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Phenotypic dichotomy following developmental exposure to perfluorooctanoic acid (PFOA) in female CD-1 mice: Low doses induce elevated serum leptin and insulin, and overweight in mid-life☆

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ABSTRACT

The synthetic surfactant, perfluorooctanoic acid (PFOA) is a proven developmental toxicant in mice, causing pregnancy loss, increased neonatal mortality, delayed eye opening, and abnormal mammary gland growth in animals exposed during fetal life. PFOA is found in the sera and tissues of wildlife and humans throughout the world, but is especially high in the sera of children compared to adults. These studies in CD-1 mice aim to determine the latent health effects of PFOA following: (1) an *in utero* exposure, (2) an *in utero* exposure followed by ovariectomy (ovx), or (3) exposure as an adult. Mice were exposed to 0, 0.01, 0.1, 0.3, 1, 3, or 5 mg PFOA/kg BW for 17 days of pregnancy or as young adults. Body weight was reduced in the highest doses on postnatal day (PND) 1 and at weaning. However, the lowest exposures (0.01–0.3 mg/kg) significantly increased body weight, and serum insulin and leptin (0.01–0.1 mg/kg) in mid-life after developmental exposure. PFOA exposure combined with ovx caused no additional increase in mid-life body weight. At 18 months of age, the effects of *in utero* PFOA exposure on body weight were no longer detected. White adipose tissue and spleen weights were decreased at high doses of PFOA in intact developmentally exposed mice, and spleen weight was reduced in PFOA-exposed ovx mice. Brown adipose tissue weight was significantly increased in both ovx and intact mice at high PFOA doses. Liver weight was unaffected in late life by these exposure paradigms. Finally, there was no effect of adult exposure to PFOA on body weight. These studies demonstrate an important window of exposure for low-dose effects of PFOA on body weight gain, as well as leptin and insulin concentrations in mid-life, at a lowest observed effect level of 0.01 mg PFOA/kg BW. The mode of action of these effects and its relevance to human health remain to be explored.

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Abbreviations: ANOVA, analysis of variance; BMI, body mass index; BW, body weight; C8, eight-carbon; CV, coefficient of variation; DES, dietylstilbestrol; E_2, estradiol; GD, gestational day; $t_{1/2}$, Half-life; IACUC, Institutional Animal Care and Use Committee; LH, luteinizing hormone; LOD, limit of detection; LOQ, limit of quantitation; NHANES, National Health and Nutrition Examination Survey; NMR, nuclear magnetic resonance; NOAEL, no observable adverse effect level; ovx, ovariectomized; PFAA, perfluoroalkyl acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PND, postnatal day; PPAR, peroxisome proliferator-activated receptors; SMR, standardized mortality ratio.

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1. Introduction

Perfluorooctanoic acid (PFOA), one of the eight carbon (C8) perfluoroalkyl acids (PFAAs), is a synthetic, stable, persistent organic fluorine surfactant, used to impart water and grease resistance to various consumer products including non-stick pans, as surface treatments for clothing and food wrappers, insulation and fire-fighting foams. PFOA's high energy carbon–fluorine bonds are resistant to hydrolysis, photolysis and metabolism and thus it bioaccumulates and persists within biota and environmental matrices, including water and soil, from the Arctic to the South Pacific (Lau et al., 2007). This ubiquitous environmental contaminant has an estimated half-life ($t_{1/2}$) in humans of 3.8 years (Olsen et al., 2007) and is found in production workers' sera, as well as those of the general population.

Bio-monitoring studies show detectable levels of PFOA in human populations. The National Health and Nutrition Examination Survey (NHANES) reported that mean serum PFOA concentrations are declining in the USA population, from 5.2 ng/ml in 1999–2000 to 3.9 ng/ml, in 2003–2004 (Calafat et al., 2007). Arnsberg, Germany, an area with known drinking water PFAA contamination, had reported PFOA mean serum levels in 2006 of 25 ng/ml vs. 4 ng/ml in unaffected German provinces (Hölzer et al., 2008). The highest known non-occupational PFOA exposure via drinking water exists in the Little Hocking drinking water district where U.S. residents (Ohio and West Virginia) have mean serum PFOA concentrations of 478 ng/ml (Emmett et al., 2006).

Children may receive significant PFOA exposures via dietary and water intake. Mean serum PFAA concentrations (such as perfluorohexane sulfonic acid) were reportedly higher in children than in adult/elderly populations (Olsen et al., 2004). In the Little Hocking water district, an area of high environmental PFOA exposure, children age two to five and the elderly had significantly increased PFOA serum levels when compared with other age groups (Emmett et al., 2006). Although a bio-monitoring study in Japan found PFOA in maternal blood, but not umbilical cord blood at parturition (Inoue et al., 2004, limit of quantitation [LOQ] 35.2 ng/ml), a recent U.S. study (Apelberg et al., 2007) of human cord blood from term pregnancies reported relatively low levels of PFOA (limit of detection [LOD] 0.2 ng/ml) and another C8 compound, perfluorooctane sulfonate (PFOS). Within the reported study concentrations, the authors found that cord blood PFOA concentrations were significantly negatively associated with birth weight. A subsequent larger Danish study also found a significant negative correlation between maternal plasma PFOA and birth weight (Fei et al., 2007).

There have been no consistent adverse health effects associated with occupational exposure to PFOA, in fact, the studies to date are contradictory. In worker populations, serum cholesterol and triglycerides have been positively associated with PFOA exposure while high density lipoproteins have been negatively associated with PFOA (Olsen et al., 2001). Categorical division of workers by PFOA exposure levels showed that, although not significantly different from the other categories, body mass index (BMI) was elevated in the highest PFOA category (>30 ppm and BMIs >28, 1995 data); this trend was not seen in the 1993 data set (Olsen et al., 1998). A retrospective cohort mortality study ($n > 6000$) of PFOA-exposed employees reported significantly elevated standardized mortality ratios (SMR) in males with diabetes mellitus when compared to men residing in West Virginia (minus the PFOA manufacturing area), Ohio, Virginia, Kentucky, Indiana, Pennsylvania, Tennessee, or North Carolina; the SMR for PFOA workers was not significantly increased when compared to West Virginia alone or USA residents (DuPont, 2006). In Arnsberg, Germany, PFOA was found to have an inverse correlation with BMI in adults (Hölzer et al., 2008).

The $t_{1/2}$s for PFOA in men and women are similar (Harada et al., 2005). Unlike humans, gender differences in PFOA clearance exist in rats (Kudo and Kawashima, 2003; Vanden Heuvel et al., 1991). Mice are the preferred animal model for evaluating the effects of PFOA on the developing fetus as they do not exhibit gender-dependent $t_{1/2}$ differences (Lau et al., 2006). However, even in the rat model system where the female rat rapidly excretes the compound, PFOA readily crosses the placenta (Hinderliter et al., 2005) and PFAAs are present in rat milk after PFOA treatment (Hinderliter et al., 2005).

