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The reproductive toxicology of ammonium perfluorooctanoate (APFO) in the rat

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Abstract

Ammonium perfluorooctanoate (APFO) is a surfactant used primarily as an aid in processing various fluoropolymers. Many toxicology and epidemiological studies have been conducted with APFO; however, no specific information regarding functional reproduction was previously available. Therefore, the potential reproductive toxicity of APFO across two generations of offspring was studied using current EPA OPPTS 870.3800 guidelines. Male and female Sprague–Dawley rats were dosed orally with 0, 1, 3, 10, or 30 mg/kg APFO. Parental (P) generation rats (~6 weeks old) were dosed at least 70 days prior to mating and until sacrificed (after mating for male rats; after weaning for female rats). F_1-generation rats were dosed similarly, beginning at weaning. The F_2-generation pups were maintained through 22 days of lactation. Reproductive parameters evaluated in P- and F_1-generation rats included estrous cycling, sperm number and quality, mating, fertility, natural delivery, and litter viability and growth. Age at sexual maturation in F_1, anogenital distance in F_2, and presence of nipples (males) in F_2-generation pups were also determined. Feed consumption, body-weight gain, selected organ-weights, gross pathology and appropriate histopathology were evaluated. Reproductive endpoints including mating, fertility, and natural delivery were not affected in either generation. P- and F_1-generation male rats showed decreased body weight, and liver and kidney weight increases at all doses. The 30 mg/kg F_1-generation pups had decreased birth weight. Viability was reduced in the 30 mg/kg F_1-generation pups in apparent relationship to reduced body weight at birth and weaning; however, F_2-generation pups at 30 mg/kg, although somewhat lighter, did not show a loss in viability. Preputial separation and vaginal opening were somewhat delayed at 30 mg/kg, but these rats went on to show normal reproductive performance. No-observed-adverse-effect-levels were >30 mg/kg for reproductive function of P- and F_1-generation rats, 10 mg/kg for F_1-generation pup mortality, birth weight, and sexual maturation, and less than 1 mg/kg for male body-weight and organ-weight changes.

Keywords: Ammonium perfluorooctanoate (APFO); Perfluorooctanoate (PFOA); Reproduction; Multi-generation

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1. Introduction

Ammonium perfluorooctanoate (APFO; FC-143, C_8, $C_7F_{15}COO^-NH_4^+$, CAS Registry No. 3825-26-1) has been used as a processing aid in the production of fluoropolymers. Perfluorooctanoate (PFOA; $C_7F_{15}COO^-$), the dissociation product of APFO, is not metabolized (Pastoor et al., 1987; Vanden Heuvel et al., 1991) and has been identified in blood samples from exposed workers and the general population (Taves et al., 1976; Hansen et al., 2001; Olsen et al., 2003a). The most extensive survey of population PFOA exposure comes from three studies involving children, adults, and the elderly residing in the United States (Olsen et al., 2002a,b, 2003b, 2004) showing geometric mean serum concentrations in all three age groups of 4–5 ng/ml (ppb). The sources and pathways of exposure resulting in the presence of PFOA in general population sera have not been specifically identified. These may include: (1) emission of PFOA and its salts from industrial processes; (2) environmental or metabolic degradation of certain fluorochemical product residuals to PFOA; or (3) the distribution of PFOA as a residual in various fluorochemical products. There are marked differences in excretion of PFOA between species and between sexes in some species (Vanden Heuvel et al., 1991; Hanhijarvi et al., 1988; Ohmori et al., 2003; Burris et al., 2002). Of the species for which data have been obtained, female rats have the highest rate of elimination with half-lives measured in hours. By comparison, male rats have elimination half-lives measured in days. In humans, PFOA appears to be poorly eliminated. The human serum elimination half-life for PFOA is currently estimated to be 4.4 ± 3.5 years (Burris et al., 2002) although there are some uncertainties around this estimate. Considering the low elimination rate of PFOA in humans relative to rats and the small amount of PFOA found in sera of the general population, it is reasonable to assume that the magnitude of human exposure is quite small relative to doses typically used in toxicological studies.

A large database of experimental studies on the potential health hazards of PFOA is available, as are recent reviews (USEPA, 2002; Kennedy et al., 2004). In addition to toxicology studies in laboratory animals, the potential association of APFO exposure with health effects in fluorochemical production workers has been studied since 1976 through medical monitoring and epidemiological investigation (Ubel et al., 1980; Olsen et al., 1998, 2000). No excess cause-specific mortality has been associated with APFO exposure in this workforce (Alexander, 2001), and clinical indications of liver and hormonal function (including estradiol, testosterone, and cholecystokinin) are normal (Olsen et al., 1998, 2000). In response to toxicological findings that suggested PFOA might modulate endocrine activity in the male rat (Biegel et al., 1995, 2001), serum levels of several hormones (estradiol, 17-hydroxyprogesterone, free and bound testosterone, dehydroepiandrosterone sulfate, follicle-stimulating hormone, luteinizing hormone, prolactin, and thyroid-stimulating hormone) of 3M Company's Cottage Grove workers (predominantly male) have been analyzed. Mean estradiol levels were 10% greater among employees with the highest serum PFOA concentrations (30 ppm) (Olsen et al., 1998) but were confounded by body mass index, a factor known to be correlated positively with elevated estradiol in males (Schneider et al., 1979; Deslypere et al., 1985; Kley et al., 1980). It was concluded that there was a reasonable assurance of no significant hormonal changes associated with PFOA exposure in man (Olsen et al., 1998).

Numerous repeated-dose studies have been conducted with a variety of species (mice, rats, and monkeys), and these have recently been reviewed (Butenhoff et al., 2002a; Kennedy et al., 2004; USEPA, 2002). Together these demonstrate that liver is the primary target organ for PFOA toxicity, with liver-weight increases being observed at doses lower than those causing histological or biochemical indications of liver injury. The hepatotoxicity manifests as increased liver weights, hepatocellular hypertrophy, liver degeneration and necrosis, increases in plasma transaminases, and proliferation of smooth endoplasmic reticulum and peroxisomes in rodents. Hypolipidemia has also been reported in some rodent studies. The liver effects can be reversible on cessation of dosing. Other effects observed in one or more species include body weight decreases and increased kidney weights (without histological correlate).

Two cancer studies were conducted in rats (Riker Pharmaceuticals, 1983; Biegel et al., 2001), and increases in Leydig cell hyperplasia and benign Leydig cell tumors were observed in both studies at

dietary dose of 300 ppm. In the study by Biegel et al. (2001) increases in benign liver tumors and benign pancreatic acinar cell tumors were also observed at 300 ppm.

The potential of APFO to produce developmental effects has been evaluated in rats by the oral (Gortner, 1981; Staples et al., 1984) and inhalation (Staples et al., 1984) routes, and in the rabbit by the oral route (Gortner, 1982). No evidence of embryo-fetal toxicity or gross developmental abnormalities was noted in these studies, even at doses that produced maternal toxicity as manifested by reduced body-weight gain and increased mortality. Neonatal effects did not occur, and pups were normal throughout lactation (Staples et al., 1984). Gortner (1981) originally reported a defect of the lens of the eye in the rat; however, this was later determined to be an artifact of methodology (Staples, 1985). Also, Gortner (1982) found an increase in a normal, stress-related structural variant in the rabbit, a 13th rib. This finding is not considered a significant structural malformation per se and is not likely to be relevant to humans (Christian et al., 1987). The current practice regarding this variant is to not even count it as a variant. These studies suggest that APFO is not toxic to the embryo and fetus of rats and rabbits and does not produce structural aberrations in fetuses of either species. The morphologic effects of APFO on reproductive organs have been evaluated in a number of bioassays in multiple laboratory animal species. Results indicated no abnormalities.

