

## Effects of Perfluorooctanoic Acid Exposure during Pregnancy in the Mouse

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Perfluorooctanoic acid (PFOA), a member of the perfluoroalkyl acids that have wide commercial applications, has recently been detected in humans and wildlife. The current study characterizes the developmental toxicity of PFOA in the mouse. Timed-pregnant CD-1 mice were given 1, 3, 5, 10, 20, or 40 mg/kg PFOA by oral gavage daily from gestational day (GD) 1 to 17; controls received an equivalent volume (10 ml/kg) of water. PFOA treatment produced dose-dependent full-litter resorptions; all dams in the 40-mg/kg group resorbed their litters. Weight gain in dams that carried pregnancy to term was significantly lower in the 20-mg/kg group. At GD 18, some dams were sacrificed for maternal and fetal examinations (group A), and the rest were treated once more with PFOA and allowed to give birth (group B). Postnatal survival, growth, and development of the offspring were monitored. PFOA induced enlarged liver in group A dams at all dosages, but did not alter the number of implantations. The percent of live fetuses was lower only in the 20-mg/kg group (74 vs. 94% in controls), and fetal weight was also significantly lower in this group. However, no significant increase in malformations was noted in any treatment group. The incidence of live birth in group B mice was significantly lowered by PFOA: ca. 70% for the 10- and 20-mg/kg groups compared to 96% for controls. Postnatal survival was severely compromised at 10 or 20 mg/kg, and moderately so at 5 mg/kg. Dose-dependent growth deficits were detected in all PFOA-treated litters except the 1-mg/kg group. Significant delays in eye-opening (up to 2–3 days) were noted at 5 mg/kg and higher dosages. Accelerated sexual maturation was observed in male offspring, but not in females. These data indicate maternal and developmental toxicity of PFOA in the mouse, leading to early pregnancy loss, compromised postnatal survival, delays in

general growth and development, and sex-specific alterations in pubertal maturation.

**Key Words:** perfluorooctanoic acid; developmental toxicity.

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are members of perfluoroalkyl acids (PFAA) commonly used as surfactant processing aids in the production of fluoropolymers, coatings for clothing fabrics, upholstery, and carpets, and paper products approved for food contact. PFAA are composed of a carbon backbone (typically ranging from C-4 to C-15), full substitution of hydrogen by fluorine, and a functional group (typically carboxylic acid or sulfonic acid). These chemicals are stable and persistent in the environment, present in humans, and wide-spread in wildlife. They are readily absorbed (Johnson and Ober, 1979; Kemper 2003), not known to be metabolized (Kuslikis *et al.*, 1992; Ophaug and Singer, 1980; Vanden Heuvel *et al.*, 1991), and poorly eliminated, with half-lives in humans estimated at 3.8 years for PFOA, 5.4 years for PFOS, and 8.7 years for perfluorohexane sulfonate (PFHS) (Ehresman *et al.*, 2005; Olsen *et al.*, 2005a). In the year 2000, production of PFOS was estimated at over 3500 metric tons and PFOA in excess of 500 metric tons. Although the production of PFOS by its major manufacturer was phased out by the end of 2002, replacement PFAA chemicals (such as PFOA and perfluorobutane sulfonate, PFBS) are filling its void in the consumer and industrial markets. Indeed, preliminary results from Furdui *et al.* (2005) have indicated that, while the contaminant levels of PFOS in Great Lakes fish were still far greater than those of PFOA, those of perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) were nonetheless as high, if not higher than those of PFOA.

The toxic potentials of PFOS and PFOA have been reviewed recently (3M, 2003; Kennedy *et al.*, 2004; Lau *et al.*, 2004). In particular, developmental toxicity of PFOS has been described in rodent models (Lau *et al.*, 2003; Luebker *et al.*, 2005a,b; Thibodeaux *et al.*, 2003). In both rat and mouse, *in utero*

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exposure to PFOS compromised the survival of newborns and retarded postnatal growth and development of the survivors in a dose-dependent manner. More recent studies have examined the involvement of pulmonary complications in the neonatal mortality (Grasty *et al.*, 2004, 2005). Developmental toxicity of PFOA has also been assessed in the rat (Butenhoff *et al.*, 2004b; Hinderliter *et al.*, 2005). While results indicated that PFOA was less developmentally toxic than PFOS, this was probably due, in part, to the efficient renal elimination of PFOA in the female rat, with a half-life estimate of 3–4 h, compared to that of 6–8 days in the males (Hanhijarvi *et al.*, 1982, 1987, 1988; Kemper and Jepson, 2003; Kojo *et al.*, 1986; Kudo *et al.*, 2002; Kudo and Kawashima, 2003; Ohmori *et al.*, 2003; Uy-yu *et al.*, 1990; Vanden Heuvel *et al.*, 1991; Ylinen *et al.*, 1989, 1990). Hence, PFOA was undetectable in the serum of pregnant rats 24 h after an oral treatment (Gibson and Johnson, 1983). Thus, in rats, daily single administration of PFOA during pregnancy constitutes repeated episodic exposures to the dams, with the chemical surging to a maximum level in circulation within 1.25 h (Kemper and Jepson, 2003), but declining rapidly, reaching a nadir between 20–24 h; the level of PFOA then rises again in the succeeding treatment. Such a major gender difference in the elimination of PFOA has not been established in humans or primates (Butenhoff *et al.*, 2004c; Ehresman *et al.*, 2005; Olsen *et al.*, 2003, 2005b), thus complicating the extrapolation of findings from the rat model to humans for health risk assessment (Butenhoff *et al.*, 2004a).

On the other hand, results from studies by Uy-yu *et al.* (1990) and Sohlenius *et al.* (1992) suggest that the mouse may provide an alternative model for the evaluation of PFOA developmental toxicity. These investigators reported a lack of sex-related differences in mice for the effects of PFOA on hepatic responses. In support of these findings, studies conducted by the PFOA manufacturer indicated that retention of <sup>14</sup>C-PFOA for both sexes of mice was more like that of male rats than female rats (Kennedy *et al.*, 2004). The current study, therefore, was undertaken to ascertain whether there was a sex difference in the bioaccumulation of PFOA in the mouse and to evaluate the effects of PFOA exposure during pregnancy on prenatal and postnatal development of offspring.