Mice prenatally exposed to doses of PFOA at $\geq$1 mg/kg/day exhibit developmental toxicity including decreased litter size, neonatal death, delayed eye opening, growth deficits, stunted mammary gland development, and early onset male puberty (Lau et al., 2006; White et al., 2007; Wolf et al., 2007). At higher doses and following long-term adult exposure, cancer endpoints associated with PFOA exposure in rats include Leydig cell adenomas, pancreatic acinar cell adenoma/carcinomas, mammary fibroadenomas, and liver tumors (Biegel et al., 2001; Sibinski, 1987). PFOA increased estradiol (E_2) levels in male rats and PFOA-induced rodent Leydig cell tumors are hypothesized to arise from increased estradiol levels from aromatase induction (Liu et al., 1996; Biegel et al., 2001).

The majority of the ongoing work in the PFOA field has focused on the health effects following developmental exposure to PFOA. This study focuses on adult latent health outcomes in female offspring after developmental (gestational days (GD) 1–17) vs. adult (at 8 weeks of age, for 17 days) exposure to PFOA. Ovariectomized siblings were utilized in our second study block to address the role of the ovarian hormones in PFOA exposure-related health effects, as luteinizing hormone (LH)-overexpressing mice (Kero et al., 2003) displayed several phenotypic effects resembling those in our preliminary studies with PFOA. These studies address the role of developmental exposure and ovarian hormones in adult health effects including circulating leptin and insulin concentrations, adult body weight, and tissue and body weights in old age.

2. Materials and methods

2.1. Animals

Timed-pregnant CD-1 mice (Charles River Laboratories, Raleigh, NC) arrived on gestational day (GD)0 (sperm positive) at the US EPA where they were weighed upon arrival and randomly distributed among treatment groups. Pregnant dams were housed individually in polypropylene cages and received chow (LabDiet 5001, PMI Nutrition International LLC, Brentwood, MO) and tap water *ad libitum*. Two blocks of animals were used in these studies. Block 1 animals were dosed with vehicle (distilled water), 1, 3, or 5 mg PFOA/kg body weight (BW) ($n = 5$, 8, 7, and 5 dams, respectively); block 2 animals were dosed with vehicle, 0.01, 0.1, 0.3, 1, or 5 mg PFOA/kg ($n = 14$ dams in all groups except 5 mg PFOA/kg BW, which had 10 dams). PFOA exposures are shown in the text as mg PFOA/kg. Animal facilities were maintained on a 12:12-h light-dark cycle, at 20–24 °C with 40–50% relative humidity. Animals were humanely treated as approved under National Health and Environmental Effects Research Laboratory protocols in accordance with the US EPA Institutional Animal Care and Use Committee (IACUC). Sentinel mice, housed in the same room, were known to be free of ecto/endoparasites and antibodies to certain viruses for the duration of these studies.

2.2. Dosing solution and procedures

PFOA, as its ammonium salt (>98% pure), was acquired from Fluka Chemical (Steinhiem, Switzerland). PFOA dosing solution was prepared fresh daily in deionized water, and the dosing solution was administered at a volume of 10 μl/g. Mice received either water vehicle or PFOA at 0.01, 0.1, 0.3, 1, 3, or 5 mg/kg BW by oral gavage once daily over the dosing periods. The highest dose (5 mg PFOA/kg/day) was chosen because it was known to result in slightly reduced neonatal body weight gain with minimal postnatal mortality (Lau et al., 2006).

2.3. Experimental design

2.3.1. Developmental exposure/intact

Timed-pregnant CD-1 mice ($n = 7$–22 dams per dose group over two blocks) received 0, 0.01, 0.1, 0.3, 1, 3, or 5 mg/kg PFOA by oral gavage on the mornings of GD 1–17. Dams were weighed daily prior to dosing and throughout gestation. At birth, pups were individually weighed and sexed. Pups within a treatment group were pooled and randomly redistributed among the dams of their respective treatment groups, and litters were equalized to 10 pups (both genders represented). Dams

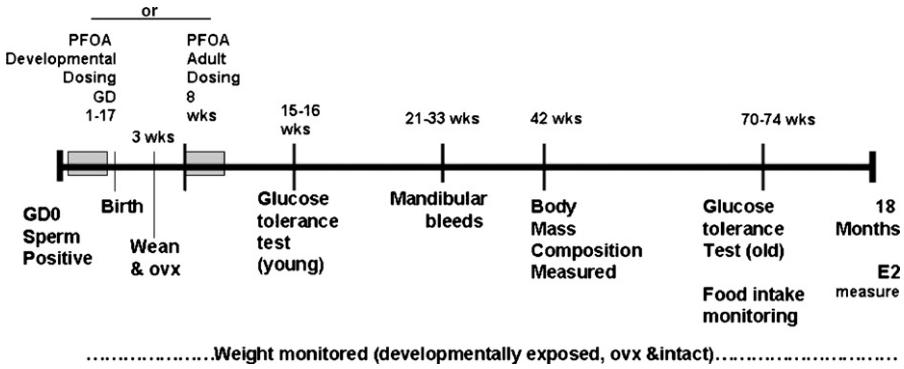

Fig. 1. Data collection schematic for study of developmentally and adult PFOA-exposed female mice.

that delivered small litters ($n < 4$ pups) were excluded from the remainder of the study. Pups were weaned at 3 weeks of age at which point females were retained and housed 3–5 mice per cage. Males were evaluated separately, at end points that varied from those reported here.

2.3.2. Developmental exposure/ovariectomy

A subset of developmentally exposed female siblings (0 mg PFOA/kg, $n = 8$; 0.01 mg PFOA/kg, $n = 15$; 0.1 mg PFOA/kg, $n = 11$; 0.3 mg PFOA/kg, $n = 14$; 1 mg PFOA/kg, $n = 6$; 5 mg PFOA/kg, $n = 7$) were ovariectomized (ovx) at 21 or 22 days of age, before the onset of puberty. Animals were sedated with ketamine/xylazine (87/13 mg/kg i.p., respectively), their ovaries surgically removed through the abdomen, sutured, and animals were placed in warming cages until they regained alertness. Buprenorphine analgesic (0.05 mg/kg) was given twice daily i.m. for 48 h in 0.1 ml volume for pain relief.

2.3.3. Adult exposure

A separate cohort of mice received PFOA starting at 8 weeks of age, for 17 days (0 mg PFOA/kg, $n = 8$; 1 mg PFOA/kg, $n = 14$; 5 mg PFOA/kg, $n = 14$).

