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Definitions:

F1	First filial generation
F2	Second filial generation
GD	Gestational day(s)
LOAEL	Lowest observable adverse effect level
P0	Parental generation
PFAA	Perfluoroalkyl acids
PFOA	Perfluorooctanoic Acid
PND	Postnatal day(s)
1 ppb	= 1 µg/L = 1 ng/ml
SE	Standard error of the mean

Abstract:

Background: Prenatal exposure to perfluorooctanoic acid (PFOA), a ubiquitous industrial surfactant, has been reported to delay mammary gland development in female mouse offspring (F1) and the treated lactating dam (P0) following gestational treatments at 3 and 5 mg PFOA/kg/day.

Objective: Our study aimed to determine the consequences of gestational and chronic PFOA exposure on F1 lactational function, and subsequent development of F2 offspring.

Methods: We treated P0 dams with 0, 1, or 5 mg PFOA/kg/day on gestation days 1-17. In addition, a second group of P0 dams treated with 0 or 1 mg/kg/day during gestation and their F1 and F2 offspring received continuous PFOA exposure in drinking water at 5 ppb. Resulting adult F1 females were bred to generate F2 offspring, whose development was monitored over postnatal days (PND) 1-63. F1 gland function was assessed on PND 10 by timed-lactation experiments. Mammary tissue was isolated from P0, F1, and F2 females throughout the study and histologically assessed for age-appropriate development.

Results: PFOA-exposed F1 dams exhibited diminished lactational morphology, though F1 maternal behavior and F2 offspring body weights were not significantly affected by P0 treatment. In addition to reduced gland development in F1 females under all exposures, F2 females with chronic low-dose drinking water exposures exhibited visibly slowed mammary gland differentiation from weaning onward. F2 females derived from 5 mg/kg PFOA-treated P0 dams displayed gland morphology similar to F2 chronic water exposure groups on PNDs 22-63.

Conclusions: Gestational PFOA exposure induced delays in mammary gland development and/or lactational differentiation across three generations. Chronic, low-dose PFOA exposure in drinking water was also sufficient to alter mammary morphological development in mice, at concentrations approximating those found in contaminated human water supplies.

Introduction

Perfluorooctanoic acid (PFOA) is a fully-fluorinated eight-carbon perfluoroalkyl acid (PFAA), with a carboxylic acid functional group. As with other PFAAs, PFOA is utilized in the production of fluorochemicals, which have extensive commercial applications (Prevedouros et al. 2006). PFOA is also a final breakdown product of certain fluorochemicals, and resists degradation in the ambient environment by biota or physical processes (Martin et al. 2005). The ubiquity of fluorochemicals in the marketplace, combined with the persistence of PFOA in the environment, may explain current widespread PFOA-contamination of humans and wildlife (Harada et al. 2004; Giesy and Kannan 2002; Martin et al. 2004).

The average non-occupationally exposed American exhibits measurable serum PFOA, varying between a mean concentration of 3.9 ng/ml among participants in the 2003-2004 National Health and Nutrition Examination Survey (Calafat et al. 2007) and 2.2 ng/ml in 2005 among a smaller group of Red Cross blood donors (Olsen et al. 2007). Occupational exposure can raise serum concentrations more than 200 times this approximate range (Emmett et al. 2006). In the Little Hocking district of Ohio and West Virginia where the municipal drinking water supply was contaminated with PFOA at 3.55 ng/ml (ppb) by nearby production plants, mean human serum concentrations were 423 ng/ml (Emmett et al. 2006). Thus, non-occupationally exposed Americans may receive substantial unforeseen exposures to PFOA. It is not known, however, whether adverse adult health effects could result from these chronic, low-level exposures beginning in early life. This is of particular interest with respect to development, because the potential toxicity of PFOA in humans remains uncharacterized.

Mouse studies have demonstrated the capacity for gestational PFOA exposure to yield developmental toxicity (Lau et al. 2004; Lau et al. 2006; Wolf et al. 2007). The mammary

gland, specifically, has proven to be a sensitive tissue with respect to the developmental endpoints addressed, including functional lactation, milk protein gene expression, and developing neonatal and peripubertal structures (White et al. 2007; White et al. 2009; Yang et al. 2009; Zhao et al. 2010). In outbred CD-1 mice, treatment with 3 mg/kg PFOA during pregnancy resulted in delayed gland development among offspring, which persisted into adulthood, even among offspring with only lactational exposures (White et al. 2009). Another laboratory examined similar dose ranges, following peripubertal exposures (21 to 50 days of age) in two inbred mouse strains, C57Bl/6 and Balb/C. They observed a similar inhibitory effect on mammary gland development in Balb/C mice (Yang et al. 2009), while C57Bl/6 females exhibited stimulatory or inhibitory effects depending upon dose (Yang et al. 2009; Zhao et al. 2010). These observations illustrate the influence not only of dose, but also of exposure timing and genetic background, while confirming that the mammary gland represents a sensitive tissue in multiple mouse strains.

To understand the extended consequences of altered mammary gland development, we performed a multigenerational study examining the ability of the developmentally exposed females to provide lactational support for their litters. To address the human relevance of the route, dose, and duration of exposures employed in our studies, we included a chronic low-dose exposure.

