate an estimated mean for each treatment group. These values were rounded down so that the values in the lowest concentration range did not appear to be 1 ng/ml (near the limit of detection of 0.1 ng/ml). PFOA ranges in dams and their pups within this study can be directly compared, and changes in burden over treatment times can be compared but absolute blood concentrations could not be determined in this study, and statistical differences were not calculated.

Liver. Livers from PND 1, 10, and 20 offspring were weighed and analyzed at the U.S. EPA National Exposure Research Laboratory for PFOA concentration using a method that is a variation of the procedure described by Hansen et al. (2001). 1,2-13C2-PFOA was purchased from Perkin-Elmer (Wellesley, MA) and used as the internal standard for quantitative analyses. Pentadecafluorooctanoic acid ammonium salt was obtained from Fluka and used as an unlabeled standard. Liver samples were homogenized in water. If the tissue sample was sufficiently large the proportions were 1 g tissue + 6 ml water. Smaller tissue samples were homogenized as 10 mg tissue + 100 µl of water. Twenty-five microliters of the homogenate was added to a 15 ml polypropylene tube along with 1 ml of 0.5M tetrabutylammonium hydrogen sulfate (pH 10) and 2 ml of 0.25M sodium carbonate. The mixture was vortexed for 20 min. Three hundred microliters of this mixture was transferred to a fresh 15 ml polypropylene tube and 25 µl of 1,2-13C2-PFOA (1ng/µl) was added. Five milliliters of MTBE [methyl tert-butyl ether] was added and the mixture was vortexed for 20 min. The tube was centrifuged for 3 min at 3500 rpm. One milliliter of the MTBE layer was withdrawn and transferred to a 5-ml polypropylene tube and evaporated to dryness at 45°C with a gentle stream of nitrogen. The residue was dissolved in 400 µl 1:1 with 2mM ammonium acetate-acetonitrile, placed into an autosampler vial and analyzed by HPLC-MS/MS. Samples were analyzed using an Agilent 1100 HPLC system coupled with a Sciex API 3000 triple quadrupole MS. The HPLC was equipped with a Phenomenex (Torrance, CA) Luna C18(2) 50 × 3.0 mm, 5-µm pore size column. Samples were chromatographically separated using an isotropic mobile phase consisting of 1:1 mix of 2mM ammonium acetate and acetonitrile at a flow rate of 200 µl/min. The Sciex API 3000 MS was operated in the MS/MS mode using negative-ion TurboIonSprayJ ionization. The transitions monitored were PFOA (m/z 413–369) and 13C2-PFOA (m/z 415–370). Area counts for each analyte are determined automatically using the Analyst software provided with the API 3000. Area ratios (AR) of analyte to internal standard were used in the construction of matrix matched calibration curves (r² > 0.99). Quantitation of PFOA in unknown samples and quality assurance/QC samples were derived from the AR predicted by the calibration curves. The lowest standard curve point was 500 ng/g for the PND 20 livers and 250 ng/g for the PND 10 and 1 liver samples (represents the limit of quantification). The amount of PFOA is presented on a liver weight basis.

**Table 1**

<table>
<thead>
<tr>
<th>Primer and probe sequences for Milk Protein Gene Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
</tr>
<tr>
<td>Gene transcript</td>
</tr>
<tr>
<td>β-Casein</td>
</tr>
<tr>
<td>EGF</td>
</tr>
<tr>
<td>β-Lac</td>
</tr>
<tr>
<td>LactoF</td>
</tr>
</tbody>
</table>

**RESULTS**

**Body Weight**

Maternal weight gain during pregnancy was similar between groups, and no effect of PFOA exposure or period of treatment...
was apparent (Table 2). Visual examination of uteri following necropsy allowed determination of total number of uterine implantation sites. No effect of treatment on mean number of implantation sites, live pups born, and embryonic/fetal loss rates was observed (Table 2).