2.3.4. Data collection

The data collection scheme for these studies is shown in Fig. 1. Blood was collected from the submandibular veins of ovx and intact mice between the ages of 21 and 33 weeks. These bleeds took place between 14:00 and 18:00, and 200 μl of blood (100 μl of serum) was collected for subsequent analyses of insulin and leptin. Females in all three exposure scenarios were weighed weekly up to 9 months of age and then monthly until 18 months. The number of intact, developmentally exposed mice weighed weekly/monthly was 10, 25, 20, 11, and 32, respectively for 0, 0.01, 0.1, 0.3, and 1.0 mg PFOA/kg. If mice became moribund before the study ended, they were euthanized in compliance with the protocol approved by the US EPA IACUC (early necropsy). Date and cause of early morbidity or mortality was recorded if known. At early necropsy (collected when necessary) or at 18 months, trunk blood, retroperitoneal abdominal white (found lying ventral to the intestines and reproductive tract) and interscapular brown fat pads, abnormal growths, and organs were collected from all exposure groups. Relative organ weight is used to express organ weight as percent of total body weight. Data are reported here as mean ± SEM.

2.4. Glucose tolerance test

Glucose tolerance tests were performed on two groups of intact developmentally PFOA-exposed animals: old adults (17 months of age with 0, 0.1, 1 or 5 mg PFOA/kg; $n = 8$–13 per dose group) and young adults (15–16 weeks old with 0, 1 or 5 mg PFOA/kg; $n = 12$ per dose group). The night before the assay, fur was shaved from the lateral area of the lower leg to expose the saphenous vein and animals were fasted. The following morning, the mice were weighed and blood glucose was measured by collecting a drop of blood from each mouse via puncture of the saphenous vein (or tail vein if necessary). The blood drop was placed on a test strip, and inserted into the calibrated glucometer (Accuchek Advantage) for baseline glucose measurement. The mice were then injected i.p. with D-glucose solution (2 g/kg body weight from a stock solution), and blood glucose concentrations were measured at 20, 40, 60 and 120 (old mice) or 180 (young mice) minutes (±1–3 min) after the initial glucose injection.

2.5. Serum leptin

Serum (10 μl) collected by mandibular venipuncture was assayed for leptin by radio-immunoassay (Linco Research, St. Charles, MO) following the manufacturer's protocol ($n = 5$, controls; $n = 18$, 0.01; $n = 16$, 0.1; $n = 11$, 0.3; $n = 24$, 1 mg PFOA/kg). The coefficient of variation (CVs) for the standards (concentration range of 0.2–20 ng/ml) ranged from 0.1% to 8.0%. The quality control standards termed QC1 (expected range 0.6–1.3 ng/ml) and QC2 (range 1.8–3.8) had a measured concentration in these assays of 0.9 and 2.9, respectively.

2.6. Serum insulin

Sera (10 μl) collected by mandibular venipuncture were assayed for insulin by the ultra-sensitive single molecule immunoassay by Singulex (Alameda, CA) following the manufacturer's protocol ($n = 9$ control, $n = 21$, 0.01 mg PFOA/kg; $n = 16$, 0.1 mg PFOA/kg; $n = 11$, 0.3 mg PFOA/kg; $n = 31$, 1 mg PFOA/kg). Samples were analyzed using a 384-well plate format with monoclonal capture and detection antibodies on the Singulex Errena equipment. The CVs for the assay standards (range 19.5–5000 pg/ml) were from 3% to 17%. The assay LOD was 16 pg/ml. All samples were run on the same day and the interassay CV was 9.4% and 5.1% for the 29 and 1745 pg/ml quality assurance standards, respectively.

2.7. Body mass composition

Whole body mass composition was measured in live, non-sedated 42-week-old mice using the Bruker Minispec mq 7.5 LF50 Live Mouse Analyzer (The Woodlands, TX). The minispec was a benchtop 7.5 MHz time-domain nuclear magnetic resonance (NMR) analyzer, which quantified body fat, lean tissue, and free body fluid in mice. The minispec was calibrated by Bruker Optics, Inc. staff prior to animal analysis with daily validations using Bruker standards. Mice were weighed and inserted into the instrument for analysis (1–2 min/animal). Intact developmentally exposed female mice that underwent body mass composition analysis included control, 0.01, 0.1, 0.3, and 1 mg PFOA/kg ($n = 9$, 23, 20, 11, and 32, respectively) dose groups. It was not possible to perform these measures with younger mice due to equipment availability.

2.8. Measurement of E_2 in serum of intact mice at 18 months

Serum E_2 (25 μl volume) from 18-month-old mice (intact developmentally PFOA-exposed animals) was measured with time resolved fluoro-immunoassay (DELFIA Estradiol Kit, Wallac Oy, Finland) following the manufacturer's recommendation using a VICTOR^{2}D 1420 Multilabel counter, PerkinElmer Precisely time-resolved fluorometer (PerkinElmer Life & Analytical Sciences, Shelton, CT). The CVs for the standards (concentration range of 6.81–142.5 pg/ml) ranged from 0.2% to 4.8%.

2.9. Feed consumption

Feed consumption in 17-month-old, developmentally exposed, intact female mice ($n = 6$ per dose group, 0, 0.1, 1 and 5 mg PFOA/kg) was measured in metabolic cages. Mice were allowed to acclimate to the cages for 1 week and food intake was monitored during the second week. Mice were individually housed and provided with a pre-weighed amount of powdered lab chow *ad libitum*. The remaining chow was measured at the end of the week and the total amount was subtracted from the starting amount to determine the total feed consumed for each mouse per week.

2.10. Measurement of serum PFOA

Trunk blood serum samples (~50 μl) from the female CD-1 offspring at 18-month necropsies or from mice terminated at earlier intervals because of illness were transferred to the CDC for PFOA measurement. Serum PFOA determination was performed as described in Kuklenyik et al. (2005) and White et al. (2009).

2.11. Statistics

Data were analyzed using SAS 9.1 (SAS Inc., Cary, NC). Body weight on PND1 was evaluated as litter means as these data were obtained prior to mixing litter offspring within a dose group.

Body weights at each time point were analyzed with mixed effects linear models (SAS Proc Mixed) to estimate means and standard errors and test for dose effects separately by time point. For each time point the model included dose as a fixed effect

and cage nested within dose as a random effect. Pairwise *t*-tests were calculated to test for any difference between each treatment group mean and the control group.

Repeated measures analysis of body weight data was evaluated two ways. First, weights were averaged by animal over eight 10-week intervals. This was done to decrease missing values in the data due to animal mortality in late life that was not equal across treatment, and to reduce the effect of large body weight variances later in life. This data smoothing method decreased uninformative short-term variations and also reduced the number of estimated parameters to a tractable value. A multivariate repeated measures analysis (SAS Proc GLM) was performed on these reduced data. Subsequent to a significant finding, comparisons were carried out as subtests of the overall analysis of variance (ANOVA) at specific times or doses.