Materials and Methods

Animals. Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). Sperm-positive females (GD 0) were weighed upon arrival at the US EPA, housed individually in polypropylene cages, and received food (LabDiet 5001, PMI Nutrition

International LLC, Brentwood, MO) and tap water *ad libitum* in polyethylene water bottles sealed with rubber stoppers and stainless-steel sipper tubes, as specified in White et al., 2009. Animal protocols were approved by the US EPA's Institutional Animal Care and Use Committee. Animals were treated humanely, with regard for alleviation of suffering.

Dosing solutions. PFOA (ammonium perfluorooctanoate; > 98% pure) was purchased from Fluka Chemical (Steinhiem, Switzerland). PFOA was dissolved by agitation in de-ionized water at concentrations of 0.1 and 0.5 mg/ml (for 1 and 5 mg/kg doses, respectively), and prepared fresh daily, immediately prior to administration. PFOA-containing drinking water was prepared similarly, by serial dilution to a final concentration of 5 ng/ml (ppb). Drinking water was prepared fresh weekly, and cage bottles were refilled weekly after rinsing.

Study design. A study timeline is shown in Supplemental Material, Figure 1. Timed pregnant P0 dams were randomly distributed amongst five treatment groups. Three groups were treated once daily by oral gavage on GD 1–17 (designated "gestational"), with 0 ("Control," N =10), 1 ("1 mg/kg," N =12), or 5 mg PFOA/kg body weight ("5 mg/kg," N =11). The remaining two groups received 0 or 1 mg/kg as described above, but additionally received PFOA in their drinking water (designated "chronic") at a concentration of 5 ppb ("Control + 5 ppb PFOA," N =7; "1 mg/kg + 5 ppb PFOA," N =10) – to approximate the 3.55 ppb PFOA present in the contaminated drinking water supply in Little Hocking, OH (Emmett et al. 2006). These two groups received PFOA-containing drinking water throughout gestation (starting on GD 7) and for the duration of the study, as did subsequent F1 and F2 offspring (except during F1 breeding and early gestation, to avoid exposing control males). Weekly water consumption was

calculated per cage by weighing bottles when filled, and again at the end of the week; the differential reflected consumption.

P0 dams were weighed daily throughout gestation. On PND 1, F1 litters were weighed and sexed. F1 neonates were pooled and randomly redistributed to dams of their respective treatment groups, consistent with previous studies (Lau et al. 2006; White et al. 2009), equalizing litters to 12-13 neonates, with similar gender representation. Litters were monitored and weighed on PND 10. On PND 22, F1 offspring were weaned, and dams and 1-2 female offspring/litter were weighed and necropsied (*N* =5-7 litters/treatment group). A subset of F1 females were maintained into adulthood, and weighed and necropsied at PND 42 and 63 (*N* =6-8 per treatment group).

Remaining adult F1 females were bred to age-matched control F1 males at 7-8 weeks of age, on the night of proestrus (determined by vaginal cytology). Breeding pairs remained together overnight only, and plug-positive females (GD 0) were housed individually and monitored over gestation (*N* =7-10 F1 dams/treatment group). On PND 1, F2 neonates were weighed and sexed. F2 litters were equalized to 10 neonates for the lactational challenge experiment. F1 dams and 3 female offspring per F2 litter were sacrificed at either PND 10 or 22. The remaining F2 females were weaned, and necropsied on either PND 42 or 63 (*N* =4-8 per treatment group).

The lactational challenge experiment was performed with F1 dams and their F2 litters on PND 10, the peak of lactation. Dams were separated from offspring for three hours, then returned to their litters and allowed to nurse for 30 minutes. The time between reunion and initiation to nurse (arched back position over the litter) was recorded to the nearest second, as was the weight of the 10-pup litter before and after precisely 30 minutes of nursing, in order to

estimate the volume of milk produced during the nursing period. Dams were euthanized and necropsied immediately following nursing.

Necropsy. All animals were terminated by decapitation and trunk blood was collected, from which serum was isolated and stored at -80° C in snap-top polypropylene tubes for PFOA analysis. Uteri were dissected from P0 and F1 dams and implantation sites were visually identified by light macroscope (Leica WILD M420 macroscope, Leica, Wetzlar, Germany), to assess post-implantation loss per dam. Mammary glands were collected as stated below.

Mammary gland preparation. Mammary glands were removed from P0 and F1 dams on PND 10 (F1 dams only) and 22 (N =4-12 per treatment group) because these times represent peak lactational output and weaning, respectively. In F1 and F2 offspring, a set of fourth and fifth glands was removed from the skin and flattened onto glass slides. Whole mounts were fixed in Carnoy's solution, stained in carmine alum, then dehydrated and cleared in xylene, as previously described (Fenton et al. 2002). From dams only, a portion of the contralateral mammary gland was removed, placed in a histology cassette, fixed in 10% neutral buffered formalin for 48 h, and stored in 70% ethanol. These 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 the aforementioned light macroscope.

Mammary gland whole mounts from F1 and F2 female offspring were scored on a 1–4 subjective, age-appropriate developmental scale (4=excellent development/structure; 1=poor development/structure). The number of primary ducts and large secondary ducts, lateral side branching, appearance of budding from the ductal tree, and longitudinal outgrowth of the

epithelia were assessed. Because estrous cycle stage at the time of necropsy was not addressed, stage-sensitive morphological traits were not included in scoring criteria. Slides were separated by score during evaluation, compared within a score for consistency, and then recorded. Two individuals, blind to treatment, scored glands. Mean scores for the various ages and treatment groups were calculated and analyzed statistically for treatment and time-related differences.