A comparison of pup BW over time and exposure period is also shown in Table 2. On PND 1, BWs among prenatally PFOA-exposed pups were significantly reduced in an exposure duration-dependent manner by 3% (GD 12–17), 7% (GD 8–17), and 12% (GD 1–17), compared to controls ($p < 0.001$). On PND 5, mean BWs for PFOA-exposed pups were further reduced compared to controls (by 23, 35, and 40%, respectively; $p < 0.001$), also in an exposure duration-dependent manner. This effect was sustained throughout the lactational period in all PFOA-treated groups, as treated offspring remained smaller than controls at both PND 10 (by 25, 31, and 39%, respectively; $p < 0.05$) and PND 20 (by 26, 27, and 33%, respectively; $p < 0.001$). The BW deficit compared to controls, within each treated group, was maximal by PND 5. Taken together, the lack of effect of PFOA exposure on the mean number of implantation sites, numbers of live pups born, percent preimplantation loss, and maternal weight gain suggest that the observed reductions in neonatal BW at birth and throughout lactation were not due to general maternal toxicity (as defined in Case et al., 2001) or to a difference in pup number per dam.

Lactating Mammary Gland Development

Mammary glands from dams on PND 10 and 20 were examined for morphological differentiation, which included subjective scoring of H & E–stained sections, as well as a comparison of mammary whole mounts. On PND 10, typically the peak of lactation in rodents, dams treated during GD 8–17 and GD 1–17 exhibited significant visible delays in epithelial differentiation (Fig. 2A; GD 8–17 not shown) and developmental scores compared to controls (Table 3, $p < 0.05$ and $p < 0.001$, respectively), and these glands morphologically resembled those of dams days earlier in lactation. Altered differentiation was visible in glands of GD 12–17 exposed dams, however, these changes were not statistically significant with respect to score. In the affected glands, excessive adipose tissue remained, whereas in the control dam the gland was fully occupied by well differentiated, extensive milk-filled alveoli. In lactating dams examined at PND 20, control glands displayed signs of involution, normally found at this developmental time point, immediately prior to weaning. By contrast, glands from dams in all PFOA exposure groups at PND 20 had few apoptotic bodies, little visible adipose tissue, and displayed milk-filled alveoli, appearing quite similar to the glands of control animals at PND 10 (peak lactation). Therefore, in addition to a treatment-related effect ($p < 0.05$), there was also a significant effect of time within exposure parameter ($p < 0.05$, significant time $\times$ treatment interaction) on the postnatal course of mammary gland differentiation and lactation (evident from mean scores in Table 3). These data suggest a substantial delay (possibly up to 10 days) in differentiation of the glands from dams exposed to PFOA throughout gestation.

To determine if delayed differentiation of the mammary glands in PFOA-exposed dams resulted from their gestational exposure, or from an effect of PFOA on the thriftiness of pups—that is, their ability to suckle and sufficiently stimulate lactation—glands of GD 1–17 dams were evaluated on GD 18 by whole mount analysis (mean score $\pm$ SE: control $= 3.9 \pm 0.1$; GD 1–17 $= 2.0 \pm 0.6$; $p < 0.015$). As shown in Figure 2B, control glands were saturated with milk-filled alveoli, whereas

## TABLE 2
Maternal Indices and Offspring BW

<table>
<thead>
<tr>
<th>PFOA exposure periods</th>
<th>Control</th>
<th>GD 12–17</th>
<th>GD 8–17</th>
<th>GD 1–17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal indices and offsprin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gestational weight gain (g)</td>
<td>24.8 ± 1.9</td>
<td>23.5 ± 1.4</td>
<td>25.6 ± 0.9</td>
<td>27.1 ± 0.8</td>
</tr>
<tr>
<td>Implants (# per live litter)</td>
<td>11.7 ± 1.0</td>
<td>10.9 ± 0.9</td>
<td>11.8 ± 0.7</td>
<td>14.0 ± 0.9</td>
</tr>
<tr>
<td>Live fetuses (# per live litter)</td>
<td>10.8 ± 1.0</td>
<td>10.2 ± 0.8</td>
<td>11.1 ± 0.6</td>
<td>11.1 ± 1.0</td>
</tr>
<tr>
<td>Neutal BW (g) at age</td>
<td>7.7 ± 3.0</td>
<td>5.7 ± 2.2</td>
<td>5.9 ± 2.0</td>
<td>20.3 ± 5.1***</td>
</tr>
<tr>
<td>PND 1</td>
<td>1.65 ± 0.02</td>
<td>1.60 ± 0.01***</td>
<td>1.53 ± 0.02***</td>
<td>1.45 ± 0.03***</td>
</tr>
<tr>
<td>PND 5</td>
<td>3.8 ± 0.1</td>
<td>2.9 ± 0.1***</td>
<td>2.5 ± 0.1***</td>
<td>2.3 ± 0.2***</td>
</tr>
<tr>
<td>PND 10</td>
<td>6.8 ± 0.3</td>
<td>5.1 ± 0.4*</td>
<td>4.7 ± 0.2**</td>
<td>4.1 ± 0.4*</td>
</tr>
<tr>
<td>PND 20</td>
<td>11.6 ± 0.2</td>
<td>8.6 ± 0.6***</td>
<td>8.4 ± 0.4***</td>
<td>7.7 ± 0.4***</td>
</tr>
</tbody>
</table>