Second, SAS Proc Mixed was used to perform a univariate repeated measures analysis of the weights across time up until 37 weeks (latest weight point at which no animals had died). The model estimated a separate fixed quadratic curve across time for each dose group and included a random effect for cage nested within dose. Correlation within animals was modeled with a random effect for animal nested within cage and dose in addition to an autoregressive covariance structure within each animal. In this way, the covariance matrix for each animal's measurements included a constant covariance component at all time points in addition to a component which decreased as time points grew farther apart.

Tissue weight, relative tissue weight, body composition, food consumption, and body weight measurements were analyzed using a one-way ANOVA (Dunnett's post hoc tests), with dose being the independent variable. A blocking variable was included to adjust for the group difference. No adjustment was made for multiple comparisons. Glucose tolerance was compared at individual collection times by one-way *t*-test and over time by repeated measures and area under the curve comparisons according to the trapezoidal rule. Hormone (insulin, E_2 and leptin) concentrations were analyzed using ANOVA followed by Tukey's post hoc test.

Mortality data were analyzed with product limited survival estimates; log-rank and Wilcoxon tests were used to test for differences among the treatment groups in survival across time (SAS Proc Lifetest). The level of significance for all tests was $p < 0.05$.

3. Results

3.1. Developmental exposure

3.1.1. Early and mid-life body weight effects

There were no significant differences in live pup number at birth by dose group ($p < 0.05$) and postnatal mortality was not addressed in this study as litters were equalized at birth. On postnatal day (PND) 1, the average weight of the developmentally exposed 5 mg PFOA/kg offspring was significantly less than controls (Fig. 2A); no other dose group demonstrated significant litter weight effects at PND1. At weaning, mean female body weights were still significantly decreased in the 5 mg PFOA/kg (13.9 g ± 0.8) compared to 18.4 g ± 0.4 in control untreated pups. At this time, the 1 mg PFOA/kg exposed animals were also significantly smaller than controls ($p < 0.05$; 16.4 g ± 0.3).

Time-grouped mean body weights of the female offspring over their lifetime are shown in Fig. 2B. Beginning at 10–19 weeks of age, there was an increase in weight in the 0.1 and 0.3 mg PFOA/kg groups compared to controls; by 20–29 weeks of age, females developmentally exposed to PFOA showed significant dose-dependent increases in body weight at 0.01, 0.1, and 0.3 mg PFOA/kg which extended to 40 weeks of age in the 0.01 and 0.1 mg PFOA/kg when compared with control ($p \leq 0.05$). This is specifically shown at 20–29 weeks (Fig. 2C), where the 0.01–0.3 mg PFOA/kg groups had average weights 11–15% higher than controls.

Continuous analysis of repeated measures of body weight over time demonstrated that the five dose groups were similar in intercept using a quadratic fit; however, the 0.01, 0.1 and 0.3 groups had a significantly greater week effect than control, indicating that their weights were changing at a more rapid rate than control or 1 mg/kg. This is shown in Fig. 2D for weeks 6–37 (the latest weight collection time point prior to death of any study animals). Additionally, the 0.1 mg/kg ($p = 0.056$) and 0.3 mg/kg ($p = 0.046$) groups had larger negative coefficients for week^2 (week squared), suggesting that their weights were starting to fall off more quickly at the later time points than the control groups (not shown). The estimated weight curve for the 1 mg PFOA/kg dose group was not significantly different from the control curve. Data from 5 mg PFOA/kg exposed

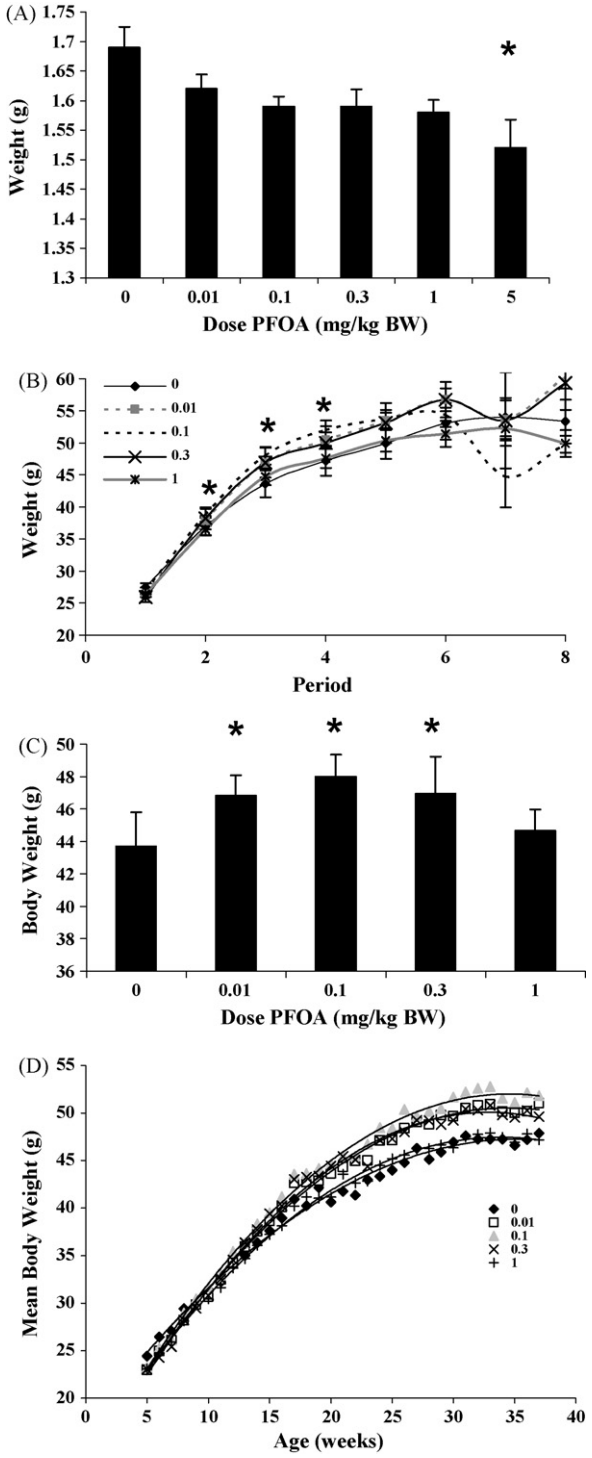

Fig. 2. Body weights of developmentally PFOA-exposed female offspring. Data are shown as mean ± SEM with $^{*}p < 0.05$ vs. control. (A) Pup weight at PND1 after developmental PFOA exposure. (B) Body weight of female CD-1 mice over their lifetime, following developmental PFOA exposure over 8 periods of time [period 1 (0–9 weeks old), period 2 (10–19 weeks old), period 3 (20–29 weeks old), period 4 (30–39 weeks old), period 5 (40–49 weeks old), period 6 (50–59 weeks old), period 7 (60–69 weeks old), and period 8 (70–79 weeks)]. (C) Group mean body weights of female offspring at 20–29 weeks of age demonstrating excessive weight gain at low doses. (D) Dose-dependent quadratic regression fit to repeated measures of body weight in female mice. An increased rate of weight gain was seen in 0.01, 0.1, and 0.3 mg PFOA/kg dose groups compared to control and 1 mg PFOA/kg.