The reproductive system has not been the principal target of investigation of APFO toxicity in past studies; therefore, past studies have not looked at the functional capability of the reproductive systems. Two somewhat related compounds, perfluorooctanesulfonate (PFOS) and *N*-ethyl-*N*-(2-hydroxyethyl)-perfluorooctanesulfonamide (which converts metabolically to PFOS and the *N*-ethyl-*N*-(2-acetyl)-perfluorooctanesulfonamide), have been shown to cause an increase in perinatal mortality in reproduction studies (Lau et al., 2003; York, 1999a,b; Butenhoff et al., 2002b). The extent to which the postnatal effects seen with PFOS and *N*-EtFOSE are transferable to another perfluorinated acid, such as PFOA, was not known. The purpose of the present study was to evaluate the potential effects of APFO on functional reproduction and postnatal development across two generations of offspring.

2. Materials and methods

2.1. Animals

Crl:CD(SD)IGS BR VAF/Plus® (Sprague–Dawley) rats were supplied by Charles River Laboratory (Kingston, NY—male rats; and Raleigh, NC—female rats). Rats were housed individually except during mating and lactation. All cage sizes and housing conditions were in compliance with the "Guide for the Care and Use of Laboratory Animals". Rats were maintained on a 12:12 h light–dark cycle and fed ad libitum Certified Rodent Diet® #5002 (PMI Nutrition International, St. Louis, MO). Water was provided ad libitum from individual bottles and/or from an automatic watering access system. Bed-o'cobs® bedding (Anderson's Industrial Products, Maumee, OH) was used as the nesting material. The study was conducted in an AAALAC accredited facility.

2.2. Test substance

Ammonium perfluorooctanoate (97.99% pure), was provided by 3M Company, Specialty Materials Manufacturing Division (St. Paul, MN). The sample was 77.6% straight chain. Mole percent of branched content was as follows: 12.6% internal monomethyl (non-alpha); 9% isopropyl; 0.2% *tert*-butyl; 0.1% gem-dimethyl; and 0.1% alpha monomethyl. The certificate of analysis indicated 2.01% lesser-homolog (C_4–C_7) impurities (Richard Payfer, 3M, 2 March 2000). Homolog distribution was as follows: C_4, 0.01%; C_5, 0.03%; C_6, 0.43%; C_7, 0.57%; C_8, 97.99%; C_9, 0.16%. The sample also included 0.09% monohydro APFO, 0.72% monounsaturated APFO, 0.3% undefined (possibly) substituted perfluorocyclo species (0.2% cyclopentyl and 0.1% cyclohexyl). Solutions of APFO were prepared using reverse osmosis processed deionized water.

2.3. Study design

The P generation consisted of five dosage groups, 30 rats per sex per group. Two F_1-generation pups per sex per litter per group (60 per sex per group) were selected for continued evaluation (300 rats per sex total) at weaning. APFO or vehicle (water) was administered by oral gavage to the P-generation rats, beginning at

6 weeks of age and at least 70 days before cohabitation, at daily dosages of 0, 1, 3, 10, and 30 mg/kg using a constant volume of 5 ml/kg. The oral route was presumed to be most relevant, and doses were selected based on the results of previous oral toxicity studies in rats with APFO in which doses >30 mg/kg were not tolerated in male rats. Gavage dosing was chosen due to the fact that the dose can be delivered more accurately than by other non-parenteral methods. The F_1-generation rats were individually identified and given the same dosage level and volume of the test substance as their respective sires and dams beginning at weaning (days of lactation 22—DL 22). The P- and F_1-generation rats were observed for clinical observations, abortions or premature deliveries, and deaths before and 60 min postdosage. Body weights and feed consumption values of the male and female rats were recorded weekly and on days of gestation (DG) 0, 7, 10, 14, 18, 21 and on DL 1, 5, 8, 11, 15, and 22 for the dams. Feed consumption values were not recorded during cohabitation. Feed consumption values were not tabulated after DL 15 because pups begin to consume maternal feed on or about this time.

F_1-generation female and male rats were examined for age of vaginal patency or preputial separation beginning on day 28 or 29 postpartum, respectively. Body weights were recorded when rats reached sexual maturity. Following sexual maturation, 30 mating pairs were randomly selected, excluding sibling pairs, and non-selected pups were sacrificed and necropsied. Estrous cycling was evaluated by examination of vaginal cytology beginning 21 days before cohabitation and until mating. The cohabitation period was a maximum of 14 days. Evidence of mating was determined by spermatozoa observed in a vaginal smear or a copulatory plug observed in situ (DG0). On the day of sacrifice, stage of estrous was again determined. The female rats were evaluated for duration of gestation, fertility and gestation indices, number and sex of offspring, number of implantation sites, condition of dam and litter, litter size, viability index, lactation index, percent survival and sex ratio. F_2-generation pup body weights were recorded on DL 1, 5, 8, 15, and 22. On DL 12 all F_2-generation males were examined for the presence of nipples. Anogenital distance was measured for F_2-generation pups on DL 1 and 22.

P- and F_1-generation rats were euthanized by carbon dioxide asphyxiation, necropsied and examined for gross lesions. Blood samples were collected from the inferior vena cava and serum shipped frozen for APFO analysis by HPLC electrospray MS/MS (Hansen et al., 2001). The brain, kidneys, spleen, ovaries, testes, thymus, liver, adrenal glands, pituitary, uterus with oviducts and cervix, left epididymis, right epididymis, prostate and seminal vesicles were individually weighed. The pituitary, adrenal glands, vagina, uterus with oviducts, cervix and ovaries, right testis (initially fixed in Bouin's solution), seminal vesicles (with coagulating glands), right epididymis and prostate were retained for histology. Histological examination by a Board Certified Veterinary Pathologist was performed on tissues from 10 randomly selected rats per sex from the control and high dosage groups. All gross lesions and reproductive organs of the low and intermediate dosage group rats with reduced fertility were histologically evaluated. Histopathological examination of the testis was conducted in order to identify retained spermatids, missing germ cell layers or types, multinucleated giant cells or sloughing of spermatogenic cells into the lumen. Examination of the intact epididymis (including the caput, corpus and cauda) was conducted in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or absence of clear cells in the cauda epididymal epithelium.

Male rats were sacrificed after cohabitation. The P-generation male rats were 106–110 days of age and the F_1-generation male rats were 109–120 days of age. A portion of the left cauda epididymis was used for evaluation of cauda epididymal sperm concentration and motility using computer assisted sperm analysis (CASA). The motility was evaluated by the Hamilton Thorne IVOS. The remaining left cauda epididymis was used to manually evaluate sperm morphology. Sperm morphology evaluations included both the determination of the percentage of normal sperm (in a sample of at least 200) and qualitative evaluation of abnormal sperm. The left testis was used for evaluation of testicular spermatid concentration (10 fields) using the CASA. The left testis was weighed both before and after removal of the tunica albuginea and then homogenized; a sample from the homogenate was stained with an IDENT stain before IVOS analysis.

Female rats were euthanized by carbon dioxide asphyxiation on DL 22. The number and distribution

of implantation sites were recorded. Uteri of apparently nonpregnant rats were examined while being pressed between glass plates to confirm the absence of implantation sites. All F_1-generation pups culled on DL 22 were euthanized by carbon dioxide asphyxiation. Three randomly selected pups per sex per litter were examined for gross lesions. The necropsy included a single cross-section of the head at the level of the frontal–parietal suture and examination for hydrocephaly. The brain, spleen and thymus from one of the three selected F_1-generation pups per sex per litter were weighed and retained for histological evaluation.

2.4. Statistical analyses

Clinical observations and other proportional data were analyzed using the Variance Test for Homogeneity of the Binomial Distribution (Snedecor and Cochran, 1967a). Continuous data (body weights, body weight changes, feed consumption data, organ weights, duration of gestation, litter averages for pup body weights, percent male pups, pup viability and cumulative survival) were analyzed using Bartlett's Test of Homogeneity of Variances (Sokal and Rohlf, 1969a) and the analysis of variance (ANOVA; Snedecor and Cochran, 1967b), when appropriate (when Bartlett's Test was not significant; $P > 0.001$). If the ANOVA was significant ($P \leq 0.05$), Dunnett's Test (Dunnett, 1955) was used to identify the statistical significance of the individual groups. If the ANOVA was not appropriate (when Barlett's Test was significant; $P \leq 0.001$), the Kruskal–Wallis Test (Sokal and Rohlf, 1969b) was used, when 75% or fewer ties were present. When more than 75% ties were present, Fisher's exact test (Siegel, 1956) was used. In cases where the Kruskal–Wallis was statistically significant ($P \leq 0.05$), Dunn's method of multiple comparisons (Dunn, 1964) was used to identify the statistical significance of the individual groups. All other natural delivery data involving discrete data were evaluated using the Kruskal–Wallis Test procedures.