Note. Data presented are mean ± SE. Maternal weight gain is shown for entire period of GD 1–17. N = 7–11 litters per treatment group at PND 1 and 5. N = 4–6 dams per litters per treatment group at PND 10. N = 3–6 dams per litters per treatment group at PND 20.

*Significant treatment effect by ANOVA; $p < 0.05$.

**Significant treatment effect by ANOVA; $p < 0.01$.

***Significant treatment effect by ANOVA; $p < 0.001$. 

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the PFOA-exposed glands were not, and exhibited stunted alveolar development (spiked appearance). These observations suggest that this alteration in functional mammary gland differentiation occurs prior to stimulation of the gland by offspring.

Milk Protein Gene Expression

To assess the association of altered milk content with reduced weight gain among PFOA-exposed offspring during lactation, and to evaluate physiologic aspects of lactation in dams, gene expression profiles of several milk protein genes were evaluated at PND 10 and 20 in maternal mammary tissue. Gene expression patterns for milk proteins in maternal mammary glands, shown as percentage difference from control levels at the same time point, can be seen in Figures 3 and 4. For comparative purposes, the number of molecules of each milk protein transcript is reported in Table 4. β-Casein and EGF are proteins normally present in milk, reaching their peak concentrations by PND 10 and PND 6, respectively (Baruch et al., 1995; Grueters et al., 1985). Although isolated significant differences were apparent, there was no clear and consistent pattern of effect of PFOA exposure on gene expression levels for these two milk proteins. The differences that were apparent were greatest between the intermediate-duration treatment exposure group (GD 8–17) and the control group, and did not occur in an exposure duration-dependent manner.

α-Lac is a common whey protein in milk, reaching peak concentrations between PND 6 and 10 (Grigor et al., 1990). On PND 10, mammary tissue from dams with the shortest exposures (GD 12–17) exhibited significantly reduced expression of α-Lac (p < 0.001). On PND 20, however, α-Lac expression was recovered in this treatment group. LactoF is a protein with antimicrobial properties that is commonly present in milk, with highest levels early and late in lactation (when the risk of infection is highest; Grigor et al., 1990). On PND 10, expression of LactoF was elevated in gland extracts from dams with the longest exposures (p < 0.01), suggesting that the early lactation peak of this milk protein was delayed in the GD 1–17 treatment group. By PND 20, however, this

FIG. 2. Development of maternal mammary glands of mice exposed to PFOA on GD 1–17. Glands were removed and prepared on PND 10 and 20, or GD 18. (A) H & E–stained sections of mammary glands at ×70 showing significantly (p < 0.001 at PND 10) delayed development stemming from PFOA exposure (N = 4–6 dams per treatment group at PND 10, N = 3–6 dams per treatment group at PND 20). (B) Whole mount preparations of mammary tissue from dams on GD 18 at ×20 (N = 4 control dams, N = 3 GD 1–17 PFOA dams). Glands shown are representative of mean respective scores (Table 2). Arrows point to adipose tissue.
pattern was reversed, and GD 1–17 dams, as well as dams with shorter exposures (GD 8–17, GD 12–17), exhibited significantly lower LactoF expression compared with control animals, suggesting again that the late lactation peak of this milk protein had not yet taken place. While this may initially appear to conflict with the histologic findings, a closer examination suggests that LactoF levels on PND 20 in exposed animals are quite similar to control levels on PND 10 (Table 4), which is consistent with the postulated 10-day delay in mammary gland development.