## MATERIALS AND METHODS

**PFOA.** Perfluorooctanoic acid (PFOA, ammonium salt; >98% pure) was purchased from Fluka Chemical (Steinheim, Switzerland). NMR analysis kindly provided by 3M Company (St. Paul, MN) indicated that approximately 98.9% of the chemical was straight-chain and the remaining 1.1% was branched isomers. 1,2-<sup>13</sup>C-Pefluorooctanoic acid was purchased from Perkin-Elmer (Wellesley, MA) and used as an internal standard in the quantitative analysis. For all studies, PFOA was dissolved in deionized water and prepared fresh daily.

**Animal treatment.** All animal studies were conducted with approval by the U.S. EPA ORD/NHEERL Institutional Animal Care and Use Committee; procedures and facilities were consistent with the recommendations of the 1996 NRC "Guide for the Care and Use of Laboratory Animals," the Animal

Welfare Act, and Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Timed-pregnant CD-1 mice were obtained from Charles River Laboratories (Raleigh, NC). Females were bred overnight, and those with spermatozoa in a vaginal smear and/or with a copulatory plug were considered to be at gestational day (GD) 0. Animals were shipped by truck to our facilities in the afternoon of the same day. In a separate study, mature male and female CD-1 mice and Sprague-Dawley rats (about 10 weeks old) were obtained from the same supplier. Animals were randomly assigned to treatment groups, housed individually in polypropylene cages, and provided pellet chow (LabDiet 5001, PMI Nutrition International) and tap water *ad libitum*. Animal facilities were controlled for temperature (20–24°C) and relative humidity (40–60%), and operated under a 12-h light-dark cycle.

PFOA (1, 3, 5, 10, 20, or 40 mg/kg) was given to pregnant mice by gavage once daily from GD 1 through GD 17. Controls received an equivalent volume (10 ml/kg) of water. Maternal weight was monitored daily throughout gestation. Some mice were sacrificed on GD 18 (24 h after last treatment) by CO<sub>2</sub> asphyxiation for teratological evaluation. Blood was collected from the descending aorta, and serum samples were prepared and analyzed for PFOA concentration. Maternal liver was dissected and weighed. The gravid uterus was then removed and examined; the numbers of the live or dead fetuses as well as resorptions were recorded. Live fetuses were weighed individually, sex-determined, and examined for external abnormalities. All fetuses were killed with an overdose of pentobarbital; one half of each litter was prepared for skeletal examination, while the other half was prepared for visceral evaluation. For skeletal evaluation, the fetuses were eviscerated, fixed in 95% ethanol, and subsequently stained with Alizarin red and alcian blue to visualize bone and cartilage, respectively. Skeletal morphology was evaluated as described previously (Narotsky and Rogers, 2000). For visceral evaluation, fetuses were fixed in Bodian's solution (2% formaldehyde, 5% acetic acid, 72% ethanol, 21% water). Examination of the head, as well as thoracic and abdominal viscera was carried out using free-hand razor dissection.

The remaining pregnant mice received an additional PFOA treatment on GD 18. On GD 19, these mice were monitored hourly; the day was divided into four 6-h quarters, and mice giving birth during each quarter were tabulated. The quarter in which the first control mouse gave birth was arbitrarily assigned as parturition time zero. Time of parturition for each animal, condition of the newborns, and number of live offspring were noted. The following day was designated as postnatal day (PD) 1. The number of live pups in each litter and their body weight were tabulated daily for the first 4 days after birth and at intervals of several days thereafter. Litter size was not adjusted unless three or fewer pups survived within a litter. In such cases, the surviving pups were distributed randomly to nursing dams within the same dosage group, with litter size maintained at 10 or less. The age at which the mouse neonates opened their eyes was tracked beginning on PD 12. All pups were weaned on PD 23 and separated by sex. The age at which the mouse offspring reached puberty was determined by monitoring vaginal opening in females and preputial separation in males beginning on PD 24. Representative pups (2–4 for each sex) from each litter were randomly selected, ear-marked, and monitored; in addition, body weights of the mice when they reached puberty were noted. Following vaginal opening in female mice, the age at first detectable estrus was determined by daily evaluation of vaginal cytology, according to the method of Cooper and Goldman (1999). After weaning, the nursing dams were sacrificed, and their uteri were removed, stained with 2% ammonium sulfide, and the residual implantation sites were counted. The postnatal survival rate was calculated based on the number of implantations for each pregnant mouse.

In a separate study, adult male and female rats and mice were given PFOA daily (10 mg/kg for 20 days and 20 mg/kg for 17 days, respectively); controls received deionized water. Mice were sacrificed by CO<sub>2</sub> asphyxiation, and rats by decapitation 24 h after last treatment; blood samples were collected, and serum prepared and stored for PFOA analysis.

**Determination of PFOA concentrations.** For controls and animals receiving 1 mg/kg of PFOA, a 25- $\mu$ l aliquot of thawed serum sample was spiked with 10 ng of the <sup>13</sup>C-PFOA internal standard and added to 200  $\mu$ l of 0.1 M formic acid, followed by 2 ml of cold acetonitrile. The samples were vortex-mixed and

centrifuged at 5,000 rpm to precipitate the serum proteins. The supernatant was diluted by 20 ml of deionized water and loaded onto SPE cartridges (Waters Instruments, Bedford, MA) and eluted with 2 ml of acetone. The eluent was evaporated to dryness and reconstituted in 400  $\mu$ l of 50:50 2 mM ammonium acetate:acetonitrile for high performance liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS).