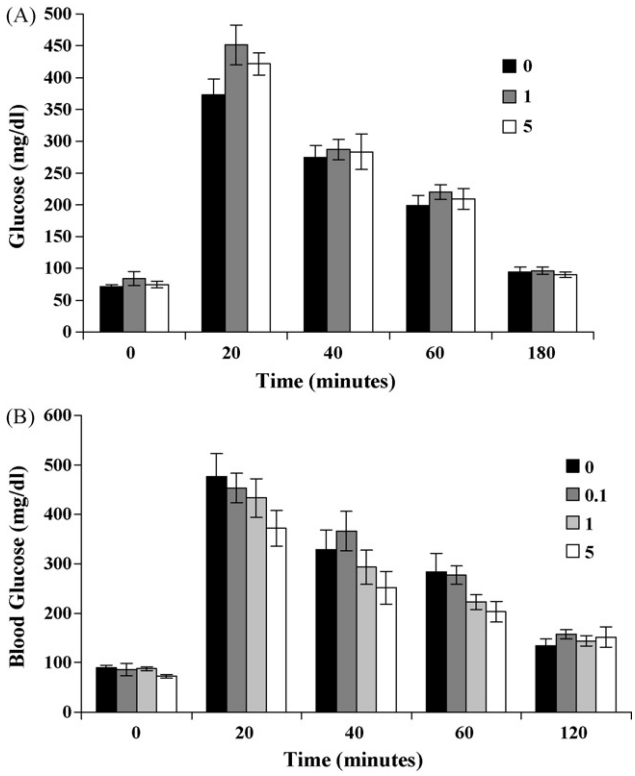

Fig. 3. Blood glucose concentrations following a glucose challenge after time 0 in (A) young (15–16 weeks old) and (B) old (70–74 weeks old) female CD-1 mice that were developmentally exposed to PFOA. Data are shown as mean ± SEM.

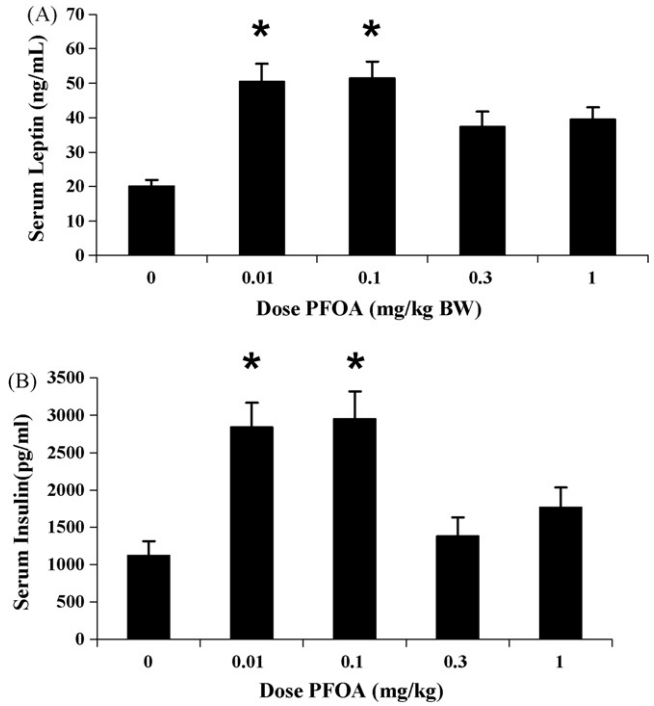

Fig. 4. Serum leptin (A) and insulin (B) in mice at 21–33 weeks of age ($^{*}p < 0.05$ vs. control). Significant elevations are seen at 0.01 and 0.1 mg PFOA/kg. Data are shown as mean ± SEM.

mice, which were decreased in BW compared to control at PND1, weaning, and 18 months, are not shown.

3.1.2. Serum glucose tolerance testing

Because of the excess weight gain in the PFOA developmentally exposed mice during mid-life, various tests were conducted on these animals (as close to the appropriate age as was possible) to examine the associated effects of these changes. No significant differences were detected in baseline glucose or serum glucose area under the curve in response to a glucose challenge in young or old mice (control, 0.1, 1, or 5 mg PFOA/kg, $p < 0.05$, Fig. 3). In a time-dependent comparison, young mice exposed to 1 mg PFOA/kg showed a nearly significant increase in blood glucose over control animals at 20 min post-glucose challenge ($p = 0.06$). In old PFOA-exposed mice, although there appeared to be dose-dependent glucose insensitivity at 20 min, this shift in response was not significant.

3.1.3. Serum insulin and leptin

Serum insulin and leptin measurements were made using blood obtained via mandibular bleeds between 21 and 33 weeks (within the time frame of greatest observed body weight increases) using intact female mice dosed with 0, 0.01, 0.1, 0.3, and 1 mg/kg PFOA. Insulin and leptin concentrations were significantly increased in mice developmentally exposed to the lowest doses of PFOA tested (0.01 and 0.1 mg PFOA/kg). Although elevated from the control mean, leptin concentrations were not significantly different from control at 0.3 or 1 mg/kg PFOA (Fig. 4).

3.1.4. Fat to lean ratio

At 42 weeks of age, mice from block 2 (control, 0.01, 0.1, 0.3, and 1 mg PFOA/kg) were evaluated using a Bruker Optics Body Mass Analyzer, which determines the amount of fat, lean and fluid in live animals. There was no significant increase detected in % body fat:body weight in PFOA-exposed mice (data not shown). Developmentally exposed mice had no significant differences in fat:lean ratio across dose groups when compared to control (means ranged from 0.75% in controls to 0.9% in 0.01 and 0.1 mg PFOA/kg). Although no dose groups were significantly different from control, there was an increase above control levels of about 12% in mean % fat:body weight ratio and 14% in mean fat:lean ratio in the dose group exhibiting the largest change in body weight at 24 weeks (0.1 mg PFOA/kg).

3.1.4.1. Feed consumption. Feed consumption was measured in 17-month-old, developmentally exposed intact mice (0, 0.1, 1 and 5 mg PFOA/kg) and no significant differences were found across dose groups when compared to controls (mean 26 g/week consumed; individual data not shown).