### Blood PFOA Concentration Range

At the time the animal experiments were conducted, no validated method for measurement of PFOA in blood was available to the authors, and thus whole blood and liver were collected at necropsy in order to preserve all possible sources of information. Although a validated method for the quantitative measurement of PFOA in serum now exists (Kuklenyik et al., 2005), a validated method for blood was not available. Because we felt it was important to compare relative levels of PFOA in the dams and offspring within this study, trunk blood, collected at necropsy, was diluted to semiquantitatively determine the concentration range of PFOA. PFOA concentration ranges on PND 10 and 20 in maternal and neonatal blood are shown in Figure 5A. Instead of decreasing over time as is seen in the serum of adult, nonlactating females (Lau et al., 2006), maternal blood PFOA concentration ranges remained fairly constant between PND 10 and 20, within a treatment group, and increasing duration of gestational exposure was correlated with increased blood concentration ranges postnatally. However, a drop in maternal blood PFOA in our study may have been disguised by the concentration ranges to which values were assigned. On PND 10, offspring consistently exhibited higher blood PFOA concentrations than dams but by PND 20 dams exhibited similar or higher (for GD 1–17 only) blood PFOA concentration ranges than offspring. Therefore, unlike their dams, the offspring in these studies decreased their blood PFOA concentrations between PND 10 and 20 by nearly half. Our blood PFOA estimations were consistent with the concentrations in mouse serum under the same dose and exposure length (Wolf et al., in press; at weaning, 5 U + L dam = 36,900 ng/ml and pup = 22,114 ng/ml vs. our PND 20 dam = 45,000 ng/ml and pup = 33,000 ng/ml), and obtained

### TABLE 3

Mammary Gland Developmental Scores at PND 10 and 20

<table>
<thead>
<tr>
<th>PFOA exposure parameters</th>
<th>Control</th>
<th>GD 12–17</th>
<th>GD 8–17</th>
<th>GD 1–17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactating maternal MG at time</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 10</td>
<td>4.0 ± 0.0</td>
<td>3.7 ± 0.1</td>
<td>3.2 ± 0.2*</td>
<td>1.8 ± 0.5***</td>
</tr>
<tr>
<td>PND 20</td>
<td>2.7 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Developing neonatal MG at age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 10</td>
<td>3.1 ± 0.2</td>
<td>1.7 ± 0.1***</td>
<td>1.4 ± 0.1***</td>
<td>1.6 ± 0.2***</td>
</tr>
<tr>
<td>PND 20</td>
<td>3.3 ± 0.2</td>
<td>1.4 ± 0.1***</td>
<td>1.5 ± 0.1***</td>
<td>1.8 ± 0.3***</td>
</tr>
</tbody>
</table>

*Significant treatment effect by ANOVA; *p < 0.05.
***Significant treatment effect by ANOVA; **p < 0.001.

*Note.* Data presented are mean ± SE. Scores are on 1–4 scale; criteria adjusted for stage of development and age. N = 4–6 dams per litters per treatment group at PND 10. N = 3–6 dams per litters per treatment group at PND 20. MG = mammary gland.

**FIG. 3.** PND 10 milk protein gene expression in mammary gland tissue of dams exposed to PFOA for various periods ending on GD 17. Gene expression is depicted as percent difference from mean control tissue expression of respective genes. Statistical significance of number of starting molecules (Table 3) is depicted by asterisks, where *p < 0.05, and ***p < 0.001 (N = 4–6 dams per treatment group).

**FIG. 4.** PND 20 milk protein gene expression in mammary gland tissue of dams exposed to PFOA for various periods ending on GD 17. Gene expression is depicted as percent difference from mean control tissue expression of respective genes. Statistical significance of number of starting molecules (Table 3) is depicted by asterisks, where ***p < 0.001, and where ‡p < 0.055 (N = 3–6 dams per treatment group).
TABLE 4
Milk Protein Gene Expression in Lactating MG on PND 10 and 20