Lactating mammary gland H&E-stained sections from P0 and F1 dams, were similarly scored on a 1-4 subjective scale. A value of 4 represented a well-differentiated, functionally lactating tissue characterized by extensive epithelium, reduced adiposity, and presence of secretory alveoli, consistent with the peak of lactation (PND 10, as previously described in Vorderstrasse et al. 2004). A value of 1 represented little or diminishing presence of lobuloalveoli, and extensive involution and regression of the tissue, with the presence of apoptotic bodies, increasing adiposity, and regressing alveoli, as anticipated at weaning (PND 22). At both time points dams were euthanized immediately following removal from litters, to ensure comparable lactational morphology. Mammary glands representing the mean score or observation for each treatment group were photographed using the described scope and mounted camera (Photometrics CoolSNAP, Roper Scientific, Inc., Tucson, AZ).

Measurement of PFOA in serum. Serum samples from the P0 and F1 dams at PND 22, F1 and F2 offspring at PND 22, 42, and 63 were stored frozen in snap-top polypropylene vials, until they were shipped on dry ice to the Centers for Disease Control and Prevention (CDC) laboratory. Serum PFOA measurements were performed by the CDC using the methodology described in detail in White et al. 2009.

Data analysis. Data were evaluated for dose effects using mixed-model analysis of variance (ANOVA) in SAS 9.1 (SAS Institute, Inc. Cary, NC). For both generations, treatment-specific mean gestational weight gain was calculated for dams between GD 1 and 17, and treatment-specific mean body weights were determined for F1 and F2 offspring on PND 22, 42, and 63. F2 offspring body weight means were calculated also at PND 1, 3, 5, 10, 14, and 17, based on whole litter weights (divided by number of pups; litter used as the unit of measure prior to weaning). For all three generations, mean mammary gland lactational or developmental scores were calculated. Scores were analyzed using body weight at time of collection as a random effect, with litter as the unit of measure for neonatal scores. For both P0 and F1 dams, mean implant number, percentage of postimplantation (prenatal) loss, and percentage of postnatal survival was calculated. Differences between treatment groups were determined using Dunnett's, Tukey's, or Student's t-tests (significance at the level of $p < 0.05$ for all comparisons, in text and figures).

Results

P0 dams and F1 offspring. There was no significant effect of PFOA on P0 dam gestational weight gain or implant number (Table 1). Consistent with previous studies (White et al. 2007; White et al. 2009; Wolf et al., 2007), gestational 5 mg/kg PFOA significantly reduced the number of live fetuses, prenatal survival, and postnatal offspring growth and survival, but similar effects were not observed with 1 mg/kg PFOA, or drinking water treatment (Table 1). Given these observations in P0 dams – and in agreement with the conclusions of prior studies (Lau et al. 2006; Wolf et al., 2007) – maternal toxicity was not responsible for F1 developmental deficits seen at low exposures.

As evidenced by significantly elevated histological scores at PND 22, normal weaning-induced mammary involution was compromised among all PFOA-treated P0 dams, including those with only low-dose exposures via drinking water (Table 1). In contrast with the extensive gland regression observed in control dams at weaning, glands in PFOA-treated dams at PND 22 demonstrated structural similarity to normal dam mammary tissue at or near the peak of lactation at PND 10, including the presence of functional lobuloalveolar units (not shown). This observation was consistent with our previous finding that gestational PFOA exposure delays lactational differentiation and eventual involution in the exposed dam (White et al. 2007), but here we also observed the effect with exposure to 5 ppb PFOA in drinking water for a total of 34 days (Supplemental Material, Table 1 provides dose estimates).

F1 offspring body weights and adjusted body weights (body weight less liver weight) between PND 22 and 63 were not consistently associated with PFOA treatment (Table 2). Liver-to-body weight ratios at PND 22 were significantly elevated among F1 females exposed to 1 or 5 mg/kg, consistent with hepatomegaly. At PND 42, F1 females exposed to 5 mg/kg had a significantly increased liver-to-body weight ratio and significant reductions in total and adjusted body weight, but all three parameters were similar to controls by 9 weeks of age (PND 63). Chronic 5 ppb PFOA exposure in drinking water did not affect the liver-to-body weight ratio in F1 offspring. In contrast, developmental mammary scores of F1 offspring were significantly reduced among all treatment groups (including 5ppb in water) until at least 9 weeks of age (PND 63; Table 2, Figure 1), suggesting that delayed mammary gland development is a more sensitive and persistent endpoint than hepatomegaly.

F1 dams and F2 offspring. Maternal toxicity was not observed in F1 dams with developmental or chronic low-level PFOA exposures. Interestingly, the number of uterine

implants was significantly reduced among F1 dams developmentally exposed to 5 mg/kg, resulting in litters with significantly fewer offspring (Table 3). As previously described, postnatal survival of 5 mg/kg F1 females was significantly decreased, however no effect on this end point was observed with respect to postnatal survival of F2 offspring. This suggests that both F2 thriftiness – specifically referring to the ability to suckle with sufficient vigor and frequency, so as to yield nourishment -- and F1 lactational competency were sufficient to support litters.