3. Results

3.1. P generation—reproductive outcome

There were no changes in any of the reproductive performance and outcome parameters of either male or female rats exposed to up to 30 mg/kg APFO (Table 1). In male rats, fertility was normal as were all sperm parameters. Pairings of treated male and female rats resulted in normal fertility, pregnancy, and natural delivery including length of gestation. Estrous cycling in treated females prior to mating was normal. The only statistically significant difference was that the days in cohabitation prior to mating were reduced in the group given 1 mg/kg (1.9 days versus 3.3 days in the control).

The numbers of pups delivered live or stillborn were unaffected by treatment with APFO (Table 2). The survival and body weight of pups delivered to female rats exposed to 1–10 mg/kg of APFO was similar to those of controls. In the 30 mg/kg group, the pups were somewhat lighter throughout lactation. At DL 22, the difference in mean pup weights (30 mg/kg versus 0 mg/kg) was approximately 3%; whereas, this difference was about 10% from DL 1–5. Although statistically significant only on DL 6–8, the total number of dead pups DL 1–22 from the 30 mg/kg group was increased compared to any of the other groups including the controls (26/388 deaths at 30 mg/kg versus 10/397 in the controls). There was no apparent sex predilection for these pup deaths. The ratio of male to female offspring was unaltered, and there were no structural malformations/variations that could be related to APFO treatment.

3.2. F_1 generation—reproductive outcome

As in the P-generation rats, there were no reproductive effects in the F_1 generation that could be attributed to APFO treatment (Table 3); male and female reproductive performance was normal. Female rats given 30 mg/kg had a slight but statistically significant increase in the mean number of complete estrous cycles (5.4 versus 4.7) per 21 days. However, the number of female rats with normal estrous cycles was similar between treated and control groups (Table 3), and the stages of estrus at study termination were comparable (data not shown).

The numbers of pups (F_2 generation) delivered and their survival through lactation were unaffected by APFO treatment (Table 4). Again, pup weights at 30 mg/kg tended to be somewhat lower (6–9%) than

Table 1
Reproductive endpoints in a two-generation reproduction study of ammonium perfluorooctanoate (APFO) in rats, P-generation data

	Dose group (mg/kg)				
	0	1	3	10	30
Males					
No. cohabitating	29	29	30	30	29
Fertility index[a] (%)	96.6	93.1	100	100	96.6
Motile sperm	354 ± 99	346 ± 118	373 ± 102	338 ± 89	327 ± 130
Motile (%)	82.6 ± 11.1	84.7 ± 7.0	86.8 ± 6.7	87.4 ± 4.0	83.9 ± 8.8
Total count	427 ± 106	410 ± 136	430 ± 113	387 ± 101	386 ± 138
% Abnormal	6.4 ± 12.2	4.3 ± 3.5	4.4 ± 2.6	4.6 ± 1.8	9.7 ± 21.0
Male/female					
Days in cohabitation	3.3 ± 2.6	1.9 ± 1.2*	2.4 ± 1.7	3.1 ± 2.4	2.6 ± 1.4
Rats mating days 1–7	27	29	28	27	28
Rats mating days 8–14	2	0	1	0	0
Females					
No. cohabitating	29	29	30	30	30
Estrous stages/21 days	5.2 ± 1.0	5.2 ± 0.6	5.0 ± 0.8	4.9 ± 1.0	5.2 ± 0.7
Fertility index[a] (%)	96.6	93.1	100	100	100
Deliveries (%)	100	100	96.7	100	100
Length of gestation (days)	22.6 ± 0.5	22.5 ± 0.5	22.8 ± 0.5	22.6 ± 0.6	22.7 ± 0.5
Litters					
Implantation sites	15.3 ± 1.9	15.6 ± 2.1	15.2 ± 2.3	15.6 ± 2.0	15.3 ± 2.0
Pups delivered	14.4 ± 2.1	14.6 ± 2.2	13.6 ± 3.6	13.9 ± 3.0	13.6 ± 3.3
Liveborn	14.2 ± 2.2	14.2 ± 2.6	13.4 ± 3.6	13.8 ± 3.0	13.4 ± 3.3
Stillborn	0.1 ± 0.3	0.4 ± 1.4	0.2 ± 0.4	0.1 ± 0.4	0.2 ± 0.6

[a] Fertility index (%) = (number of pregnancies/number of rats cohabitating) × 100.
* Significantly different from controls ($P < 0.05$).

those of the controls; although, the differences were not statistically significant.

In the F_1 generation, an increase in mortality of both male and female rats was seen in the 30 mg/kg groups, primarily within the first few days post-weaning (Fig. 1A and B). Most deaths appeared to be related to a failure of the pups to thrive within a few days of weaning and were seen almost exclusively in rats that were small at weaning.

3.3. Sexual maturation

Sexual maturation was delayed with statistical significance as compared to controls in F_1 male and female rats in the 30 mg/kg dose group (Table 5). In male rats, preputial separation was delayed by 3.7 days on average, and in female rats, vaginal patency was delayed 1.7 days on average. These delays in maturation were considered treatment-related, and were not seen at doses of 10 mg/kg or lower.

3.4. P-generation males—toxicology endpoints

3.4.1. Clinical observations

Low incidences of dehydration (4/30), urine-stained abdominal fur (3/30) and ungroomed fur (3/30) were present in the 30 mg/kg group of P-generation male rats. No treatment-related clinical signs were present in P-generation males administered 10 mg/kg or less of APFO.

3.4.2. Body weight and food consumption

At the end of the exposure period, body weights (Tables 6 and 7) were statistically decreased in P-generation male rats administered APFO at dosages of 3 mg/kg and above. However, during the peripubertal (through test day 15; approximately 57 days of age) and early postpubertal periods (Ojeda and Urbanski, 1994), statistically significant effects on body weight were present only at 10 and 30 mg/kg (Table 6). Mean absolute body-weight gains at

Table 2
Postnatal survival and body weights through lactation in a two-generation reproduction study of ammonium perfluorooctanoate (APFO) in rats, P-generation litters/F_1-generation pups

	Dose group (mg/kg)				
	0	1	3	10	30
No. of litters	28	27	29	29	29
PND[a] 1					
Liveborn/litter	14.2 ± 2.2	14.2 ± 2.6	13.4 ± 3.6	13.8 ± 3.0	13.4 ± 3.3
Mean pup weight/litter (g)	6.3 ± 0.5	6.0 ± 0.4*	6.2 ± 0.5	6.2 ± 0.4	5.7 ± 0.5*
PND5					
Survivors/litter	13.8 ± 2.1	13.9 ± 2.5	13.0 ± 3.4	13.6 ± 2.8	12.9 ± 3.7
Mean pup weight/litter (g)	9.4 ± 1.3	8.8 ± 0.8	9.5 ± 1.5	9.3 ± 0.9	8.5 ± 1.3*
PND8					
Survivors/litter	13.9 ± 2.0	13.8 ± 2.5	12.9 ± 3.4	13.6 ± 2.8	12.6 ± 3.6
Mean pup weight/litter (g)	13.3 ± 2.0	12.7 ± 1.3	13.5 ± 2.2	13.0 ± 1.6	11.9 ± 2.1*
PND15					
Survivors/litter	13.9 ± 2.0	13.9 ± 2.5	12.8 ± 3.3	13.4 ± 2.8	12.5 ± 3.6
Mean pup weight/litter (g)	25.0 ± 4.0	24.2 ± 2.8	26.4 ± 4.7	24.8 ± 4.0	22.9 ± 4.3*
PND22					
Survivors/litter	13.9 ± 2.0	13.9 ± 2.5	12.8 ± 3.4	13.4 ± 2.8	12.5 ± 3.6
Mean pup weight/litter (g)	37.4 ± 6.5	36.7 ± 5.5	39.7 ± 8.3	38.8 ± 6.1	35.7 ± 7.0
Viability index[b] (%)	97.7	98.2	97.4	98.8	96.9
Lactation index[c] (%)	99.7	98.9	98.1*	98.7	96.3*
Pre-weaning mortality[d] (%)	2.6	3.0	4.4	2.5	6.7
Post-weaning mortality[e] (%)					
Males ($N = 60$)	5.0	5.0	5.0	3.3	12
Females ($N = 60$)	0.0	3.3	1.7	1.7	10*