3.1.5. Late life organ and body weight effects

A noted loss of animals after 36 weeks of age was further evaluated (Fig. 5). At 51 weeks old, when there was no mortality in controls there were 20%, 10%, 36%, and 6% mortality rates in 0.01, 0.1, 0.3, and 1 mg PFOA/kg groups, respectively. By 76 weeks, there was a 40% mortality rate in controls, and 32%, 63%, 60%, and 44% in 0.01, 0.1, 0.3 and 1 mg PFOA/kg groups, respectively. However, there were no significant differences between control and any treatment group at specific times in late life or in survival across time.

Among those mice surviving to 18 months, body weight of PFOA-exposed females was no longer elevated compared to controls. Furthermore, a significant decrease in body weight at the 5 mg PFOA/kg dose was noted (Table 1). At that time, all remaining females were necropsied. Trunk blood, tissues (affected or of interest) and abnormal masses were collected, weighed and fixed for future study. Serum was collected and PFOA levels were measured. The majority of the samples across dose groups had PFOA concentrations lower than the limit of detection (0.5 ng/ml) with detectable values at maximum concentrations of 3.5 ng/ml, and

Table 1
Mean or relative body and tissue weights at 18 months of age in intact and ovariectomized (ovx) female CD-1 mice.

PFOA dose (mg/kg)	Body weight (g)		Abdominal white fat		Interscapular brown		Relative spleen		Relative liver	
			Weight (g)		Fat weight (g)		Weight (%)[c]		Weight (%)[c]	
	Intact	Ovx	Intact	Ovx	Intact	Ovx	Intact	Ovx	Intact	Ovx
0	54.90 ± 1.83	52.73 ± 5.67	7.07 ± 0.56	3.83 ± 1.03	0.73 ± 0.04	0.37 ± 0.09	0.39 ± 0.05	0.52 ± 0.16	4.20 ± 0.10	4.80 ± 0.44
0.01	56.56 ± 1.48	52.61 ± 3.63	6.68 ± 0.41	5.08 ± 1.00	0.80 ± 0.04	0.36 ± 0.04	0.30 ± 0.03	0.29 ± 0.04	3.99 ± 0.11	4.12 ± 0.35
0.1	54.60 ± 1.17	52.76 ± 1.98	5.91 ± 0.37	4.84 ± 0.71	0.82 ± 0.04	0.46 ± 0.06	0.45 ± 0.12	0.40 ± 0.08	4.20 ± 0.41	4.18 ± 0.21
0.3	56.00 ± 1.74	49.36 ± 3.53	5.96 ± 0.63	4.57 ± 0.46	0.79 ± 0.06	0.39 ± 0.02	0.45 ± 0.10	0.33 ± 0.08	4.30 ± 0.31	4.07 ± 0.22
1	56.15 ± 1.35	61.47 ± 3.53	5.82 ± 0.43[a]	5.82 ± 0.77	0.89 ± 0.04[a]	0.69 ± 0.06[a]	0.30 ± 0.03	0.22 ± 0.02[b]	4.02 ± 0.10	3.55 ± 0.29[b]
3	53.69 ± 2.27	nc	nc	nc	1.22 ± 0.10[a]	nc	0.18 ± 0.03[a]	nc	3.98 ± 0.23	nc
5	49.37 ± 1.51[a]	55.13 ± 5.76	4.48 ± 0.65[a]	5.86 ± 1.67	0.86 ± 0.05	0.62 ± 0.20	0.52 ± 0.24	0.21 ± 0.04[b]	4.37 ± 0.34	3.65 ± 0.33

nc, Denotes not collected from this dose group.
[a] $p < 0.01$ vs. control.
[b] $p = 0.05–0.07$.
[c] Relative weight (organ weight as percent of body weight).

there was no significant difference in serum PFOA concentrations across dose groups (data not shown). There were no significant differences in serum estradiol levels in developmentally exposed females at 18 months when compared to controls (non-cycling; mean range across doses from 12.9 to 15.8 pg/ml).

Tissue weights from 18-month-old animals (intact and ovx) are shown in Table 1. To determine if the weight of fat depots was altered in old animals due to developmental PFOA exposures, the retroperitoneal abdominal white and interscapular brown fat pads were collected and weighed. Abdominal white fat weight and relative white fat weight both showed significant decreases vs. control ($p < 0.05$) at 1 and 5 mg PFOA/kg. White fat weights were not collected for 3 mg/kg PFOA animals. At 18 months, interscapular brown fat weight and relative brown fat weight both showed significant increases above control ($p < 0.05$) at 1 and 3 mg PFOA/kg. The spleen was quite variable in weight among the different treatment groups, but there was a significant difference in spleen weight and relative spleen weight vs. control at 3 mg PFOA/kg ($p < 0.05$). Finally, at 18 months, no significant differences in liver weight or relative liver weight were detected.

3.1.6. Effect of ovariectomy on tissue and body weight gain

A group of developmentally PFOA-exposed animals (0, 0.01, 0.1, 0.3, 1, and 5 mg PFOA/kg) were ovx at weaning and their body weight gain and adult health was assessed until they reached 18 months of age. At mid-life the weight of the control ovx females was expected to be greater than that of the sham-operated, intact controls (Fig. 6A; set of bars at 0 mg/kg), but the variance in the animal weights was appreciable and therefore the differences did not reach statistical significance. When comparing the body weights of animals in the ovx study by treatment group, over time (4 weeks to 18 months), using statistical methods consistent with those used for intact animals, there was no effect of PFOA (Fig. 6B). Comparison of ovx animals to intact animals at 20–29 weeks, as shown in Fig. 6A, demonstrates an absence of body weight gain over control in the ovx animals treated with PFOA. PFOA exposure did not stimulate increased weight gain (above that of control ovx) at any developmental exposure level in the absence of the ovaries (also seen in Fig. 6B). The ovx animals were siblings to the intact animals in this study.

The ovx animals were also assessed at 18 months. Developmentally PFOA-exposed ovx animals showed no significant differences in body weight when compared to control ovx females (control mean = 52.7 ± 5.67; highest mean, 1 mg PFOA/kg = 61.5 ± 3.3; Table 1).

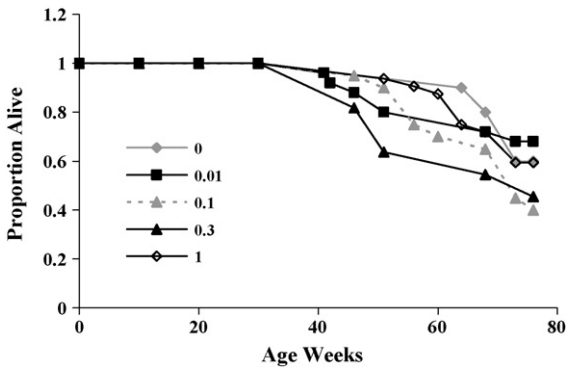

Fig. 5. Survival curves for developmentally PFOA-exposed female mice (0–1 mg PFOA/kg). Although a fair number of PFOA-exposed animals die early, a Lifetest (SAS) analysis detected no significant decrease in time to death. The reasons for early life mortality are under investigation.