In the lactational challenge on PND 10, neither milk volume nor timed nursing behavior was significantly different from controls with gestational (P0) or chronic, low-level PFOA exposure of the F1 dams (Table 3). Although large differences in mean values were noted (i.e., 1/3 reduction in milk transferred to offspring as measured by litter weight and an 84 second longer time to suckling in 1 mg/kg + water exposure group compared to controls), high variability in these responses limited the power to detect a significant difference. Nevertheless, F1 lactational morphology was significantly compromised among all treatment groups at PND 10 (Table 3 and Figure 2). By PND 22, most morphological delays were no longer evident, and only F1 dams with the highest developmental exposure (i.e. 5 mg/kg PFOA) still exhibited morphology that was significantly different from controls, with little evidence of normal regression. Consistent with this, we observed productive spherical alveoli in the 5 mg/kg group, in contrast with the regressing alveoli and apoptotic bodies observed in controls. Of note, at the time F1 dams became pregnant and underwent lactational differentiation, their virgin siblings still exhibited stunted mammary gland development in all exposure groups compared with controls (PND 63, Table 2 and Figure 1).

Despite striking morphological abnormalities in the lactating glands of PFOA-exposed F1 dams on PND 10, there was no clear evidence of diminished nutritional support provided by these dams based on F2 body weights (Table 4). These data suggest that nursing behavior of the neonates may have changed (i.e., increased number of nursing events/day or longer nursing/event) to compensate for the decreased potential in milk production by the F1 dam, but these end points were not evaluated in this study. Adjusted body weights and liver-to-body weight ratios did not demonstrate clear differences by treatment group in the F2 offspring (Table 4).

Unlike F1 females, developmental mammary gland scores in F2 females did not differ in association with maternal exposure, however, control F2 females exhibited unusually low mammary gland scores at PND 10 and 22 which might have reduced the statistical ability to detect effects in other treatment groups at these time points (Table 4). At PND 22, scores were consistent with developmental delays in all treatment groups relative to controls, but contrasts were not statistically significant. By PND 42, both groups with chronic drinking water exposures (control + 5 ppb PFOA, 1 mg/kg + 5 ppb PFOA) displayed significantly reduced gland development relative to controls (Table 4) that was characterized by an excess of terminal end buds (TEBs) (Figure 3). Furthermore, mammary gland scores for the F2 offspring of gestationally-exposed F1 females in the 5 mg/kg group were generally consistent with delayed differentiation (Table 4), with histological evidence of postponed lobule formation (note arrows in Figure 3). A more sparse appearance was frequently observed in F2 mammary tissue from these three groups (data not shown), resulting from delayed ductal outgrowth and persistence of TEBs in adults (arrows in Figure 3).

Water consumption. Average daily intake for the two groups receiving chronic drinking-water exposures was calculated from measurements of weekly water consumption, and is shown in Supplemental Material, Figure 2. There was no difference in water intake between groups (as a function of P0 treatment), and daily estimated PFOA intake for drinking-water groups ranged from approximately 50 to 100 ng, excepting anticipated changes in water intake depending on life stage (i.e. increased intake during lactation, lower intake in early life; see Supplemental Material, Table 1 and Figure 2).

Serum PFOA analyses. In F1 offspring at 9 weeks of age (PND 63, Table 5), serum PFOA concentrations in the 5 mg/kg group were only an order of magnitude greater than the levels exhibited in the chronic drinking water 5 ppb PFOA exposure-only group. When F1 dams (then 13 weeks old) were weaning their litters (F2 at PND 22, Table 5), serum PFOA concentrations in the F2 drinking water exposure groups had surpassed those of the F2 offspring of F1 dams developmentally exposed to 1 and 5 mg/kg PFOA during their gestation. The control + 5 ppb PFOA group was particularly interesting, as averaged over their lifetimes (PND 22, 42, and 63, and ~PND 91 for F1 dams, means averaged, for each respective generation), the F1 and F2 generations exhibited nearly identical average serum PFOA concentrations, at 59.5 and 50.8 ng/ml, respectively. Furthermore, because the final serum measurement taken on the F1 generation was at 13-weeks postnatally (~PND 91), as compared to only 9-weeks for the F2 generation, the lifetime average may have been skewed slightly higher for the F1 generation. Serum PFOA concentrations did not differ significantly at any time point between the two drinking-water treatment groups in the F2 generation.

Discussion

Our prior studies identified morphological delays in mammary gland development that resulted from gestational PFOA exposure (White et al. 2007; White et al. 2009), but we did not previously determine whether such morphological effects persisted and were associated with functional consequences, nor did we evaluate the effects of low-level, chronic exposures, similar to non-occupational exposures in humans. Here, we have shown evidence that the previously reported effects on F1 offspring mammary development -- resulting from treatment of P0 dams with 1 and 5 mg/kg PFOA during pregnancy -- did persist, and that these histopathological diminishments in the developing gland translated to altered lactational morphology, when F1 females were bred and challenged to lactationally support F2 litters. However, these effects were not associated with an overt reduction in the nutritional support provided by the F1 dam, as F2 offspring demonstrated normal postnatal survival and weight gain. Among F1 females that received only chronic low-level 5ppb PFOA exposure, comparable and significant diminishments were also observed in developmental morphology between PND 22 and 63, as well as in later, adult lactational morphology at the peak of lactation, suggesting a far greater sensitivity of the tissue than previously identified.. F2 offspring of these F1 dams with only chronic low-dose exposures also displayed a trend toward delayed development, and exhibited significantly stunted morphology at PND 42.

The degree to which these persistent alterations in F1 mammary gland morphology are associated with functional consequences is difficult to determine, as impaired weight gain in F2 offspring was the only relevant endpoint assessed. The morphological effects of PFOA exposure in the F1 glands did not translate to significant decreases in growth and survival of F2 litters, as opposed to the case with F1 offspring of P0 dams. Nonetheless, an increase in the

thriftiness of offspring from the F1 to F2 generations or an increase in F2 nursing frequency could have masked effects on milk production in affected lactating F1 glands.