For animals receiving greater than 1 mg/kg of PFOA, an additional dilution of the serum samples was required. A 25- $\mu$ l aliquot of thawed serum was added to 5 ml of 0.1 M formic acid and sonicated for 30 min. A 50- $\mu$ l aliquot of the diluted sample was removed and added to 5 ml of acetonitrile containing 20 ng of  $^{13}$ C-PFOA internal standard and sonicated for another 30 min. A 200- $\mu$ l aliquot of the acetonitrile extract was then added to an equal volume of 2 mM ammonium acetate for LC-MS/MS analysis.

**Data analysis.** Data are presented as means and standard errors. Statistical significance was determined by the analysis of variance (ANOVA), using the individual litter as the statistical unit. Maternal weight gain was analyzed by ANOVA with repeated measures. When a significant treatment effect or interaction was detected, Duncan's multiple range test was performed *post hoc*. Incidence of full litter resorptions was analyzed by Fisher's Exact test. Statistically significant differences were determined at  $p \leq 0.05$ .

The U.S. Environmental Protection Agency now uses the benchmark dose (BMD) approach (Barnes *et al.*, 1995; Crump, 1984) for noncancer risk assessment (U.S. EPA, 1995). This approach is designed to provide a more quantitative alternative to dose-response assessment than the no-observed-adverse-effect-level (NOAEL) process, by constructing mathematical models to fit all data points in the dose-response study, and to take data variance into consideration. In the current study, BMD<sub>5</sub> and BMDL<sub>5</sub> values were calculated for the maternal and developmental toxicity of PFOA. BMD<sub>5</sub> refers to the central estimate of the administered dose predicted to cause a 5% increase in response above background, and BMDL<sub>5</sub> is defined as the corresponding lower limit of the 95% confidence interval on the BMD<sub>5</sub> (Allen *et al.*, 1994). Benchmark Dose Software (U.S. EPA, 2000) was used to calculate the BMD<sub>5</sub> values. Selection of a specific curve-fitting model for the BMD determination was based on the Akaike's Information Criterion (AIC) value. The AIC is equal to  $-2L + 2p$ , where L is the log-likelihood at the maximum likelihood estimates for the parameters, and p is the number of model parameters estimated. The model that demonstrates "goodness-of-fit" with the lowest AIC value is presumed to be the most appropriate.

## RESULTS

An experiment was carried out to compare the body burdens of PFOA between rat and mouse after subchronic exposure. A clear sex difference in PFOA accumulation was observed in the rat (Table 1); a serum level of 111  $\mu$ g PFOA/ml was reached in male rats after 20 daily treatments, while only traces of PFOA were detectable in female rats. In contrast, no significant differences were seen between serum PFOA concentrations of male and female mice. Additionally, a steady-state level of serum PFOA was apparently reached in mice within 1 week of chemical exposure, as serum levels in both male and female mice did not change appreciably between 7 and 17 days of treatment. It is also noteworthy that, when the male animals are compared, doubling the administered dose of PFOA in the mouse versus the rat (20 mg/kg vs. 10 mg/kg, respectively) yielded approximately twice the amount of the chemical in circulation, suggesting that steady-state levels of PFOA are proportional to administered dose and are amenable to extrapolation between males of these two species.

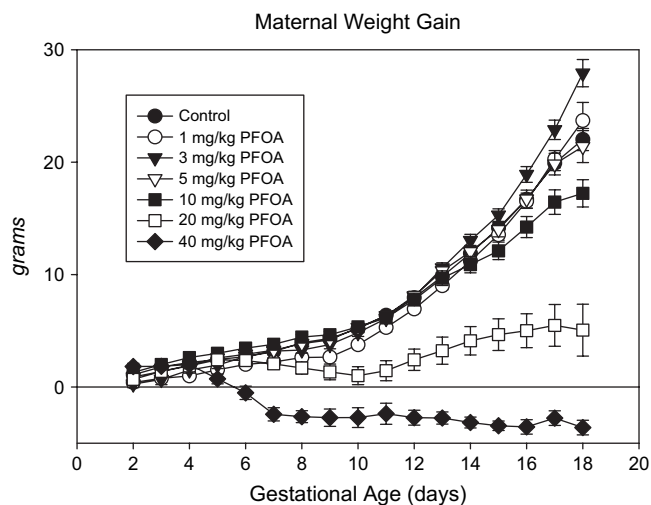
**TABLE 1**  
Serum Levels of PFOA in Adult Sprague-Dawley Rats and CD-1 Mice after Receiving Daily Oral Gavage

Species	Dose	Days of treatment	Males	Females
Rat	10 mg/kg	20	111 $\pm$ 10 $\mu$ g/ml (8)	0.69 $\pm$ 0.18 $\mu$ g/ml* (8)
Mouse	20 mg/kg	7	181 $\pm$ 34 $\mu$ g/ml (6)	178 $\pm$ 19 $\mu$ g/ml (7)
Mouse	20 mg/kg	17	199 $\pm$ 19 $\mu$ g/ml (8)	171 $\pm$ 15 $\mu$ g/ml (5)

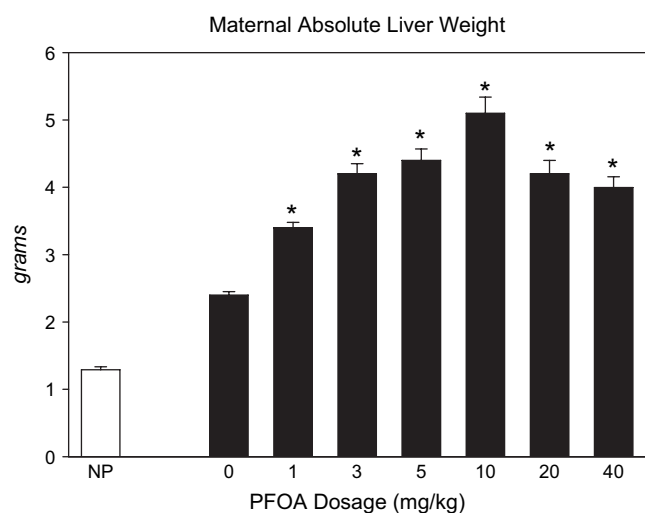
*Note.* Trunk blood was collected 24 h after the last treatment. Data are expressed as means  $\pm$  SE of the numbers of animals indicated in parentheses. Asterisk denotes significant difference between the sexes ( $p < 0.05$ ).