[a] Postnatal day.
[b] Viability index (%) = (number of live pups on PND5/number of liveborn pups) × 100.
[c] Pre-weaning mortality (%) = (number of live pups on PND22/number of liveborn pups on PND5) × 100.
[d] Mortality PND1–22.
[e] Mortality after weaning on PND22.
* Significantly different from controls ($P < 0.05$).

terminal sacrifice were (mean grams ± S.D., $N = 30$) 400 ± 37, 395 ± 46, 362 ± 48, 335 ± 53, and 253 ± 63 for the 0, 1, 3, 10, and 30 mg/kg dose groups, respectively, and were significantly lower ($P < 0.01$) at doses of 3 mg/kg and above. Mean absolute feed consumption was decreased in the 30 mg/kg dose group male rats to 91% of control values ($P < 0.001$) for study days 1–106, and this decrease was present at all time intervals, with statistical significance in all time intervals except days 8–22. In contrast, mean feed consumption relative to body weight was increased in a dose-dependent manner in all treated groups for study days 1–106 with statistical significance at 3 mg/kg and above. Mean relative feed consumption values as a percent of control for study days 1–106 were 101, 105, 110, and 118% for the 1, 3, 10, and 30 mg/kg dose groups, respectively. No effects on either body weight (Table 6) or feed consumption parameters were observed in P-generation male rats administered 1 mg/kg.

3.4.3. Necropsy, histology and organ weight changes

There were no treatment-related gross findings in P-generation male rats. No treatment-related microscopic changes were present in reproductive organs of P-generation male rats. Hypertrophy and/or vacuolation of the zona glomerulosa of the adrenal gland was present in 2/10 and 7/10 P-generation male rats in the

Table 3
Reproductive endpoints in a two-generation reproduction study of ammonium perfluorooctanoate (APFO) in rats, F_1-generation data

	Dose group (mg/kg)				
	0	1	3	10	30
Males					
No. cohabitating	30	29	30	30	30
Fertility index[a] (%)	96.7	100	100	100	100
Motile sperm	250 ± 81	266 ± 95	272 ± 74	259 ± 73	257 ± 87
Motile (%)	83 ± 9	84 ± 7.0	84 ± 7	83 ± 7	80 ± 11
Total count	301 ± 78	318 ± 109	324 ± 87	315 ± 89	322 ± 104
% Abnormal	2.4 ± 0.9	2.4 ± 1.3	2.6 ± 1.2	2.4 ± 1.0	2.9 ± 1.3
Male/female					
Days in cohabitation	3.0 ± 2.4	3.0 ± 1.1	2.4 ± 1.2	2.7 ± 1.6	2.5 ± 1.2
Rats mating days 1–7	29	29	30	30	30
Rats mating days 8–14	0	0	0	0	0
Females					
No. cohabitating	30	29	30	30	30
Estrous stages/21 days	4.7 ± 0.9	5.0 ± 0.9	5.0 ± 0.8	5.0 ± 1.1	5.4 ± 0.7*
Rats with >6 days diestrus	1	0	0	0	1
Rats with >6 days estrus	0	0	0	0	0
Fertility index[a] (%)	93.3	96.6	93.3	93.3	96.6
Deliveries (%)	100	100	100	100	100
Length of gestation (days)	22.8 ± 0.4	22.6 ± 0.6	22.7 ± 0.5	22.8 ± 0.6	22.7 ± 0.5
Litters					
Implantation sites	14.8 ± 3.0	15.8 ± 2.1	14.7 ± 2.8	15.0 ± 2.9	14.5 ± 2.9
Pups delivered	13.8 ± 2.9	13.8 ± 2.5	14.1 ± 3.0	13.8 ± 3.1	13.4 ± 2.8
Liveborn	13.6 ± 2.9	13.7 ± 2.4	13.8 ± 3.0	13.5 ± 3.1	13.4 ± 2.8
Stillborn	0.2 ± 0.5	0.2 ± 0.5	0.2 ± 0.6	0.2 ± 0.5	0.1 ± 0.2

[a] Fertility index (%) = (number of pregnancies/number of rats cohabitating) × 100.
* Significantly different from controls ($P < 0.05$).

10 and 30 mg/kg groups, respectively. These effects were not observed in the lower dose groups or in the control group.

All liver weight parameters (absolute and relative to both brain and terminal body weight) were increased in all APFO-treated groups of male rats (Table 7). Liver weight increases were dose-related in most instances. Kidney weights relative to body weight were also statistically increased in all APFO-treated groups, and the magnitude of this effect was similar across groups administered 3 mg/kg and above. Variable statistical significance (increased or decreased) was also present in absolute kidney weights and kidney to brain weight ratios in APFO-treated groups. This variability was likely the result of body weight decrements in these groups, as absolute kidney weights, unlike brain weight, typically decrease in association with body weight decrements (Table 7).

Statistically significant changes (increases or decreases) in several other organ weight parameters occurred at 30 mg/kg. These changes occurred in a pattern typically associated with decrements in body weight (Oishi et al., 1979; Feron et al., 1973). Thus, for those organs whose weights are typically stable during moderate body weight decrements (testis, brain), absolute weights were unaffected while organ-to-body weight ratios were increased. For body-weight-dependent organs, which tend to decrease to some degree with body weight decrements (endocrine organs, lymphoid organs, accessory sex glands), absolute organ weight and/or organ-to-brain weight ratio were decreased and organ weight to body weight ratios were unchanged or increased. All other statistically significant organ weight changes did not occur in a dose-response fashion and were considered to be spurious findings.

Table 4
Postnatal survival and body weights through lactation in a two-generation reproduction study of ammonium perfluorooctanoate (APFO) in rats, F_1-generation litters/F_2-generation pups

	Dose group (mg/kg)				
	0	1	3	10	30
No. of litters	30	29	30	30	30
PND[a]1					
Liveborn/litter	13.6 ± 2.9	13.7 ± 2.4	13.8 ± 3.0	13.5 ± 3.1	13.3 ± 2.8
Mean pup weight/litter (g)	6.5 ± 0.5	6.3 ± 0.5	6.4 ± 0.7	6.4 ± 0.7	6.0 ± 0.5
PND5					
Survivors/litter	13.5 ± 2.8	13.5 ± 2.3	13.5 ± 2.9	13.2 ± 3.0	13.2 ± 2.8
Mean pup weight/litter (g)	10.2 ± 1.2	10.0 ± 1.2	10.1 ± 1.6	10.2 ± 1.5	9.6 ± 1.2
PND8					
Survivors/litter	13.5 ± 2.8	13.5 ± 2.3	13.3 ± 2.9	13.2 ± 2.9	13.1 ± 2.8
Mean pup weight/litter (g)	14.5 ± 2.1	14.4 ± 1.8	14.3 ± 2.3	14.3 ± 2.3	13.5 ± 2.0
PND15					
Survivors/litter	13.4 ± 2.8	13.4 ± 2.2	13.3 ± 2.9	13.2 ± 2.9	13.1 ± 2.8
Mean pup weight/litter (g)	25.4 ± 4.1	25.3 ± 3.4	25.8 ± 4.4	24.8 ± 3.7	23.2 ± 3.7
PND22					
Survivors/litter	13.3 ± 2.7	13.4 ± 2.2	13.3 ± 2.9	13.1 ± 2.9	13.0 ± 2.7
Mean pup weight/litter (g)	38.7 ± 7.1	38.4 ± 5.4	39.6 ± 7.1	39.1 ± 6.6	36.5 ± 6.7
Viability index[b] (%)	99.5	98.7	97.4	98.1	99.0
Lactation index[c] (%)	98.7	98.9	98.7	99.2	98.7

[a] Postnatal day.
[b] Viability index (%) = (number of live pups on PND5/number of liveborn pups) × 100.
[c] Lactation index (%) = (number of live pups on PND22/number of liveborn pups on PND5) × 100.