<table>
<thead>
<tr>
<th>PFOA exposure parameters</th>
<th>Control</th>
<th>GD 12–17</th>
<th>GD 8–17</th>
<th>GD 1–17</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Casein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 10</td>
<td>62.3 ± 4.8E + 07</td>
<td>55.1 ± 5.9E + 07</td>
<td>73.3 ± 5.1E + 07*</td>
<td>68.0 ± 5.9E + 07</td>
</tr>
<tr>
<td>PND 20</td>
<td>8.7 ± 0.8E + 07</td>
<td>10.7 ± 1.9E + 07</td>
<td>8.2 ± 0.6E + 07</td>
<td>11.2 ± 1.7E + 07</td>
</tr>
<tr>
<td>EGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 10</td>
<td>2.5 ± 0.8E + 04</td>
<td>8.4 ± 1.4E + 04</td>
<td>16.4 ± 1.6E + 04***</td>
<td>10.7 ± 0.7E + 04*</td>
</tr>
<tr>
<td>PND 20</td>
<td>4.0 ± 0.5E + 04</td>
<td>0.0 ± 0.2E + 04</td>
<td>4.0 ± 0.2E + 04</td>
<td>8.4 ± 0.2E + 04‡</td>
</tr>
<tr>
<td>α-Lac</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 10</td>
<td>42.1 ± 2.3E + 06</td>
<td>30.7 ± 4.0E + 06***</td>
<td>38.5 ± 4.3E + 06</td>
<td>42.7 ± 3.0E + 06</td>
</tr>
<tr>
<td>PND 20</td>
<td>2.4 ± 0.3E + 06</td>
<td>2.5 ± 0.8E + 06</td>
<td>1.7 ± 0.2E + 06</td>
<td>2.1 ± 0.4E + 06</td>
</tr>
<tr>
<td>LactoF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 10</td>
<td>11.8 ± 2.5E + 06</td>
<td>9.4 ± 1.3E + 06</td>
<td>16.6 ± 3.0E + 06</td>
<td>26.3 ± 7.3E + 06*</td>
</tr>
<tr>
<td>PND 20</td>
<td>41.1 ± 10.5E + 06</td>
<td>19.4 ± 8.3E + 06***</td>
<td>8.6 ± 2.1E + 06***</td>
<td>10.4 ± 4.0E + 06***</td>
</tr>
</tbody>
</table>

MG = mammary gland.

*Significant treatment effect by ANOVA; p < 0.05.
‡p = 0.055.
***Significant treatment effect by ANOVA; p < 0.001.

using the validated serum method (Kuklenyik et al., 2005). However, we expected that the serum levels reported in Wolf et al. (in press) would slightly exceed our estimates of PFOA in blood (and they did not), in accordance with reports of direct comparisons of those media in humans (Ehresman et al., 2006). This may be due not to the matrix but to the high dilution factor involved in obtaining the blood PFOA concentrations (making it necessary to group the data, and possibly forcing the means slightly higher than they would be if grouping hadn’t been required).

Due to the semiquantitative nature of the blood data, we also quantitatively analyzed the PFOA content in livers of the PND 1, 10, and 20 offspring. This was done to (1) clarify the relationship between blood and liver PFOA levels within offspring of a particular treatment group and (2) determine that the liver PFOA levels in the offspring followed the same trend as the blood PFOA levels within and between treatment groups, thereby lending credence to the blood data. Measured accuracy for matrix-matched high QC samples was 98.1% (cv. 5.8%) and for low QC samples 107.3% (cv. 4.1%). Replicate analysis of unknown liver samples (n = 26) over the course of analysis had a cv. of 6.3%. Values that fell below the level of quantitation are reported as 0 (controls only). Figure 5B graphically demonstrates that the liver PFOA concentration in prenatally exposed female offspring was very large and higher than the blood concentrations regardless of time since exposure (PND 1, 10, or 20) or amount of time exposed (exposure period). Further, the liver PFOA concentrations on PND 1 and 10 are statistically similar within an exposure group, and decreased significantly to nearly ½ their original value by PND 20 in all exposure groups. The mean liver:blood PFOA concentration ratios were 2.5 at PND 10 (for all exposure groups) and varied between 1.9 and 3.0 at PND 20.