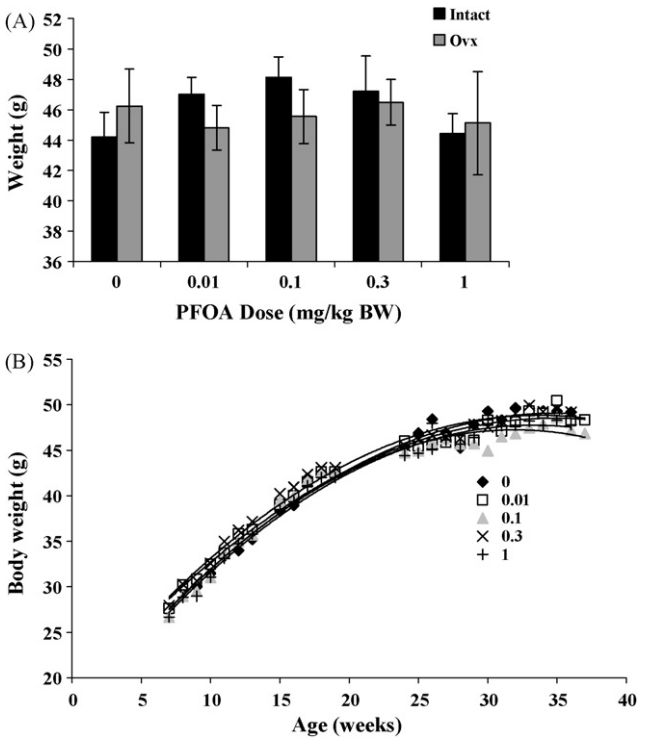

Fig. 6. (A) PFOA-dependent changes in group mean body weight of intact and ovx female offspring at 20–29 weeks of age. There was no change in body weight of ovx animals across PFOA exposures. (B) Dose-dependent quadratic regression fit to repeated measures of body weight in ovx female mice. Unlike intact siblings, no significant differences were seen between dose groups in the ovx animals.

As with intact siblings, the tissue weights of ovx animals are reported in detail in Table 1. In ovx animals, neither abdominal white fat pad weight, nor relative abdominal white fat pad weight, were significantly different from ovx or intact control levels. This varies slightly from intact siblings, where the white fat pad was significantly decreased in size; although, in animals that weighed significantly less than intact controls. Among PFOA-exposed ovx animals, both interscapular brown fat weight and relative brown fat weight (data not shown) showed significant increases above control ovx levels at 1 mg PFOA/kg ($p < 0.05$); no other dose groups showed a significant increase. This is similar to the effect seen in intact animals, and was significant at the same dose. Spleen weight (data not shown) and relative spleen weight in ovx animals was highly variable at 18 months, and showed decreases, albeit not highly significant, at the 1 and 5 mg PFOA/kg doses ($p = 0.06$ and $p = 0.05$, respectively; Table 1). 1 and 3 mg PFOA/kg (not 5 mg/kg) were the doses in the intact animals showing the largest decreases in relative spleen weight compared to controls. Finally, relative liver weight showed no significant differences across dose groups when compared to ovx control.

3.1.7. Lack of effects from adult PFOA exposure

At 18 months of age, body and tissue weights were recorded in adult PFOA-exposed mice. Adult PFOA exposure had no effect on terminal body or organ weights. When a comparison of data from 18-month-old adult intact and developmentally exposed animals in the 0, 1 and 5 mg PFOA/kg dose groups was made, body weight, brown fat weight, and white fat weight of the 1 mg PFOA/kg developmentally exposed animals were significantly higher than the same dose in adult-exposed animals (data not shown).

4. Discussion

These studies demonstrated the effects of developmental PFOA exposure on CD-1 female mouse body and organ weight, as well as serum leptin and insulin in adulthood. In the developmental PFOA studies, a dose-dependent dichotomy of phenotypes was present in intact female mice; latent effects present following high doses were not present in mice exposed to low-dose PFOA and *vice versa*. Although there was no detectable change in body weight neonatally, low-dose PFOA exposures (0.01, 0.1, or 0.3 mg PFOA/kg) led to significantly increased mean weight and rate of weight gain in mid-life (up to and including 37 weeks of age) and a coincident significant elevation of serum leptin and insulin values between 21 and 33 weeks (0.01 and 0.1 mg PFOA/kg).

Our low-dose hormone data indicate potentially important metabolic changes that mechanistically support the findings of increased weight in the lower dose groups. Previous dosimetry work in our lab has shown that *in utero* exposure to PFOA in the mouse translates into an extended developmental exposure period via lactational exposure (all of gestation and nearly 3 months postnatally; White et al., 2009; Wolf et al., 2007; Fenton et al., 2009). This long exposure may lead to reprogramming/metabolic events that govern fat metabolism or appetite control. Although we were unable to perform some of the other end points of interest during this time period of greatest weight gain, our findings relating leptin and insulin concentrations to the time of overweight in PFOA-exposed mice support our theory. Other environmental chemicals, termed environmental obesogens (dietylstibestrol (DES), 2OH-E_2, 4OH-E_2, genestein and bisphenol A), have been shown to induce obesity in adulthood after low-dose developmental exposure, while inducing weight loss at higher doses (Grün et al., 2006; Newbold et al., 2005; Miyawaki et al., 2007) and are reviewed further within this issue.

Serum leptin was significantly elevated in mid-life in the low-dose PFOA-exposed groups. This effect occurred at the same PFOA dose range as overweight in these animals, congruent with a leptin-resistance mechanism of action for overweight, as previously reported in humans (Considine et al., 1996). Others have reported increased leptin with developmental exposure to environmental obesogens including DES (Newbold et al., 2007).

Low-dose (0.01 and 0.1 mg PFOA/kg) developmental PFOA exposure that led to increased serum leptin and body weight also increased insulin values at 21–33 weeks. This suggests that the insulin resistance mechanistic pathway could also be affected and play a role in developmental PFOA exposure-induced overweight in mice. In an insulin resistance scenario, there are raised plasma glucose levels (elevated, but not significant, at 15–16 weeks in our study), reflecting the loss of a post-challenge peak in insulin response (reviewed in Montecucco et al., 2008). Insulin resistance is known to be associated with excess abdominal fat in normal and overweight women (Carey et al., 1996). High plasma levels of insulin and glucose, due to insulin resistance, are often associated with type II diabetes and metabolic syndrome in humans, and thus this effect of low-dose PFOA developmental exposure and its association with increased serum insulin are important.