3.5. P-generation females—toxicology endpoints

3.5.1. Clinical signs

No treatment-related clinical signs were observed in P-generation female rats at any of the APFO dosages tested.

3.5.2. Body weight and feed consumption

No statistically significant changes in body weight (Tables 8 and 9) or feed consumption parameters occurred at APFO dosages up to 30 mg/kg. All statistically significant changes observed were either transient or did not occur in a dose-response manner.

3.5.3. Necropsy, histology and organ weight changes

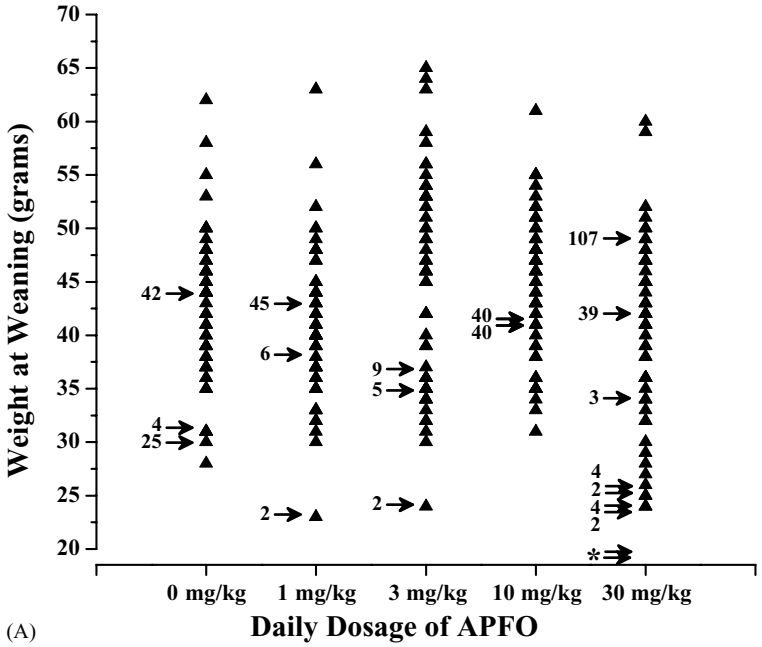
There were no treatment related gross or microscopic effects in P-generation female rats at any of the AFPO doses tested.

Statistically significant decreases in kidney weight parameters were observed in the 30 mg/kg group of the female P-generation rats (Table 7). These decreases were slight (<10%), were not repeated in the F_1-generation females, and were inconsistent with increased kidney weights noted in males. Therefore, these kidney weight changes were likely spurious. Statistically significant decreases in liver-to-body weight ratio were present in the 3 and 10 mg/kg groups but not in the 30 mg/kg group. These changes were likely not treatment-related as they were not dose-related, were not associated with changes in absolute liver weight or liver to brain weight ratio, and are inconsistent with the known effects of APFO on liver weight. All other mean organ weight values in APFO-treated groups were comparable to controls.

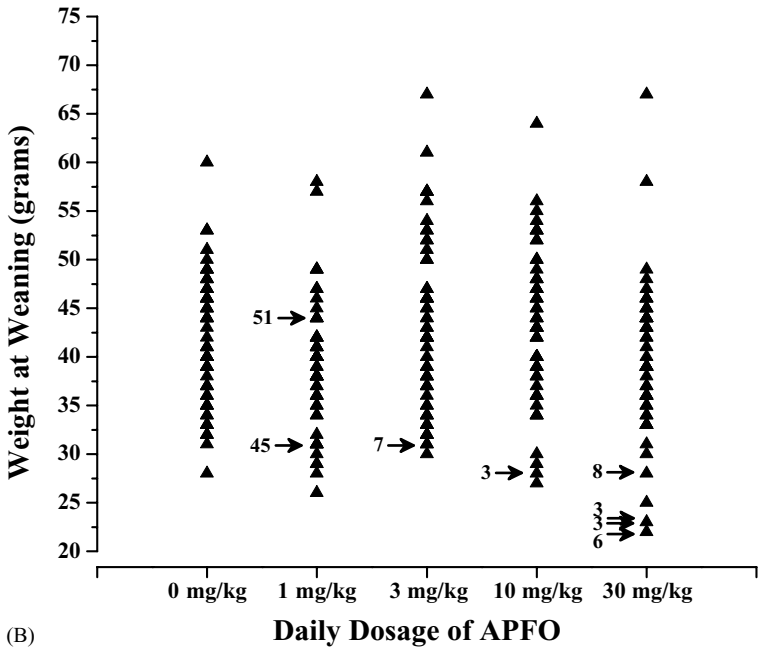
3.6. F_1-generation males—toxicology endpoints

3.6.1. Clinical signs

Dermatitis of the tail, diagnosed as annular constriction of the tail, was observed in all groups, including controls, with statistically significant increases in F_1-generation male rats in all dosed groups.



(A)



(B)

Fig. 1. Body weight and mortality in male (A) and female (B) F_1 offspring at weaning. Arrows indicate animal that died during treatment. The numbers next to the arrows indicate the post-weaning day that death occurred. (*): Two male offspring found dead on post-weaning day 2 were not weighed.

Table 5
Average postnatal day for sexual maturation of F_1 male and female rat pups in a two-generation reproduction study of ammonium perfluorooctanoate (APFO)

	Dose group (mg/kg)				
	0	1	3	10	30
Males					
N	52	58	57	58	54
Days to preputial separation	48.5 ± 2.0	49.5 ± 2.7	49.4 ± 3.4	49.7 ± 3.1	52.2 ± 3.1*
Females					
N	60	58	58	58	54
Days to vaginal patency	34.9 ± 2.1	35.3 ± 2.3	34.1 ± 2.6	34.8 ± 2.1	36.6 ± 2.4*

* Significantly different from controls ($P < 0.05$).

However, a clear dose response was observed only at the 30 mg/kg dose. This condition was transient in the individual rats affected, is often associated with low relative humidity (ILAR, 1991) and is not considered toxicologically significant. Other statistically significant observations in F_1-generation male rats were emaciation in the 10 and 30 mg/kg dosage groups, and urine-stained abdominal fur, decreased motor activity, and abdominal distention in the 30 mg/kg dosage group.

3.6.2. Body weight and food consumption

During the juvenile (through 35 days of age) and peripubertal (through 55–60 days of age) growth phases (Ojeda and Urbanski, 1994), statistically significant decreases in body weight and body weight gain were observed in the 30 mg/kg dose group (Table 10). Decreases in body-weight gain in the 10 mg/kg group began to be apparent at the end of the peripubertal period. In sexually mature (adult) male rats of the F_1 generation, statistically significant

Table 6
Mean post-weaning body weights as a percent of control in P-generation male rats

Development period[a]	Days of treatment	Approximate age (days)	Dose group (mg/kg)			
			1	3	10	30
Peripubertal	1	43	100[b]	99	99	99
	8	50	100	101	98	87*
	15	57	100	99	95*	87*
Adult	22	64	101	99	94*	74*
	29	71	102	98	93*	81*
	36	77	101	97	91*	79*
	43	84	101	96	91*	77*
	50	91	100	95*	90*	76*
	57	98	100	95*	90*	76*
	64	105	100	94*	89*	75*
	70	112	99	94*	89*	75*
	85	126	100	94*	89*	74*
	92	133	100	93*	88*	74*
	99	140	99	93*	88*	74*
	106	147	99	93*	88*	74*

[a] From Ojeda and Urbanski (1994).
[b] Values given as percent of control group mean.
* Significantly different from control ($P < 0.05$).