These data suggest that chronic developmental exposure to environmentally-relevant levels of PFOA may not interfere with lactation *per se*, but may reduce the number and density of alveoli available to produce milk, and increase latency to peak milk output, delaying offspring maturation as seen in our previous work (White et al 2007). In the case of humans, where viable alternatives to breast milk are available, low-level functional effects on lactation that cause even a short delay in substantial milk output might result in formula-feeding instead of breast-feeding, despite the established health benefits of breast-feeding. In mammalian wildlife species, critically reliant upon lactation to raise their offspring, responsiveness of the gland to PFOA might lead to delays in milk production resulting in malnourishment or possibly starvation of offspring, in a manner similar to the effects of PCBs on wild mink reproduction in the past (Aulerich and Ringer, 1977).

Chronic, low-dose PFOA exposure in drinking water at human-relevant levels (5 ppb) delayed mammary gland development in F1 offspring. This exposure yielded serum PFOA levels which ranged between 50-100 ng/ml after approximately 6 weeks (Table 5; lifetime averages: F1 females = 59.5 ng/ml, F2 females = 50.8 ng/ml; data not shown). If these approximate serum concentration ranges represent those of an animal reaching a steady state burden, it should be noted that they are approximately an order of magnitude lower than that seen in some chronically exposed human populations. For example, communities exposed to PFOA in municipal supply drinking water at 3.55 ppb exhibited mean serum PFOA concentrations of 423 ng/ml (Emmett et al., 2006), compared to the national average of 3.9 ng/ml (Calafat et al. 2007). While it is understood that the pharmacokinetics of PFOA in the

mouse differ from those in the human – the half-life being approximately 17 days in the mouse, and 3.8 years in the human (Calafat et al. 2007; Lou et al. 2009) – it remains disconcerting that the effective circulating dose sufficient to yield histopathologic changes in the mouse mammary gland is approximately an order of magnitude lower than the mean serum concentration in certain human populations.

These low serum concentrations were associated with alterations in mouse mammary gland morphology in three generations, although it was not possible to separate the effects of post-gestational chronic exposure in each generation from gestational exposure in some instances, thus it should not be construed that the effects observed in these treatment groups were transgenerational transmitted. Because humans with exposures under similar conditions (contaminated drinking water) exhibit higher circulating serum concentrations of PFOA, by an order of magnitude – and approximately two orders of magnitude above the concentration of PFOA in their exposure source – the data presented here may actually under-represent human-relevant exposure conditions, with respect to internal circulating dose. However, it is not known whether effects of PFOA on the mouse mammary gland translate to effects in humans; research is on-going to discern a mammary-specific mode of action for PFOA, and to determine its relevance to human breast health.

Conclusion

Our studies identified a gestational exposure LOAEL of 1 mg/kg PFOA for altered lactational morphology in treated P0 dams and altered mammary gland development in their F1 offspring. Additionally, our employment of a non-traditional treatment regimen using low-dose

continual exposure has generated data that will allow others to calculate a lower, chronic exposure LOAEL or benchmark dose.

Delays in mammary epithelial growth in F1 females developmentally exposed to PFOA reported in this study and others (White et al. 2007; White et al. 2009) translated to histopathologic changes in subsequent lactational morphology. However, this did not result in functional deficits in lactation when F2 offspring growth and survival were used as proxy measures of nutritional support. We observed sparse branching morphology and delayed differentiation in three generations of CD-1 mice, but the global scoring method did not indicate consistent differences from controls across F2 time points.

While the chronic low-dose PFOA supplied in drinking water in these studies and similar concentrations reported in municipal drinking water supplies near fluorochemical plants are not representative of drinking water supplies in the US in general, PFOA is not regularly monitored in drinking water, and thus national averages cannot be well-estimated. It is concerning, however, that the chronic low dose employed here was sufficient to produce changes in the development of the mouse mammary gland; similar developmental changes are physiologically possible in girls, but would likely not be realized until they enter puberty or attempt lactation. Therefore, if human exposures in distinct populations are approximating those provided in this study, concern over human breast health and lactational competency are justified.

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Table 1. P_0 maternal indices.

	PFOA exposure parameters				
	Control	Control + 5 ppb PFOA	1 mg/kg	1 mg/kg + 5 ppb PFOA	5 mg/kg
Maternal indices, P_0 (n = 7-11)					
Gestational weight gain (g)	24.8 ± 1.2	25.0 ± 1.2	26.0 ± 1.2	27.0 ± 1.2	27.7 ± 1.2
Implants (# per live litter)	12.8 ± 0.5	12.7 ± 0.4	13.5 ± 0.7	14.0 ± 0.4	13.7 ± 0.6
Live fetuses (# per live litter)	12.0 ± 0.5	11.7 ± 0.4	12.9 ± 0.7	13.3 ± 0.5	10.0 ± 0.8*
Prenatal loss (% per live litter)	6.1 ± 1.8	7.8 ± 1.7	4.5 ± 1.7	5.1 ± 1.6	25.8 ± 5.6*
Postnatal survival (% per live litter)	96.1 ± 1.3	100 ± 0.0*	98.8 ± 0.8	89.5 ± 6.4	72.7 ± 5.8*
Mammary gland score (1-4 scale)					
PND 22	2.4 ± 0.2	3.4 ± 0.1*	3.0 ± 0.2*	3.2 ± 0.2*	3.9 ± 0.1*

Data presented as means ± SE. * $p < 0.05$ compared to Control.