The ovx data were difficult to interpret. The lack of additional weight gain with developmental PFOA and ovx may reflect a "ceiling effect" or that ovx-induced weight increases may have masked any effect of PFOA. Alternatively, as weight gain and metabolic hormones can be regulated by estrogens, the role of the ovaries in developmental effects of PFOA was explored by using ovx animals. The potential importance of the ovary in the effects of PFOA was based on the observation that LH-transgenic (overexpressing) mice (Kero et al., 2003) were phenotypically similar to ours (increased body weight, increased brown fat depots, and predominant ovarian cysts not discussed in this paper). We hypothesized that removal of the LH target (the ovary) in our study may reveal the mode of action for PFOA effects for the increase in brown fat and possibly the excessive weight gain. Ovx animals typically gain body weight in excess vs. intact animals (Kamei et al., 2005). The critical role of the ovary in weight gain of intact PFOA-exposed females beyond that of ovx treatment-matched siblings in the 0.01 and 0.1 mg PFOA/kg groups was novel and signifies the ovarian axis as a potential mediator of PFOA-dependent mid-life weight changes.

Another potential mediator of these intertwined low-dose PFOA-induced effects is the peroxisome proliferator-activated receptor (PPAR) activation pathway. PPAR gamma (PPAR-γ) and PPAR alpha (PPAR-α) are involved in lipid metabolism in adipocytes and liver/skeletal muscle, respectively (reviewed in Medina-Gomez et al., 2007; Abbott, 2009). These PPAR isoforms are known to influence lipogenesis/weight gain and have been shown to be regulated by environmental compounds such as tributyltin (Grün et al., 2006; reviewed in this issue). Weight loss events in leptin-deficient, obese, and insulin-resistant mouse models have coincided with PPAR-regulated changes in gene expression (Holvoet, 2008). A down-regulation of PPAR isoforms involved in energy expenditure, lipogenesis or fatty acid synthesis have been reported in adipose and skeletal muscle of ovariectomized mice (Kamei et al., 2005). PFOA has been shown to be a PPAR activator in liver tissue (high doses) and cell lines, and to be required for PFOA-induced developmental toxicity in mice (Takacs and Abbott, 2007; Abbott et al., 2007; Abbott, 2009). If PPAR activation via receptor binding is a primary mode of action for body weight effects following PFOA exposure, the decrease in the PPAR receptors following ovariectomy and decreased circulating estrogens may explain the lack of effect of PFOA in ovx mice. However, PFOA-induced consequences of PPAR activation following a developmental exposure are just beginning to be evaluated.

After 40 weeks of postnatal age, an increase in mortality was detected in all animals. There are previous reports in the literature of increased mortality in non-treated CD-1 mice, attributed primar-

ily to thymic lymphomas (Son, 2004; Taddesse-Heath et al., 2000). Because of this confounding circumstance, repeated measures of body weight were only followed out to 37 weeks of age.

The other half of the phenotypic dichotomy caused by developmental PFOA exposure was also novel. Developmental exposure to higher doses of PFOA (1, 3 and 5 mg PFOA/kg) led to a vastly different phenotype from low-dose PFOA exposure. This effective PFOA dose dichotomy may manifest itself in our study via unique modes of action; the animals with highest dose(s) of developmental PFOA exposure have decreased early life body weight and terminal body weight (5 mg PFOA/kg) with significant decreases in white fat weight at 18 months (1 and 5 mg PFOA/kg), significant increases in brown adipose (1 and 3 mg PFOA/kg), and significant decreases in spleen weight (3 mg PFOA/kg) findings that are absent with the lower doses of PFOA.

Others have reported dose-dependent loss of white tissue adiposity in adult male mice after PFOA exposure (0.02% PFOA weight/chow weight, which translated to approximately 32 mg PFOA/kg BW daily) with fat loss, without fat cell number loss, that is PPARγ-independent with β-adrenergic activation (Xie et al., 2002). In that same study, investigators also reported white fat and body weight decrements at higher doses that were absent at lower doses. Yang et al. (2002) showed PFOA-dependent weight loss was abrogated in PPAR-α null mice, indicating that PPAR-α is a probable regulator of weight loss in the high dose animals. In subsequent studies, Xie et al. (2003) showed that after cessation of exposure of adult male animals to PFOA (0.02% PFOA weight/chow weight, 32 mg PFOA/kg BW) daily for 7 days followed by 10 days recovery, weight loss and white adipose levels returned to baseline, which confirms the importance of developmental exposures for the latent effects reported here. In our model with developmental PFOA exposure we see permanent weight loss and white adipose tissue loss at the high dose of PFOA. However, there may be merit in further exploring these mechanisms of action, as β-adrenergic receptor upregulation is also associated with increased brown fat mass in winter-acclimated animals (Feist, 1983), and this tissue was associated with high dose (and not low dose) effects in both intact and ovx animals in this study. Although we suspected alleviation of effect in the brown fat pad by eliminating the ovary (based on phenotypes in Kero et al., 2003), significant increases in brown fat were seen at 1 mg PFOA/kg in both intact and ovx animals.

At the 18-month time point, some endpoints remained unchanged across dose groups including liver size. Earlier work has shown significant hepatomegaly after developmental PFOA exposure (1 and 3 mg PFOA/kg) observed out to at least 3 weeks after birth (the latest time point evaluated; Wolf et al., 2007; White et al., 2007). The transient nature of hepatomegaly has been illustrated in other acute adult exposure studies (reviewed by Lau et al., 2007), and is further confirmed in these studies (intact and ovx).

A final important component of these studies evaluated adult vs. developmental exposure to PFOA on body tissue weights. These data suggest that the timing of dosing (adult vs. developmental 17-day PFOA exposure) was critical for latent effects. There was no effect of 17-day adult PFOA exposure on any endpoint in this study (early life or latent) when compared to age-matched, vehicle-gavaged controls.

In conclusion, the timing and dose of PFOA exposure for induction of dichotomous, persistent, adult health effects in CD-1 female mice are critical. Developmental, low-dose PFOA exposure led to increased weight in adults, with increased serum insulin and leptin, a health effect not seen in high dose animals. No observable adverse effect levels (NOAEL) for body weight gain, serum leptin and insulin concentrations were not determined in this study; but 0.01 mg PFOA/kg had a significant impact on these particularly sensitive end points. The ovary appeared to play an important role in the overweight effect in mid-life, and it is proposed that there is a common mode of action, potentially dysregulation of PPAR and its signaling through ovarian hormones, that may be responsible for these low-dose health effects. Further studies addressing long-term PFOA-induced health outcomes in mice should focus attention on internal dose relative to the low-dose health effects seen in this study, as well as the mechanisms of action, so that any relevance to human health effects can be addressed.

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