Table 7
Body weights and absolute and relative organ weights in P- and F_1-generation male and female rats at terminal sacrifice

	Dose group (mg/kg)				
	0	1	3	10	30
P-generation males					
N	30	30	30	30	29
Body weight (g)	581 ± 40	575 ± 48	542 ± 47**	513 ± 54**	432 ± 64**
Brain weight (g)	2.26 ± 0.17	2.28 ± 0.10	2.26 ± 0.12	2.24 ± 0.12	2.20 ± 0.14
Liver weight (g)	20.3 ± 2.5	24.3 ± 3.2**	27.7 ± 2.7**	28.7 ± 3.9**	27.5 ± 3.7**
Liver/body (%)	3.49 ± 0.29	4.22 ± 0.50**	5.13 ± 0.47**	5.61 ± 0.51**	6.42 ± 0.73**
Liver/brain (%)	903 ± 119	1066 ± 154**	1230 ± 120**	1285 ± 183**	1248 ± 144**
R. kidney weight (g)	2.19 ± 0.18	2.54 ± 0.30**	2.50 ± 0.18**	2.36 ± 0.25**	2.06 ± 0.20*
R. kidney/body (%)	0.379 ± 0.030	0.443 ± 0.048**	0.463 ± 0.039**	0.462 ± 0.034**	0.481 ± 0.051**
R. kidney/brain (%)	97.5 ± 9.9	111.6 ± 13.5**	111.0 ± 9.5**	105.6 ± 12.4**	93.5 ± 8.7
L. kidney weight (g)	2.19 ± 0.20	2.51 ± 0.28**	2.51 ± 0.21**	2.34 ± 0.24*	1.99 ± 0.19**
L. kidney/body (%)	0.378 ± 0.036	0.437 ± 0.047**	0.465 ± 0.043**	0.457 ± 0.040**	0.466 ± 0.054**
L. kidney/brain (%)	97.5 ± 10.7	110.1 ± 12.6**	111.7 ± 10.5**	104.6 ± 11.7*	90.4 ± 8.7*
F_1-generation males					
N	30	29	30	30	29
Body weight (g)	560 ± 60	527 ± 55*	524 ± 48*	499 ± 64**	438 ± 42**
Brain weight (g)	2.34 ± 0.13	2.28 ± 0.16	2.31 ± 0.12	2.28 ± 0.10	2.18 ± 0.14**
Liver weight (g)	21.7 ± 3.2	24.6 ± 4.0**	28.2 ± 4.2**	29.3 ± 4.1**	29.7 ± 4.0**
Liver/body (%)	3.86 ± 0.32	4.65 ± 0.51**	5.41 ± 0.75**	5.90 ± 0.70**	6.79 ± 0.55**
Liver/brain (%)	927 ± 136	1075 ± 150**	1224 ± 179**	1285 ± 159**	1364 ± 166**
R. kidney weight (g)	2.24 ± 0.21	2.34 ± 0.28	2.48 ± 0.24**	2.33 ± 0.25	2.04 ± 0.21**
R. kidney/body (%)	0.402 ± 0.034	0.446 ± 0.041**	0.474 ± 0.041**	0.469 ± 0.050**	0.467 ± 0.036**
R. kidney/brain (%)	95.9 ± 9.1	102.6 ± 7.7**	107.4 ± 10.2**	102.3 ± 9.8*	93.6 ± 7.9
L. kidney weight (g)	2.21 ± 0.20	2.35 ± 0.26*	2.46 ± 0.20**	2.30 ± 0.22	2.03 ± 0.22**
L. kidney/body (%)	0.396 ± 0.031	0.446 ± 0.042**	0.472 ± 0.045**	0.464 ± 0.046**	0.465 ± 0.038**
L. kidney/brain (%)	94.8 ± 7.9	102.8 ± 7.6**	106.6 ± 9.1**	101.0 ± 7.9*	93.3 ± 10.0
P-generation females					
N	27	26	29	29	28
Body weight (g)	345 ± 29	340 ± 24	351 ± 23	342 ± 26	345 ± 18
Brain weight (g)	2.22 ± 0.13	2.21 ± 0.14	2.22 ± 0.13	2.22 ± 0.08	2.17 ± 0.11
Pituitary weight (g)	0.018 ± 0.005 (26)	0.019 ± 0.005	0.017 ± 0.004	0.016 ± 0.006	0.016 ± 0.006
Liver weight (g)	18.7 ± 2.5	18.0 ± 2.1	17.9 ± 1.8	17.3 ± 1.9	18.1 ± 1.7
Liver/body (%)	5.41 ± 0.43	5.28 ± 0.42	5.09 ± 0.48*	5.05 ± 0.46**	5.24 ± 0.46
Liver/brain (%)	840 ± 101	814 ± 94	806 ± 99	777 ± 91	834 ± 81
R. kidney weight (g)	1.57 ± 0.18	1.61 ± 0.14	1.62 ± 0.16	1.55 ± 0.13	1.49 ± 0.10*
R. kidney/body (%)	0.456 ± 0.044	0.474 ± 0.042	0.461 ± 0.037	0.454 ± 0.032	0.431 ± 0.029*
R. kidney/brain (%)	70.8 ± 7.6	73.1 ± 7.4	73.0 ± 7.2	69.8 ± 6.0	68.6 ± 4.9
L. kidney weight (g)	1.57 ± 0.16	1.58 ± 0.14	1.58 ± 0.18	1.52 ± 0.14	1.44 ± 0.10**
L. kidney/body (%)	0.455 ± 0.042	0.465 ± 0.041	0.449 ± 0.040	0.443 ± 0.032	0.418 ± 0.032**
L. kidney/brain (%)	70.6 ± 7.5	71.7 ± 7.9	71.2 ± 8.3	68.4 ± 6.2	66.4 ± 4.3*
F_1-generation females					
N	28	28	28	28	29
Body weight (g)	323 ± 23	322 ± 24	329 ± 22	325 ± 24	316 ± 21
Brain weight (g)	2.07 ± 0.09	2.09 ± 0.12	2.08 ± 0.10	2.10 ± 0.12	2.07 ± 0.10
Pituitary weight (g)	0.017 ± 0.004	0.016 ± 0.003	0.015 ± 0.003*	0.015 ± 0.002*	0.015 ± 0.003**
Liver weight (g)	16.6 ± 1.6	16.0 ± 1.8	16.4 ± 1.4	16.7 ± 1.7	16.8 ± 1.4
Liver/body (%)	5.13 ± 0.31	4.96 ± 0.34	4.99 ± 0.38	5.14 ± 0.41	5.33 ± 0.46
Liver/brain (%)	803 ± 86	765 ± 82	791 ± 67	798 ± 92	811 ± 72
R. kidney weight (g)	1.38 ± 0.12	1.41 ± 0.14	1.42 ± 0.10	1.42 ± 0.16	1.40 ± 0.20
R. kidney/body (%)	0.427 ± 0.030	0.438 ± 0.037	0.432 ± 0.034	0.435 ± 0.046	0.444 ± 0.058
R. kidney/brain (%)	66.8 ± 6.1	67.4 ± 5.8	66.4 ± 6.2	67.6 ± 7.1	67.6 ± 9.5
L. kidney weight (g)	1.36 ± 0.13	1.39 ± 0.14	1.41 ± 0.09 (27)	1.38 ± 0.14	1.34 ± 0.18
L. kidney/body (%)	0.421 ± 0.038	0.432 ± 0.040	0.426 ± 0.036 (27)	0.426 ± 0.038	0.426 ± 0.055
L. kidney/brain (%)	65.8 ± 6.0	66.5 ± 6.5	67.6 ± 5.3 (27)	66.1 ± 6.6	64.9 ± 8.5

*Significantly different from control ($P < 0.05$). **Significantly different from control ($P < 0.01$).