Table 2. F_1 developmental indices.

	PFOA exposure parameters				
	Control	Control + 5 ppb PFOA	1 mg/kg	1 mg/kg + 5 ppb PFOA	5 mg/kg
Developmental indices, F_1 (n = 4-10)					
Body weight (g) at age					
PND 22	12.70 ± 0.69	12.69 ± 0.87	13.40 ± 0.49	13.20 ± 0.37	11.28 ± 0.45
PND 42	25.65 ± 0.43	24.28 ± 0.57	24.24 ± 0.74	24.90 ± 0.62	22.28 ± 0.60*
PND 63	28.77 ± 0.96	26.23 ± 1.81	29.93 ± 0.97	26.35 ± 0.84#	27.88 ± 1.25
Liver:body weight ratio (x100%)					
PND 22	5.56 ± 0.16	5.29 ± 0.13	6.35 ± 0.08*	5.96 ± 0.12	7.81 ± 0.34*
PND 42	5.19 ± 0.24	5.75 ± 0.22	5.32 ± 0.10	5.26 ± 0.13	5.79 ± 0.09*
PND 63	4.85 ± 0.17	4.99 ± 0.12	4.97 ± 0.13	4.82 ± 0.15	5.24 ± 0.28
Body weight excluding liver weight (g)					
PND 22	11.99 ± 0.64	11.16 ± 0.86	12.55 ± 0.46	12.55 ± 0.36	10.39 ± 0.39
PND 42	24.32 ± 0.44	22.89 ± 0.54	22.94 ± 0.69	23.59 ± 0.58	20.99 ± 0.57*
PND 63	27.38 ± 0.94	24.92 ± 1.74	28.49 ± 1.12	24.43 ± 1.09	26.43 ± 1.24
Mammary gland score (1-4 scale)					
PND 22	3.8 ± 0.1	2.5 ± 0.2*	2.3 ± 0.2*	2.2 ± 0.1*	1.6 ± 0.1*
PND 42	3.8 ± 0.1	3.3 ± 0.2*	2.6 ± 0.4*	2.2 ± 0.3*	2.3 ± 0.2*
PND 63	3.8 ± 0.2	2.6 ± 0.4*	2.9 ± 0.2*	2.0 ± 0.3*#	2.2 ± 0.2*

Data presented as means ± SE. * $p < 0.05$ compared to Control; # $p < 0.05$ when 1 mg/kg + 5 ppb PFOA compared to 1 mg/kg.

Table 3. F_1 maternal indices.

	PFOA exposure parameters				
	Control	Control + 5 ppb PFOA	1 mg/kg	1 mg/kg + 5 ppb PFOA	5 mg/kg
Maternal indices, F1 (n = 4-10)					
Implants (# per live litter)	14.9 ± 0.4	14.6 ± 0.5	14.1 ± 0.4	13.4 ± 0.9	12.3 ± 0.2*
Live fetuses (# per live litter)	13.6 ± 0.6	13.1 ± 0.6	12.8 ± 0.6	12.1 ± 0.9	12.0 ± 0.3*
Prenatal loss (% per live litter)	8.6 ± 2.5	9.8 ± 3.2	10.0 ± 3.2	6.7 ± 2.5	2.7 ± 1.4
Postnatal survival (% per live litter)	100 ± 0.0	100 ± 0.0	98.1 ± 1.4	97.9 ± 1.5	100 ± 0.0
Lactational challenge					
Milk produced in 30 min (g)	2.10 ± 0.20	1.80 ± 0.35	2.08 ± 0.25	1.40 ± 0.44	1.73 ± 0.51
Time to initiate (s)	267 ± 38	384 ± 55	307 ± 114	351 ± 86	279 ± 30
Mammary gland score (1-4 scale)					
PND 10	4.0 ± 0.0	2.8 ± 0.5*	2.5 ± 0.2*	2.0 ± 0.2*	2.5 ± 0.2*
PND 22	2.1 ± 0.3	2.2 ± 0.2	1.9 ± 0.4	1.5 ± 0.2*	3.2 ± 0.3*

Data presented as means ± SE. * $p < 0.05$ compared to Control.

Table 4. F_2 developmental indices.