Control CD-1 mice gained approximately 22 g from days 2 to 18 of pregnancy. PFOA treatment altered maternal weight gain in a dose-dependent manner (Fig. 1), as pronounced weight deficits were seen in the 20- and 40-mg/kg dosage groups. In addition, PFOA treatment led to an increase of liver weight in a dose-related fashion (Fig. 2). Analysis of maternal serum demonstrated a dose-dependent increase in the accumulation of PFOA at term (Fig. 3).

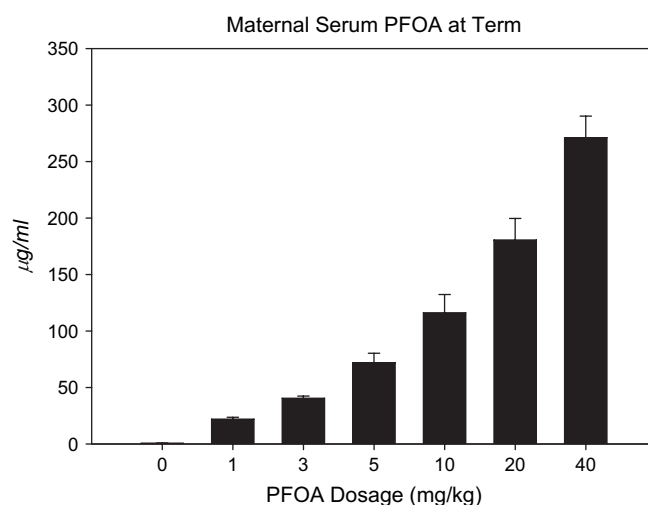
Exposure of pregnant mice to PFOA throughout gestation did not alter the number of implantations. However, a significant increase in the incidence of full-litter resorptions was seen in the 5-mg/kg and higher dose groups; indeed, all pregnancies were lost in the 40-mg/kg group (Table 2). Among litters with viable fetuses at term, significant prenatal loss was observed only in the 20-mg/kg group. Similarly, weights of the live fetuses at term were not affected significantly by PFOA at  $\leq 10$  mg/kg, although a 20% reduction was detected in the 20-mg/kg group. Fetal examination revealed enlarged fontanel



**FIG. 1.** Effects of PFOA on weight gain in pregnant mice. Data represent mean  $\pm$  SE of determinations from 9–57 animals. Two-way ANOVA indicated a significant treatment effect and a time  $\times$  treatment interaction ( $p < 0.0001$ ). Duncan's multiple-range test indicated that the 40-mg/kg dose was significantly different from the control group from GD 5 to 18, and the 20-mg/kg dose from GD 8 to 18.



**FIG. 2.** Effects of PFOA on liver weight of pregnant mice at term (GD 18). Data are expressed as means  $\pm$  SE of 9–45 determinations. For comparison, liver weight of nonpregnant female mice handled daily and treated with water as in the control group is illustrated as the open bar labeled NP. Asterisks denote significant differences from pregnant controls ( $p < 0.05$ ).



**FIG. 3.** Serum concentrations of PFOA in pregnant mice at term (GD 18). Bars represent means  $\pm$  SE of 6–58 determinations.

**TABLE 2**  
Mouse Reproductive Outcome and Fetal Teratology, Examined at Term

	PFOA dosage (mg/kg)						
	0	1	3	5	10	20	40
Dams examined (#)	45	17	17	27	26	42	9
Dams with FLR (#)	3	2	1	7	12	37	9
Dams with FLR (%)	6.7	11.8	5.9	25.9*	46.1*	88.1*	100*
Implants (# per litter with FLR)	7.0 $\pm$ 4.0	10.0 $\pm$ 3.0	13.0	11.6 $\pm$ 1.2	10.8 $\pm$ 1.2	11.5 $\pm$ 0.6	11.9 $\pm$ 0.5
Implants (# per live litter)	12.9 $\pm$ 0.4	13.1 $\pm$ 0.4	11.6 $\pm$ 0.9	11.5 $\pm$ 0.5	12.6 $\pm$ 0.6	10.2 $\pm$ 2.1	—
Live fetuses (# per live litter)	12.5 $\pm$ 0.4	13.0 $\pm$ 0.4	10.8 $\pm$ 0.9	11.1 $\pm$ 0.4	11.7 $\pm$ 0.8	7.2 $\pm$ 2.0*	—
Prenatal loss (% per live litter)	4.1 $\pm$ 1.4	1.0 $\pm$ 0.7	7.4 $\pm$ 2.5	2.4 $\pm$ 0.8	7.7 $\pm$ 3.3	25.9 $\pm$ 11.7*	—
Fetal body weight (g)	1.05 $\pm$ 0.02	0.98 $\pm$ 0.03	1.03 $\pm$ 0.04	1.03 $\pm$ 0.04	0.98 $\pm$ 0.05	0.86 $\pm$ 0.11*	—
Notable skeletal findings (n)	13	6	7	11	5	5	—
<b>Ossification (number of sites):</b>							
<b>Sternebrae</b>	5.9 $\pm$ 0.1	6.0 $\pm$ 0.1	6.0 $\pm$ 0.1	5.5 $\pm$ 0.3	5.7 $\pm$ 0.2	4.0 $\pm$ 1.1*	—
Caudal vertebrae	4.3 $\pm$ 0.3	4.1 $\pm$ 0.1	4.0 $\pm$ 0.2	4.3 $\pm$ 0.3	3.7 $\pm$ 0.2	2.1 $\pm$ 0.7*	—
Metacarpals	7.7 $\pm$ 0.2	7.3 $\pm$ 0.3	7.6 $\pm$ 0.2	6.6 $\pm$ 0.5	6.8 $\pm$ 0.4	5.2 $\pm$ 1.4*	—
Metatarsals	9.3 $\pm$ 0.3	8.9 $\pm$ 0.4	9.1 $\pm$ 0.3	8.2 $\pm$ 0.6	8.6 $\pm$ 0.4	6.2 $\pm$ 1.6*	—
Proximal phalanges (forelimb)	4.8 $\pm$ 0.8	1.8 $\pm$ 1.0*	2.2 $\pm$ 0.9*	2.9 $\pm$ 0.9	1.0 $\pm$ 0.6*	0.0 $\pm$ 0.0*	—
Proximal phalanges (hindlimb)	3.9 $\pm$ 0.9	0.4 $\pm$ 0.3*	1.5 $\pm$ 1.0	2.8 $\pm$ 0.9	1.0 $\pm$ 0.6*	0.0 $\pm$ 0.0*	—
<b>Reduced ossification (%):</b>							
Calvaria	13.5 $\pm$ 9.2	62.5 $\pm$ 15.5*	66.7 $\pm$ 13.0*	22.7 $\pm$ 10.4	35.0 $\pm$ 12.7	55.0 $\pm$ 20.0*	—
Supraoccipital	14.7 $\pm$ 4.0	33.3 $\pm$ 10.5	28.6 $\pm$ 8.5	27.3 $\pm$ 9.2	45.0 $\pm$ 9.4*	90.0 $\pm$ 10.0*	—
Unossified hyoid	0	0	0	0	0	26.7 $\pm$ 19.4*	—
Enlarged fontanel	17.3 $\pm$ 9.1	66.7 $\pm$ 21.1*	53.6 $\pm$ 15.8*	18.2 $\pm$ 9.6	45.0 $\pm$ 20.0	95.0 $\pm$ 5.0*	—
Notable visceral findings (n)	10	6	6	11	5	5	—
Tail defects (curly, bent) (%)	0	0	0	20.5 $\pm$ 5.7*	5.0 $\pm$ 5.0*	11.7 $\pm$ 7.3*	—
Limb defects (club, bent) (%)	0	0	0	5.7 $\pm$ 2.8*	0	5.8 $\pm$ 3.9*	—
Microcardia (%)	0	0	0	0	5.0 $\pm$ 5.0*	30.0 $\pm$ 18.3*	—