Table 8
Mean precohabitation body weights as a percent of control in P-generation female rats

Study day	Approximate age (days)	Dose group (mg/kg)			
		1	3	10	30
1	43[a]	95[b]	104	105	95
8	50	93	103	101	93
15	57	94	101	98	92
22	64	96	101	99	94
29	71	96	102	99	95
36	78	97	102	99	95
43	85	97	103	100	95
50	92	96	105	100	95
57	99	98	102	99	95
64	106	98	103	100	96
70	113	99	104	100	96
85	127	101	104	100	95

Note: There were no statistically significant changes.
[a] P-generation females were post-pubertal at study start, as the peripubertal period ends at around 38 days of age in females (Ojeda and Urbanski, 1994).
[b] Values given as percent of control group mean.

decreases in mean body weights were observed in all APFO groups by the end of the study. However, these decreases were small (consistently <10%) in groups dosed with either 1 or 3 mg/kg. Mean absolute body-weight gains at terminal sacrifice were (mean grams ± S.D. (*N*)) 512 ± 55 (30), 473 ± 52 (29), 468 ± 50 (30), 445 ± 58 (30), and 388 ± 37 (30) for the 0, 1, 3, 10, and 30 mg/kg dose groups, respectively, and were significantly lower than controls ($P < 0.01$) at all APFO treatment levels. Mean absolute feed consumption was decreased with statistical significance over the cohabitation period (treatment days 1–70) in the 1 and 30 mg/kg dose groups and was 94 and 92% of control values, respectively. In the 30 mg/kg dose group, this decrease was statistically significant during weeks 1–5, 7, and 8. For the 1 mg/kg dose group, this decrease was statistically significant during weeks 4, 5, and 7. Mean feed consumption relative to body weight was increased over the cohabitation period in the 10 and 30 mg/kg dose groups relative to controls by 104 and 110%, respectively. This increase was statistically significant during all treatment weeks except weeks 2 and 3 for the 30 mg/kg dose group, and, for the 10 mg/kg dose group, was only statistically significant during treatment week 5.

Table 9
Mean body weights as a percent of control during gestation and lactation in P-generation female rats

	Dose group (mg/kg)			
	1	3	10	30
Gestation day				
0	97[a]	101	100	96
7	97	101	100	95
10	97	101	101	96
14	96	102	100	95
18	98	102	100	96
21	96	102	102	94
Lactation day				
1	97	103	100	96
5	96	101	99	96
8	96	99	100	95
11	97	100	98	95
15	97	101	99	97
22	99	102	99	100

Note: There were no statistically significant changes.
[a] Values given as a percent of control group mean.

3.6.3. Necropsy, histology and organ weight changes

At necropsy, ten areas of discoloration in the liver were present in 6/60, 10/60, and 9/60 F_1-generation male rats in the 3, 10, and 30 mg/kg dose groups, respectively. Treatment-related microscopic changes in these livers were hepatocellular hypertrophy and, less commonly, focal to multifocal hepatocellular necrosis. All other gross observations occurred in single instances or did not occur in a dose-related fashion and were not considered treatment related.

No treatment-related microscopic lesions were observed in the reproductive organs of rats administered APFO. Hypertrophy and vacuolation of the zona glomerulosa of the adrenal gland was present in 7/10 F_1-generation male rats administered 30 mg/kg. Unlike the P-generation males, no adrenal changes were seen at 10 mg/kg. No other treatment-related microscopic changes were observed in APFO-treated groups of the F_1 generation.

All liver weight parameters (absolute and relative to both brain and terminal body weight) were increased in all APFO-treated groups (Table 7). Liver weight increases were dose-related and were similar in magnitude to that observed in the P-generation male rats. Kidney weights relative to body weight were also statistically increased in all APFO-treated groups and, as with the P generation, the magnitude

Table 10
Mean post-weaning body weights as a percent of control in F_1-generation male rats

Development period[a]	Days post-weaning	Approximate age (days)	Dose group (mg/kg)			
			1	3	10	30
Juvenile	1	21	96[b]	103	104	95
	8	28	96	103	102	93*
	15	35	96	102	99	88*
Peripubertal	22	42	95	101	99	88*
	29	49	96	101	97	88*
	36	56	97	101	94	84*
Adult	43	63	96	98	95	85*
	50	70	94	96	93	83*
	57	77	94	97	92*	82*
	64	84	94	95	91*	81*
	70	90	94	95	91*	80*
	PC[c]	92–106	95	96	91*	80*
	99	119	94*	95	90*	79*
	106	126	94*	94*	89*	77*
	113	133	93*	92*	88*	77*

[a] From Ojeda and Urbanski (1994).
[b] Values given as percent of control group mean.
[c] Precohabitation.
* Significantly different from control ($P < 0.05$).

of this effect was similar across groups administered 3 mg/kg and above. Variable statistical significance (increased or decreased) was also present in absolute kidney weights and kidney to brain weight ratios for rats in APFO-treated groups. This variability was likely the result of body weight decrements in this group. As was observed in the P-generation male rats, statistically significant changes in a number of other organ weight parameters followed a pattern consistent with effects occurring secondary to body weight decrements.

3.7. F_1-generation females—toxicology endpoints

3.7.1. Clinical signs

No treatment-related clinical signs were observed in F_1-generation female rats at any of the APFO dosages tested.

3.7.2. Body weight and food consumption

Body weights and body weight gains were significantly reduced in the 30 mg/kg group during precohabitation (Table 11). In addition body weights were decreased in this group during the gestation and lactation periods (Table 12). Statistically significant decreases in absolute food consumption values occurred in the 30 mg/kg dosage group at various intervals in the precohabitation, gestation and lactation periods (data not shown). However, relative feed consumption was similar across treated and control groups.

3.7.3. Necropsy, histology and organ weight changes

No treatment-related gross, microscopic, or organ weight changes (Table 7) were present in female rats in APFO-treated groups. Pituitary weight parameters were statistically decreased in F_1-generation female rats administered 3 mg/kg and above (Table 7). These decreases were not considered to be treatment-related based on the following: the pituitary weights for individual animals in the higher dosage groups were within the range of values for study controls; a similar pattern of pituitary weight changes was not observed in the P-generation female rats; a similar pattern of pituitary weight changes was not observed in either generation of male rats, which are typically more sensitive than females to APFO-induced effects; and no microscopic changes were seen in the pituitary in either sex of either generation; the statistics are based on an change in the mean weight value of control of

Table 11
Mean post-weaning body weights as a percent of control in F_1-generation female rats

Development period[a]	Days post-weaning	Approximate age (days)	Dose group (mg/kg)			
			1	3	10	30
Juvenile	1	21	95[b]	104	105	95
	8	28	93	103	101	93
Peripubertal	15	35	94*	101	98	92*
Adult	22	42	96*	101	99	94*
	29	49	96*	102	99	95*
	36	56	97	102	99	95
	43	63	97	103	100	95
	50	70	96	105	100	95*
	57	77	98	102	99	95*
	64	84	98	103	100	96
	70	90	99	104	100	96
	PC[c]	92–106	101	104	100	95*

[a] From Ojeda and Urbanski (1994).
[b] Values given as percent of control group mean.
[c] Precohabitation.
* Significantly different from control ($P < 0.05$).

2 mg (17 mg versus 15 mg) with standard deviations of 2–4 mg.

3.8. Serum PFOA concentrations

Serum PFOA concentrations were determined in the 0, 10, and 30 mg/kg dose-group male and female rats from blood samples taken on the day of sacrifice (approximately 24 h after the last dose) to confirm that maternal PFOA doses were in line with expectations based on previous studies. Male rats in the 0, 10, and 30 mg/kg dose group had serum PFOA concentrations of 0.034 ± 0.015 mg/ml, 51.5 ± 9.3 mg/ml, and 45.3 ± 12.6 mg/ml, respectively. Females in the 0,

Table 12
Mean body weights as a percent of control during gestation and lactation in F_1-generation female rats

	Dose group (mg/kg)			
	1	3	10	30
Gestation day				
0	100[a]	104	100	95*
7	100	103	100	95*
10	100	103	99	95*
14	100	103	99	95*
18	100	104	100	96
21	100	102	99	96
Lactation day				
1	100	104	100	96
5	100	103	99	95*
8	101	103	99	94*
11	102	103	100	96*
15	100	102	99	96*
22	100	102	101	98

[a] Values given as a percent of control group mean.
* Significantly different from control ($P < 0.05$).