	PFOA exposure parameters				
	Control	Control + 5 ppb PFOA	1 mg/kg	1 mg/kg + 5 ppb PFOA	5 mg/kg
Developmental indices, F2 (*n* = 4-10)					
Body weight (g) at age					
PND 1	1.71 ± 0.03	1.61 ± 0.03*	1.63 ± 0.05	1.68 ± 0.05	1.65 ± 0.04
PND 3	2.27 ± 0.05	2.22 ± 0.05	2.25 ± 0.09	2.30 ± 0.09	2.22 ± 0.06
PND 5	3.24 ± 0.07	3.35 ± 0.10	3.38 ± 0.11	3.42 ± 0.15	3.34 ± 0.09
PND 10	5.69 ± 0.22	5.83 ± 0.23	6.00 ± 0.19	5.96 ± 0.18	5.87 ± 0.20
PND 14	6.26 ± 0.06	6.34 ± 0.05	7.30 ± 0.25*	7.54 ± 0.33	6.85 ± 0.26
PND 17	6.64 ± 0.13	7.05 ± 0.06	8.15 ± 0.31*	8.19 ± 0.39	7.42 ± 0.37
PND 22	10.80 ± 0.28	11.41 ± 0.26	13.00 ± 0.50*	13.29 ± 0.61	11.60 ± 0.54
Liver : body weight ratio (x100%)					
PND 10	2.94 ± 0.15	2.94 ± 0.12	3.08 ± 0.14	2.73 ± 0.14	2.91 ± 0.09
PND 22	5.43 ± 0.14	5.25 ± 0.25	5.10 ± 0.21	5.18 ± 0.23	5.11 ± 0.15
PND 42	5.43 ± 0.13	5.47 ± 0.10	5.78 ± 0.12	5.36 ± 0.19	5.63 ± 0.21
PND 63	5.28 ± 0.25	5.13 ± 0.19	5.05 ± 0.11	5.10 ± 0.15	4.79 ± 0.25
Body weight excluding liver weight (g)					
PND 10	6.20 ± 0.18	6.15 ± 0.20	6.16 ± 0.14	5.72 ± 0.29	6.44 ± 0.36
PND 22	9.75 ± 0.58	10.10 ± 0.18	10.58 ± 0.54	11.29 ± 0.73	10.41 ± 0.78
PND 42	22.28 ± 0.79	24.07 ± 0.32	24.12 ± 0.68	25.78 ± 0.55*	24.12 ± 0.51
PND 63	27.41 ± 0.76	27.59 ± 1.22	25.98 ± 1.29	28.83 ± 0.90	29.66 ± 2.10
Mammary gland score (1-4 scale)					
PND 10	2.8 ± 0.3	3.0 ± 0.2	1.9 ± 0.3	2.6 ± 0.2	2.0 ± 0.2
PND 22	3.1 ± 0.4	1.9 ± 0.3	2.3 ± 0.1	2.3 ± 0.2	2.0 ± 0.2
PND 42	3.5 ± 0.2	2.5± 0.4*	3.4 ± 0.2	2.4 ± 0.2*#	3.3 ± 0.4
PND 63	3.4 ± 0.2	3.5 ± 0.2	2.4 ± 0.2*	2.6 ± 0.5	2.6 ± 0.4

Data presented as means ± SE. * $p < 0.05$ compared to Control; # $p < 0.05$ when 1 mg/kg + 5 ppb PFOA compared to 1 mg/kg.

Table 5. Serum PFOA concentrations (ng/ml) over three generations.

	PFOA exposure parameters				
	Control	Control + 5 ppb PFOA	1 mg/kg	1 mg/kg + 5 ppb PFOA	5 mg/kg
Generations at given ages					
P0 Dams at Weaning (PND 22)	4.0 ± 0.3	74.8 ± 11.3	6,658.0 ± 650.5	4,772.0 ± 282.4	26,980.0 ± 1,288.2
F1 Pups at PND 22	0.6 ± 0.3	21.3 ± 2.1	2,443.8 ± 256.4	2,743.8 ± 129.4	10,045 ± 1,125.6
F1 Pups at PND 42	1.4 ± 0.4	48.9 ± 4.7	609.5 ± 72.2	558.0 ± 55.8	1,581.0 ± 245.1
F1 Pups at PND 63	3.1 ± 0.2	66.2 ± 4.1	210.7 ± 21.9	187.0 ± 24.1	760.3 ± 188.3
F1 Dams at Weaning (PND 22)	2.0 ± 0.6	86.9 ± 14.5	9.3 ± 2.6	173.3 ± 36.4	18.7 ± 5.2
F2 Pups at PND 22	0.4 ± 0.0	26.6 ± 2.4	4.6 ± 1.2	28.5 ± 3.7	7.8 ± 1.9
F2 Pups at PND 42	0.7 ± 0.3	57.4 ± 2.9	0.4 ± 0.0	72.8 ± 5.8	0.4 ± 0.0
F2 Pups at PND 63	1.1 ± 0.4	68.5 ± 9.4	1.1 ± 0.5	69.2 ± 4.3	1.2 ± 0.5

Data presented as means ± SE.

Figure 1. F_1 female mammary gland development. Mammary whole mounts illustrate morphology representative of treatment groups at ages shown (n = 6-7 females/treatment/age). Scale bar is 1,000 µm at PND 22 and 2,000 µm at PND 42 and 63.

Figure 2. Histological sections of F_1 dam lactating mammary glands. Glands pictured illustrate morphology representative of respective treatment at given times (n = 4 dams/treatment/time point). Scale bar is 100 µm at PND 10 and 22. Arrows indicate presence of alveoli.

Figure 3. F_2 female mammary gland development. Mammary whole mounts illustrate morphology representative of respective treatment groups at ages shown (n = 4-5 females/treatment/age). Scale bar is 100 µm at PND 10, 1,000 µm at PND 22 and 2,000 µm at PND 42 and 63. Arrows indicate remaining TEBs.