*Note.* Data represent means  $\pm$  SE of litters examined as indicated. One-way ANOVA indicates significant differences ( $p < 0.05$ ) in number of live fetuses and prenatal loss. Asterisks denote significant differences from controls ( $p < 0.05$ ) by Fisher's exact test for full litter resorptions (FLR) and by Dunnett's *t*-test for other parameters.



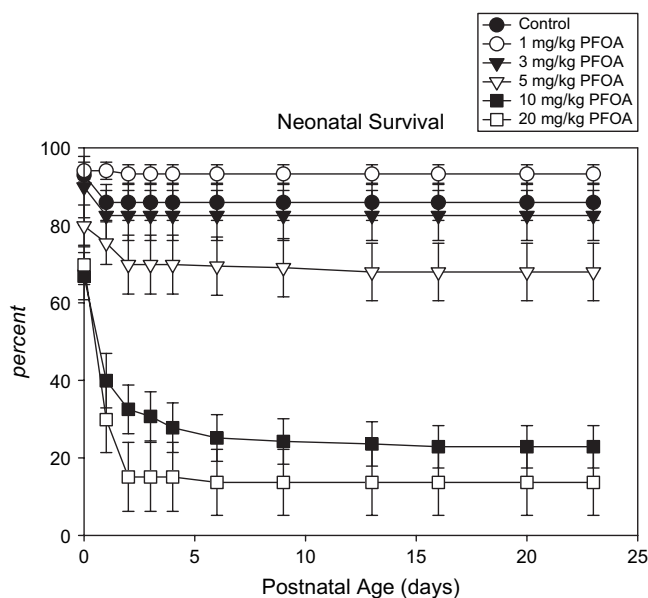
**TABLE 3**  
Effects of Gestational Exposure of PFOA on Time to Parturition

PFOA dosage (mg/kg)	<i>n</i>	Parturition delay (days)
0	23	0 ± 0.06
1	8	0.15 ± 0.12
3	8	0.31 ± 0.12*
5	19	0.14 ± 0.08
10	21	0.24 ± 0.07*
20	7	0.43 ± 0.12*

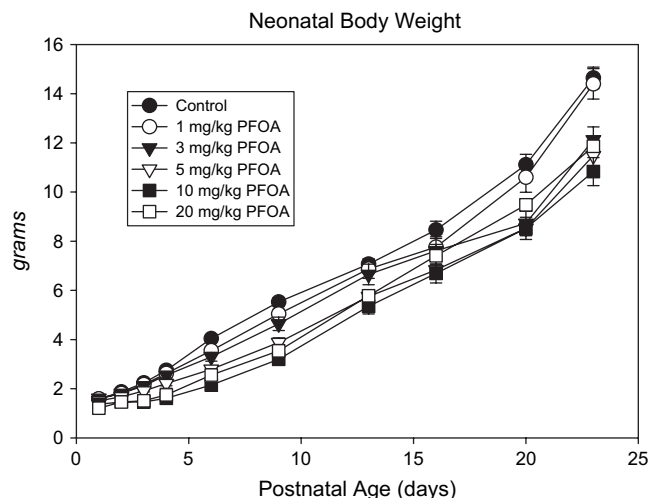
Note. On GD-19, pregnant mice were monitored hourly; the day was divided into four quarters, and mice giving birth during each quarter were noted and tabulated. Data are expressed as mean fractions of the day ± SE of PFOA treatment group compared to controls. Asterisks denote significant differences ( $p < 0.05$ ) from controls determined by Mantel-Haenszel chi-square test.

and reduced ossification of sternbrae, caudal vertebrae, metacarpals, metatarsals, phalanges, calvaria, supraoccipital, and hyoid in the 10- and 20-mg/kg dose groups. Visceral examination also revealed minor tail and limb defects and microcardia in these dose groups (Table 2).