Offspring Mammary Gland Development

To determine if prenatal exposure of pups to PFOA, even for only 6 days (1/3 of gestation), resulted in altered mammary gland development, whole mounts from female offspring at PND 10 and 20 were prepared and analyzed. The effects of exposure duration on morphological development were of particular interest. Mean developmental scores for the pups under different exposure parameters at PND 10 and 20 are shown in Table 3, and Figure 6 depicts representative glands that correspond to the mean scores within each group. Mammary gland epithelial branching and longitudinal growth was significantly stunted among all PFOA-exposed offspring at both PND 10 and 20, compared to control (Table 3, p < 0.001). Normal progression of growth was observed in control tissues between PND 10 and 20, with mean developmental scores of 3.1 and 3.3, respectively. All treated groups lacked this normal progression, and had mean scores of 1.7 or less. Despite the growth retardation noted in exposed offspring (Table 2), BW was not a significant covariate for the observed mammary effects. Furthermore, while it was reduced, neonatal growth did occur and BW was gained between PND 10 and 20 in the PFOA-exposed pups (weight increased from PND 10 to PND 20 by 1.7-, 1.7-, 1.8-, and 1.9-fold in controls, GD 12–17, 9–17, and 1–17 PFOA-exposed pups, respectively) but virtually no mammary gland development occurred during this period of normally allometric growth (there was an insignificant time × treatment interaction). In addition, the relative blood and liver
levels of PFOA in pups decreased over this time, suggesting that these PFOA concentrations were sufficient to significantly affect mechanisms necessary for early epithelial outgrowth and branching.

**DISCUSSION**

This study demonstrated that a 5 mg PFOA/kg BW/day exposure delivered throughout (GD 1–17) or during the second half of gestation (GD 8–17) was sufficient to significantly alter normal lactational development of maternal mammary glands in the absence of effects on maternal weight gain during the dosing period. Furthermore, this dose of PFOA for as short an exposure as the final 6 days of gestation (GD 12–17) was sufficient to significantly retard neonatal growth and halt female pup mammary epithelial proliferation. The delay in functional differentiation of the lactating mammary gland may be a direct effect of PFOA on the gland or may indicate that offspring are not sufficiently thrifty as to stimulate normal lactational development.

The low PND 1 neonatal BWs observed in this study are in agreement with the reports of Lau et al. (2006) and Wolf et al. (in press). Reduced PND 1 BWs among PFOA-exposed pups suggest that gestational PFOA exposure alone may be sufficient to impair neonatal growth and development. This conclusion is supported by the findings of a collaborative cross foster study (Wolf et al., in press), in which pups exposed to 5 mg PFOA/kg BW/day from GD 1–17 and fostered to a control dam exhibited significant deficits in total BW gained between PND 1 and PND 22 (although, female pups born to control dams and fostered to dams exposed to 5 mg PFOA/kg BW/day from GD 1–17 also exhibited significant BW deficits at PND 2–4 and PND 22). The effects of PFOA exposure on neonatal growth may impact the ability of offspring to nurse from dams, thus interfering with the feeding stimulation essential for optimal lactation, and impacting lactational mammary gland differentiation and nutritional transfer to the pups. Support for this idea is provided in this study by the significant delays observed in lactational mammary gland development of PFOA-exposed dams by PND 10, which is associated chronologically with the period of neonatal life that exhibited the most dramatic PFOA-induced growth retardation.

Conversely, observations of mammary glands isolated from GD 18 dams in on-going studies, under the same exposure dose and time, suggest that PFOA exposure induces delayed mammary gland differentiation prior to parturition. Thus, altered gland development cannot be entirely accounted for by underdeveloped offspring and insufficient stimulation by suckling. It is also possible that even with the morphological effects of PFOA on the lactating gland, sufficient functional capacity remains, such that the growth and development of litters may be supported. The number of molecules encoding four major milk proteins was not different in an exposure duration-dependent manner between control and PFOA-exposed animals, suggesting that the normal differentiation of the gland was not physiologically altered but that the amount of epithelium producing the proteins (and thus total milk output) was diminished due to the delayed process. The present studies have not yet addressed the volume of milk produced by PFOA-exposed dams. Unresolved issues regarding the mediation of neonatal growth deficits are under study in our laboratory and will include examination of the mammary glands from dams in a cross-foster paradigm.

A novel finding and another factor for consideration with respect to altered lactational development is the PFOA blood level of lactating dams, which remains high and relatively
unchanged between PND 10 and 20. These elevated levels of PFOA may be directly involved in delayed differentiation of the dam mammary gland. Maternal grooming practices may be responsible for steady state PFOA burden, as it is likely the cleaning of offspring and stimulating micturition results in continued maternal reingestion of PFOA excreted in the urine of the litter during the first 10–12 days of life. This recycling of PFOA, as suggested by dosimetric findings, may keep maternal blood PFOA concentrations in a near steady state, at levels correlated with exposure duration.