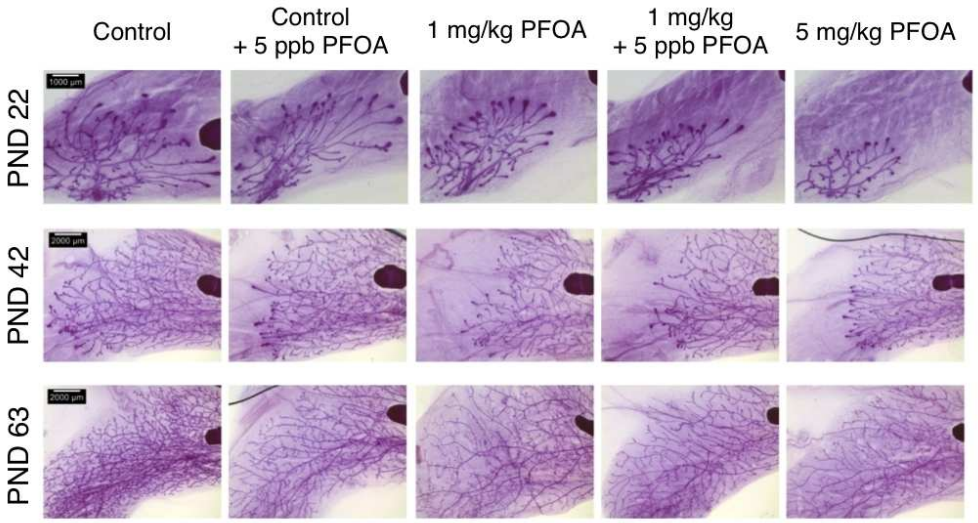

Figure 1. F1 female mammary gland development. Mammary whole mounts illustrate morphology representative of treatment groups at ages shown (n = 6-7 females/treatment/age). Scale bar is 1,000 µm at PND 22 and 2,000 µm at PND 42 and 63.
173x95mm (150 x 150 DPI)

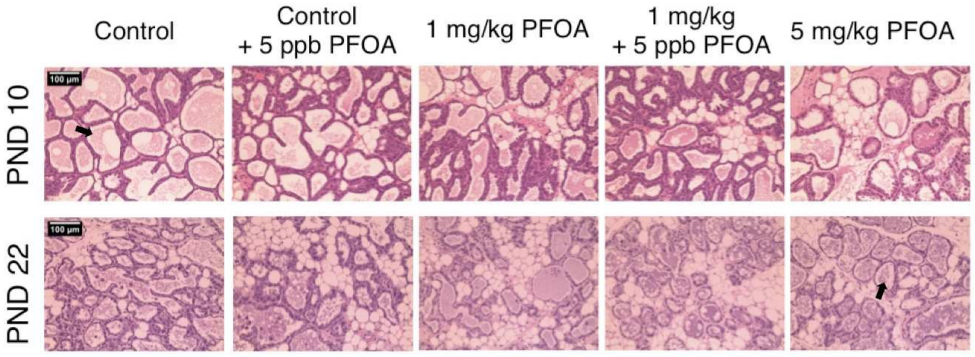

Figure 2. Histological sections of F1 dam lactating mammary glands. Glands pictured illustrate morphology representative of respective treatment at given times (n = 4 dams/treatment/time point). Scale bar is 100 µm at PND 10 and 22. Arrows indicate presence of alveoli.
474x185mm (72 x 72 DPI)

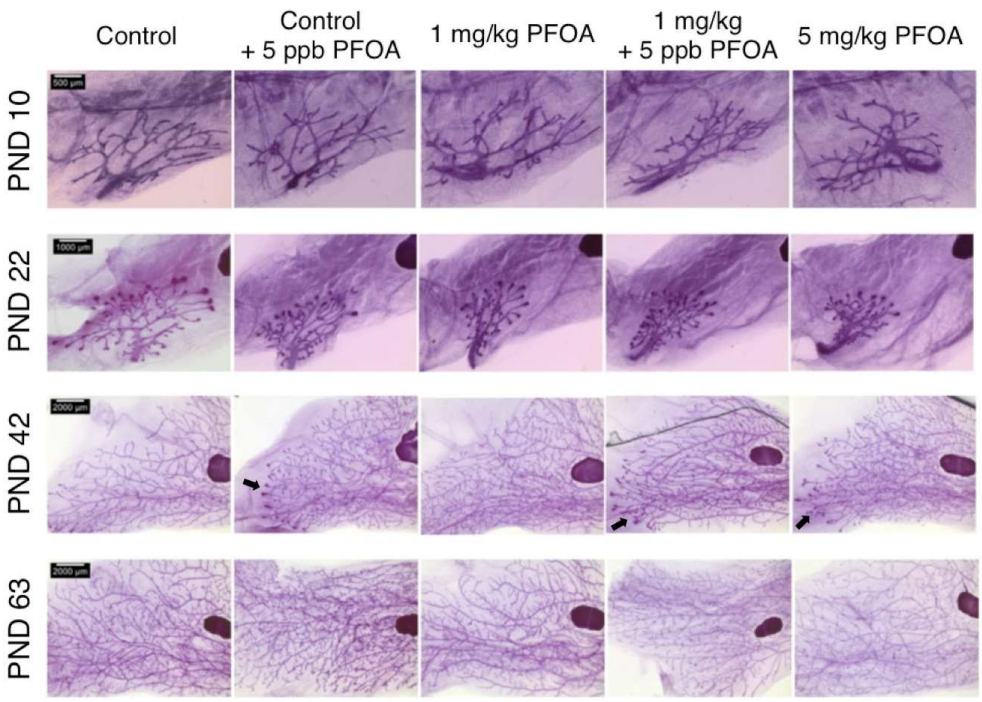

Figure 3. F2 female mammary gland development. Mammary whole mounts illustrate morphology representative of respective treatment groups at ages shown (n = 4-5 females/treatment/age). Scale bar is 100 µm at PND 10, 1,000 µm at PND 22 and 2,000 µm at PND 42 and 63. Arrows indicate remaining TEBs.
469x331mm (72 x 72 DPI)