Exposure to PFOA during pregnancy slightly increased the average time to parturition, by up to half a day in the high-dose group (Table 3). Most offspring were born alive, but the incidence of stillbirth and neonatal mortality was increased markedly by PFOA treatment, particularly in the high-dose groups (up to 30%, Fig. 4). Most of the neonates exposed to 10



**FIG. 4.** Effects of prenatal exposure to PFOA on postnatal survival in mice. The percent survival was calculated based on the number of implantations for each dam. Each data point represents mean ± SE of 8–22 litters. Two-way ANOVA indicated a significant treatment effect ( $p < 0.0001$ ). Duncan's multiple range test conducted with PD 9 data (no further changes were seen thereafter) indicated significant difference ( $p < 0.05$ ) between controls and doses of 5 mg/kg or higher, and the 10- and 20-mg/kg doses differed significantly from the 5-mg/kg dose.



**FIG. 5.** Effects of prenatal exposure to PFOA on body weight of the surviving mouse pups. Average weights of mouse pups (8–12 per litter) were determined for each litter; neonates in the high dose groups (10 and 20 mg/kg) were combined into several litters to maintain a litter size of 6–8. Each data point represents mean ± SE of 7–30 litters. Two-way ANOVA indicated significant treatment effect and an age × treatment interaction ( $p < 0.0001$ ). Duncan's multiple-range test indicated significant differences between the controls and treatment groups of 3 mg/kg or higher ( $p < 0.05$ ).

or 20 mg/kg PFOA did not survive the first day of life. Survival of the newborn mice was better in the lower-dose groups; postnatal viability in the 1- and 3-mg/kg groups was comparable to that in controls. Among survivors, a trend toward growth retardation was noted in the PFOA-treated neonates, leading to 25–30% lower body weights in the 3-mg/kg or higher dose groups at weaning (Fig. 5). Body weights subsequently recovered in these groups, reaching control levels by 6.5 weeks of age for the females and by 13 weeks for the males (Table 4). Interestingly, as the animals aged, body weights of both males and females in the PFOA-exposed groups tended to be higher (by about 10%) than those of controls. Corresponding to the early postnatal growth deficits, development of the mice exposed to PFOA *in utero* was impaired, as significant delays in eye opening were detected in the 5-mg/kg or higher dose groups, by as much as 3 days (Table 5). On the other hand, pubertal development for the female mice was not appreciably affected by prenatal PFOA treatment, as only a slight delay was noted in the highest dose group, with either age at vaginal opening or time to first estrus. In contrast, the onset of puberty for the male pups was markedly advanced by PFOA, such that the prepuce was separable in the 1-mg/kg dose group almost 4 days earlier than in the controls. It is noteworthy that this accelerated pubertal maturation took place despite a body weight deficit of 25–30%.

## DISCUSSION

Results from the current study that evaluated the developmental toxicity of PFOA in the CD-1 mouse are strikingly

TABLE 4  
Effect of Prenatal Exposure to PFOA on Mouse Body Weight at Post-Weaning Ages

A	Females (g)						
	Dose	6.5 weeks	13 weeks	18 weeks	48 weeks	55 weeks	60 weeks
Control		25.0 ± 0.6	31.0 ± 1.0	34.8 ± 1.1	49.9 ± 3.0	50.3 ± 2.5	47.6 ± 2.5
1 mg/kg		24.2 ± 0.6	—	—	51.8 ± 2.0	51.7 ± 2.1	50.5 ± 1.7
3 mg/kg		24.8 ± 0.4	—	—	56.2 ± 2.1	56.0 ± 2.2	55.6 ± 1.9
5 mg/kg		24.4 ± 0.4	33.6 ± 0.7	39.2 ± 0.9	51.0 ± 1.9	50.7 ± 1.9	50.1 ± 2.0
10 mg/kg		22.4 ± 0.2	35.3 ± 3.2	43.2 ± 4.2	49.0 ± 2.6	49.4 ± 2.8	49.7 ± 2.9

B	Males (g)						
	Dose	6.5 weeks	13 weeks	18 weeks	48 weeks	55 weeks	60 weeks
Control		34.3 ± 1.4	42.8 ± 1.5	46.5 ± 2.1	56.3 ± 3.5	55.2 ± 3.6	52.7 ± 7.0
1 mg/kg		34.5 ± 1.3	—	—	59.1 ± 2.1	58.8 ± 1.8	60.3 ± 2.0
3 mg/kg		33.6 ± 0.4	—	—	60.8 ± 2.7	60.4 ± 2.5	59.2 ± 3.0
5 mg/kg		32.6 ± 0.5	44.1 ± 1.1	49.3 ± 1.3	58.0 ± 1.5	60.2 ± 2.3	59.2 ± 1.7
10 mg/kg		29.2 ± 0.9	41.5 ± 0.2	47.9 ± 0.1	53.8 ± 3.3	52.5 ± 3.9	54.3 ± 2.0

Note. Female (panel A) and male (panel B) mice were segregated at weaning and monitored separately. Data are expressed as mean ± SE of 4–11 determinations. ANOVA indicated significant treatment effect at 6.5 weeks for males ( $p < 0.05$ ).