A comparison of liver and blood PFOA levels within the exposed pups of this study demonstrates a similar trend. High PFOA levels were detected in both on PND 10 and dropped by about half by PND 20, even though the liver concentrations were consistently higher than those in blood (2.5-fold on PND 10 and ranging from 1.9- to 3.0-fold on PND 20). These values agree with reports of the mean liver to serum ratio in human samples (mean of 1.3:1 in paired samples, but ranged from 0.2:1 to 3.4:1 in individual samples; Olsen et al., 2003b). It does appear, based on our quantitative data from pup livers, that the levels of PFOA in the pup remain elevated and steady between PND 1 and 10, the time during which the offspring require maternal assistance in elimination of body wastes. If PFOA does impact lactational development through a direct alteration of normal cell-signaling pathways, a constant high blood level could provide a substantial contribution to delayed lactational development, in addition to the contributions of gestational exposures and impaired stimulation by exposed offspring. Importantly, this may explain why delays in lactational development are seen primarily in the dams which received the longest exposures—and thus maintained the highest blood levels—even though all exposed offspring showed significant deficits in neonatal weight gain, and potentially thriftiness.

Since PFOA is a known PPARα agonist, effects on the mammary gland may involve this pathway. Overexpression of PPARα, using a keratin 5 promoter, causes severe neonatal mortality due to a lactational defect in the dam (Yang et al., 2006). The effect of this targeted overexpression to the myoepithelial cells of the mammary alveoli was most pronounced after parturition causing lobulo-alveolar units to be small and malformed, yet had no effect on expression of some of the common milk genes. Although there is a fair chance that the mode of action mediating the lactational defects seen in the current study may be through activation of PPARα, the similarities to the importance or role of PPARα in the human breast are unknown. Also, it is known that PPARα is not a critical element of mammary gland development in the neonate, as PPARα null mice exhibit normal mammary gland development and function (Lee et al., 1995). Therefore, any effects of gestational PFOA exposure on neonatal mammary tissue must not be mediated through this pathway.

The dose of PFOA used in this study, even for only the final 6 days of gestation, was also able to significantly stunt mammary gland development in female offspring. Most interestingly, development of mammary tissue between PND 10 and 20 in these offspring did not appear to be simply delayed but rather altogether arrested during a window of development where the gland should be growing at least isometrically with the body. The observation of these notable mammary gland alterations in pups under all exposure periods suggests that a critical window of sensitivity in mammary gland development may exist after GD 12 and possibly postnatally. Furthermore, because PFOA can transfer to the milk (Mylchreest, 2003) exposure of pups is presumed to continue after dosing ceases and lactation begins. Therefore, the respective contributions of late gestational and lactational PFOA exposures to these mammary alterations cannot be discerned from these data but are currently being pursued. Whether this stunting of the neonatal gland is compensated for at the conclusion of puberty, or has lasting effects on either the ability to maintain a litter or on the risk of mammary gland pathologies, are important issues being addressed in on-going studies (in collaboration with Wolf et al., in press).

While previous studies have observed effects on the mammary gland and other reproductive tissue following adult PFOA...
exposure (Sibinski, 1987), no one has yet reported effects of PFOA on reproductive tissues following gestational exposures. Further, this study is the first to report findings of PFOA-induced toxicity in mouse reproductive tissues. Additionally, this study identified that the developmental stage at the time of exposure—both in dams and offspring—determines the tissue toxicity (nonpregnant adult females that received PFOA for up to 17 days showed no changes in mammary tissue; S. Fenton, data not shown). This toxicity is not specific to females in general but is specific to lactating mothers and the developing fetus, which represent susceptible subpopulations. These findings corroborate other reports, which have identified a critical period preceding lactation in which the mammary gland is particularly sensitive to the early branching and migration in the female offspring (Enoch, R. R., Chepko, G., Clarke, R., Sholler, P. F., Lirio, A. A., Foss, C., et al. (1998)).

Our current challenge is to identify the mechanisms by which PFOA affects this dynamic tissue. As the present study has shown, gestational exposure of the mouse to PFOA clearly alters normal differentiation of the lactating gland in the dam, and the early branching and migration in the female offspring (with the latter being the more prominent in this study). However, the processes which regulate these two stages of development differ, and therefore PFOA may not mediate the observed changes by the same mechanism. Once the mechanisms which mediate these alterations in the murine mammary gland are further elucidated, the influence of PFOA on the human breast and ultimately functional lactation, may be better estimated.

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REFERENCES