different than those described previously with the rat model. Butenhoff *et al.* (2004b) conducted a comprehensive two-generation reproductive toxicity study on PFOA with Sprague-Dawley rats and reported little toxicity; small postnatal weight gain deficits, slight delays of sexual maturation, and post-weaning mortality (likely related to immaturity) were noted only in the F<sub>1</sub>-generation animals of the highest dose group (30 mg/kg). In contrast, here we report a significant increase in the incidence of full-litter resorptions and neonatal mortality in the CD-1 mouse at 5 mg/kg (Table 2), with BMD<sub>5</sub> and BMDL<sub>5</sub> estimated at 2.84 mg/kg and 1.09 mg/kg, respectively for neonatal mortality (determined by survival to weaning) (Table 6). Significant alterations of postnatal growth and development were seen at even lower doses (1 and 3 mg/kg, Fig. 5), with BMD<sub>5</sub> and BMDL<sub>5</sub> estimates of 1.07 mg/kg and 0.86 mg/kg

respectively, for pup weight at weaning, and 2.64 mg/kg and 2.10 mg/kg respectively, for eye-opening (Table 6). These disparate findings in rats and mice are likely due, at least in part, to the differential pharmacokinetic disposition of PFOA. In pregnant rats, a plasma concentration of 70–80 µg/ml was reached within 2 h after an oral treatment of 30-mg/kg dose (Hinderliter *et al.*, 2005) and declined by 98% in the ensuing 22 h (Kemper and Jepson, 2003), or became nondetectable (Gibson and Johnson, 1983). In contrast, in the current study, a dose-dependent accumulation of PFOA was noted in pregnant dams at term. Thus, daily administration of PFOA to the pregnant mouse likely led to an accumulation of the chemical until a steady state was reached (perhaps within a week, as indicated by results obtained from nonpregnant mice). Moreover, it should be noted that, although the PFOA levels in the

TABLE 5  
Developmental Landmarks of Mouse Pups Exposed to PFOA *In Utero*

PFOA (mg/kg)	Eye opening		Vaginal opening			First estrus		Preputial separation		
	N	Age (days)	N	Age (days)	Body Weight (g)	N	Age (days)	N	Age (days)	Body Weight (g)
0	22	14.8 ± 0.1 <sup>a</sup>	47 (20)	28.4 ± 0.3 <sup>a,b</sup>	18.0 ± 0.2 <sup>a</sup>	47 (20)	29.9 ± 0.4 <sup>a,b</sup>	56 (22)	30.5 ± 0.2 <sup>a</sup>	25.0 ± 0.3 <sup>a</sup>
1	8	15.2 ± 0.2 <sup>a</sup>	21 (8)	27.4 ± 0.5 <sup>b</sup>	18.2 ± 0.5 <sup>a</sup>	21 (8)	28.2 ± 0.6 <sup>b</sup>	22 (8)	26.7 ± 0.2 <sup>b</sup>	20.3 ± 0.3 <sup>b,c</sup>
3	8	15.5 ± 0.1 <sup>a,b</sup>	21 (7)	28.8 ± 0.4 <sup>a,b</sup>	17.7 ± 0.4 <sup>a,b</sup>	21 (7)	30.2 ± 0.4 <sup>a,c</sup>	20 (7)	27.1 ± 0.2 <sup>b</sup>	19.4 ± 0.6 <sup>b,c,d</sup>
5	17	16.0 ± 0.2 <sup>b</sup>	43 (16)	29.9 ± 0.4 <sup>a</sup>	17.7 ± 0.4 <sup>a,b</sup>	43 (16)	31.8 ± 0.5 <sup>c</sup>	46 (16)	28.2 ± 0.2 <sup>c</sup>	18.3 ± 0.5 <sup>c,d</sup>
10	13	17.2 ± 0.3 <sup>c</sup>	27 (12)	29.3 ± 0.3 <sup>a</sup>	16.7 ± 0.3 <sup>b</sup>	27 (12)	30.2 ± 0.3 <sup>a,c</sup>	28 (11)	28.5 ± 0.3 <sup>c</sup>	17.5 ± 0.7 <sup>d</sup>
20	3	17.9 ± 0.8 <sup>c</sup>	8 (2)	31.3 ± 0.5 <sup>c</sup>	19.3 ± 0.4 <sup>a</sup>	8 (2)	31.3 ± 0.5 <sup>c</sup>	4 (2)	31.7 ± 1.1 <sup>d</sup>	20.8 ± 1.2 <sup>b</sup>

Note. Data represent means ± SE of numbers of litters (for eye opening) or individual pups (for vaginal opening, first estrus, and preputial separation) examined as indicated. For eye opening, N = litter; for other landmarks, N = individual animal, numbers in parenthesis indicate litters represented. ANOVA indicate significant treatment effect in all parameters examined ( $p < 0.05$ ). Significant differences ( $p < 0.05$ ) between each dose group were determined by Duncan's multiple range test and are depicted by different letters (a, b, c, and d).

**TABLE 6**  
**Benchmark Dose Estimates for Various Parameters of PFOA**  
**Maternal and Developmental Toxicity in the Mouse**

	BMD <sub>5</sub> (mg/kg)	BMDL <sub>5</sub> (mg/kg)
Maternal weight gains during pregnancy	6.76	3.58
Maternal liver weight at term	0.200	0.170
Live fetus weight at term	10.3	4.3
Fetal forelimb phalanges ossification at term	0.889	0.643
Fetal hindlimb phalanges ossification at term	0.958	0.616
Neonatal survival at weaning (PD 23)	2.84	1.09
Neonatal body weight at weaning (PD 23)	1.07	0.86
Neonatal eye opening	2.64	2.10

mouse fetuses were not determined in the current study, a recent report with the rat has indicated that PFOA crossed the placenta readily (Hinderliter *et al.*, 2005).

Exposure of the mouse to PFOA during pregnancy produced developmental toxicity. Full-litter resorptions were observed in the 5-mg/kg PFOA and higher dose groups. These pregnancy losses probably took place shortly after implantation. Residual implantation sites were clearly detectable in all treatment groups, but maternal weight gains were arrested in these high-dose groups starting at GD 6–8, after the embryos were already implanted. The nature of this early pregnancy loss is not well understood, but may be related to maternal toxicity involving failure to support viability of the early embryos (Bielmeier *et al.*, 2004; Rivier and Vale, 1982; Rothchild, 1981). Consistent with a maternally mediated effect, embryo resorption appeared to be an all-or-none phenomenon. In those litters where live fetuses were found, there was little or no significant change in prenatal loss or fetal weight, with an exception of the 20-mg/kg dose group that experienced a high incidence of early pregnancy loss; in fact, fairly high values of BMD<sub>5</sub> and BMDL<sub>5</sub> for fetal weight at term were estimated (10.3 mg/kg and 4.3 mg/kg, respectively). Teratological findings (such as reduced ossification) typically reflected delays of fetal development, although a few incidences of malformed limbs and tail, and microcardia were detected at 5 mg/kg and higher dose groups. On the other hand, the BMD<sub>5</sub> estimates for phalangeal ossification were less than 1 mg/kg (Table 6), indicating the sensitivity of this PFOA effect. That reduced ossification was observed at such low doses without affecting fetal weight suggests the possibility that effects on ossification may not be a simple developmental delay. Regardless, these findings are generally comparable to those reported for two related PFAA chemicals, perfluorodecanoic acid (Harris and Birnbaum, 1989) and PFOS (Thibodeaux *et al.*, 2003).

The slight delays in fetal development might have led to a prolongation of gestational length in the PFOA-treated mice, as time of parturition in these dams was consistently later than that in controls, although a clear-cut dose-related response was lacking. In contrast, survival of the newborn mice was profoundly influenced by *in utero* exposure to PFOA. Almost 30% of the offspring were found either stillborn or died immediately after live birth in the 10- and 20-mg/kg dose groups, and less than 25% of these pups survived the ensuing days. However, those pups that survived the first postnatal week generally did reach adulthood. The profile of postnatal mortality was almost identical to that previously described for PFOS in the rat and in the mouse (Lau *et al.*, 2003; Luebker *et al.*, 2005a,b). The pathophysiological mechanism(s) for this PFOA effect remains to be elucidated, but may be, in part, related to deficits of pulmonary function as described previously for PFOS exposure (Grasty *et al.*, 2004, 2005).

Among surviving mouse pups, postnatal growth and development was significantly hampered by PFOA. Although PFOA treatment of the mouse dams ceased at the end of pregnancy, chemical exposure to the suckling pups undoubtedly continued until weaning, as a previous study with the rat reported a substantial presence of PFOA in the milk (ca. 10% of plasma level, Hinderliter *et al.*, 2005). Here, PFOA exposure in mice led to deficits of postnatal weight gain, in dose groups as low as 3 mg/kg. These shortfalls persisted into young adulthood, as female mice did not catch up to controls until 6.5 weeks of age and the males not until 13 weeks. Neonatal growth deficits may be related to the nursing dams' capability to lactate, and hence the nutritional status of the suckling pups. PFOA is a potent peroxisome proliferator-activated receptor (PPAR) agonist, and PPAR-associated proteins are known to regulate mammary gland development and function (Jia *et al.*, 2005; Qi *et al.*, 2004). Interestingly, as the mice matured, a trend toward excess weight gain (ca. 10% above control mouse weights) was noted in the PFOA-exposed mice at 48–60 weeks of age, for both sexes. However, these results were derived from a fairly small population of mice, and this observation requires confirmation. Given the propensity of PFOA to alter lipid metabolism (Kennedy *et al.*, 2004), changes of body weight and/or composition related to the chemical exposure are not beyond speculation.

Eye-opening and pubertal landmarks such as vaginal opening and preputial separation are simple but reliable indices of postnatal development in rodents (Goldman *et al.*, 2000; Stoker *et al.*, 2000). Consistent with deficits in body weight, eye-opening in the PFOA-exposed pups was delayed by as much as 3 days in the high-dose groups (10 and 20 mg/kg). On the other hand, onset of puberty in female pups was only slightly delayed (by about a day) in the high-dose groups. The effect of PFOA on pubertal attainment of the male pups was intriguing. Pups exposed to PFOA consistently displayed accelerated preputial separation (by more than 3 days). This effect was most pronounced in the lowest dose group (1 mg/kg)

and appeared to have an inverse relationship with dose. It is noteworthy that male puberty was advanced by PFOA despite growth deficits. The underlying mechanism for this PFOA action is unknown, but in a previous study, the advancement of preputial separation induced by the herbicide simazine was accompanied by elevations of circulating luteinizing hormone, testosterone, and androstenedione (Stoker *et al.*, 2005, 2006). Interestingly, these effects of simazine were also observed preferentially in the lower treatment dose groups. With the exception of male pubertal findings, the pattern of growth retardation and developmental delays seen in the PFOA-treated mice is similar to that observed with PFOS (Lau *et al.*, 2003), suggesting that this pattern of developmental toxicity may be a common feature of the PFAA.

In the present study, significant adverse outcomes were noted in the low PFOA dose groups (e.g., the effects of neonatal weight gains, eye opening, and preputial separation). Such low-dose effects were not observed with PFOS (Lau *et al.*, 2003). One potential explanation may involve differences in the pharmacokinetic handling of PFOA in the immature mice, leading to a disproportionately larger body burden of the chemical in the pups. Alternatively, PFOA may simply be more potent than other PFAA in its cellular and molecular actions.

In addition to the direct chemical insults of PFOA, contributions from maternal factors to the adverse developmental outcomes should not be overlooked. Alterations of maternal liver weight provide another indication of the maternal effects of the chemical. Indeed, liver enlargement is an exquisitely sensitive biomarker for PFOA exposure, as significant changes were observed even in the lowest dose group, with BMD<sub>5</sub> and BMDL<sub>5</sub> estimated at 0.20 mg/kg and 0.17 mg/kg, respectively (Table 6).

In summary, results from the current study present a different profile of developmental toxicity for PFOA in the mouse from that reported previously in the rat, most likely related to the pharmacokinetic differences between these two rodent species. Because of a similar lack of sex-difference in PFOA elimination among humans, primates, and mice, findings from the mouse may provide a more amenable alternative than other laboratory animal models for species extrapolation in the human health risk assessment of PFOA. Moreover, the observations of neonatal mortality, retarded postnatal growth, and delayed development in the PFOA-exposed mice closely resemble the toxicity findings of PFOS, raising concerns that these adverse outcomes may be extended to other chemicals in the PFAA family.

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