10, and 30 mg/kg dose groups had serum PFOA concentrations of <0.005, 0.37 ± 0.08 ppm and 1.02 ± 0.43 ppm, respectively. These values were consistent with expectations (Kemper, 2003).

4. Discussion

This study evaluated the male and female reproductive systems of Sprague–Dawley (CD) rats exposed orally to APFO by gavage at either 0, 1, 3, 10, or 30 mg/kg for two generations, one litter per generation. Guidelines for the study design were USEPA OPPTS 870.3800 "Reproduction and Fertility Effects" testing guidelines and FIFRA/TSCA GLP standards (40 CFR Parts 160 and 792). Parameters evaluated were gonadal function, estrous cycling, mating behavior, conception, parturition, lactation, weaning, and the growth and development of the offspring, including neonatal morbidity, target organs of the offspring, and survival. Since the study design included in utero exposure as well as postnatal exposure via lactation, the study provided the opportunity to examine the susceptibility of the immature/neonatal animal under maximum exposure conditions. There were no deviations during the conduct of the study that affected the quality or compromised the integrity of the study.

Reproductive performance was unaltered by dosing of up to 30 mg/kg APFO. Normal fertility was seen in both male and female rats. Gestation lengths were unchanged and young were present in normal numbers with no increase in either stillborn or malformed young. Treated male rats showed normal numbers of sperm with no evidence of an increase in percent abnormal and, as indicated above, were functionally capable of fertilization. Estrous cycling was also unaffected by APFO-treatment. In the F_1 generation, female rats given 30 mg/kg had a slight increase in the mean number of complete estrous cycles per 21 days (5.4 versus 4.7). The number of female rats in this group with normal estrous cycles was similar between this group and the controls and the stages of estrous at termination were comparable. The slight numerical difference is considered to be a function of the estrous stage at which the rats entered the 21-day estrous-measurement period and is not related to APFO treatment.

Decrements in body weight and weight gain in male rats was consistent with previous studies with APFO (Kennedy et al., 2004). Although statistical significance for body weight effects occurred at a lower dosage level for the F_1-generation male rats compared to the P generation (1 and 3 mg/kg, respectively), the magnitude of change relative to controls (<10%) was similar across the two generations (Tables 6 and 10). Clinical signs typically associated with poor-doing rats were also observed in P- and F_1-generation male rats administered 10 and/or 30 mg/kg. These included urine stains, ungroomed fur, and emaciation.

In this study, body weights were assessed in directly-dosed rats not only as adults but also during different periods of sexual development. Ojeda and Urbanski (1994) proposed a classification scheme for maturational stages in male and female rats based on physiological and morphological parameters of sexual development. For male rats, these phases are designated as follows: the neonatal period (birth to postnatal day 7), the infantile period (postnatal days 8–21), the juvenile period (through about day 35), and the peripubertal period (through days 55–60). In the current study, direct dosing of P-generation male rats with APFO was begun when rats were approximately 42 days of age, which corresponds to the peripubertal phase of sexual development. F_1 male rats were dosed beginning at weaning (postnatal day 21) and thus were dosed throughout the juvenile and peripubertal periods, as well into adulthood. Since body weight is a sensitive indicator of APFO exposure in rats and was measured across multiple phases of sexual maturation, this endpoint allows for a comparative assessment of APFO toxicity in sexually mature and immature rats.

In P-generation male rats, statistically significant decrements in body weights were present at 10 mg/kg and above at the end of the peripubertal period (day 57). Body weights in the 10 and 30 mg/kg groups at the end of this period were decreased to 95 and 87% of control group means, respectively (Table 6). In contrast, weight decrements were more severe throughout most of the dosing period during which male rats were sexually mature. Statistically significant effects were present in mature male rats at 3 mg/kg and above. In addition, body weight decreases compared to the control group means were greater in sexually mature male rats at a given concentration compared to sexually immature animals. Similar findings were observed

in F_1-generation males (Table 10). Statistically significant decreases in body weight were present only at the high dose of 30 mg/kg during the juvenile and peripubertal periods, but were present in all dose groups (albeit very slight at 1 and 3 mg/kg) by the last 3 weeks of dosing in sexually mature animals.

The greater sensitivity of sexually mature male rats to APFO-induced body weight effects compared to sexually immature rats may be related to differences in sex hormone levels during the different growth phases. Numerous investigators have demonstrated that the slower renal clearance (and thus longer elimination rate) of PFOA in male rats compared to females is influenced, at least in part, by differences in testosterone levels. In previous studies, castration of male rats greatly increased urinary excretion of PFOA (Vanden Heuvel et al., 1992; Kudo et al., 2001, 2002), and testosterone treatment decreased renal clearance of PFOA in castrated male and intact female rats (Kudo et al., 2002). Thus, in the current study, lower serum testosterone levels in male rats during the juvenile and peripubertal periods of growth may be associated with elimination kinetics that are more similar to female rats, which show more rapid renal clearance and shorter serum half-lifes of PFOA compared to that seen in sexually mature males. The mechanisms whereby hormones may influence elimination kinetics of perfluorinated fatty acids are currently under investigation (Kudo et al., 2002). Kudo et al. (2002), reported that the expression of mRNA for the organic anion transporter protein OAT 2 in adult male rat kidneys was only 13% of that in adult females and could be increased in the kidneys of males by treatment with estradiol or castration. Buist et al. (2003) have recently confirmed that OAT 2 is more highly expressed in female rat kidneys than in males, and that this transporter protein begins to be expressed in female rat kidney between postnatal days 35–40.

The increased mortality observed in F_1-generation pups at the 30 mg/kg doses appeared to be associated with decreased body weight. Although individual pup weights were not available until weaning, mean birth weights per litter and mean weight gains per litter were generally lower in the 30 mg/kg F_1-generation litters. The fact that 9 of 13 pup deaths in the post-weaning period involved the lightest pups and occurred in the first few days post-weaning supports the suggestion that low body weight or immaturity at weaning was an operative factor in the mortality of these pups. The mortality experiences of the four pups that died at later time periods post-weaning is comparable to the mortality experience in the control group in which three pups died. It is noteworthy that F_2-generation pups did not experience an increase in mortality in the lactation period.

Delayed age at preputial separation in male rats (mean = 3.7 days) and delayed age at vaginal opening (mean = 1.7 days) in females in the F_1-generation offspring dosed at 30 mg/kg were outside the normal range for this strain of rat in the laboratory in which the study was conducted (Lewis et al., 2002). These maturational delays may have been the result of delayed growth of the F_1-generation offspring. When co-varied with body weight at weaning, there was no statistically significant difference at the $P < 0.05$ level between treated groups and controls in days to sexual maturation; however, the male rats in the high dose were close to attaining significance, as the P value was 0.059. As noted earlier, pup weights in this group were consistently decreased throughout the lactation period. While the body weights of the F_1-generation offspring were similar to the controls at the time of sexual maturation, it is plausible that the delayed growth that was observed early in lactation may have contributed to the delays that were observed in sexual maturation of the F-generation offspring. Irrespective of the etiology of the delays in sexual maturation, these rats progressed to showing normal reproductive capacity and outcome, suggesting that these maturational delays had no consequence on reproduction.

Decreased body weights can result in non-specific delays in puberty (Carney et al., 1998; Glass et al., 1976; Glass and Swerdloff, 1980; Kennedy and Mitra, 1963; Marty et al., 1999, 2001a,b,c; Ronnekleiv et al., 1978; Stoker et al., 2000a,b; Widdowson and McCance, 1960). In a recent report by Lewis and co-workers (2002), variability of sexual maturation data was evaluated in control populations of Sprague–Dawley rats. They found that the typical variability among control groups was approximately 2 days, a finding that was also consistent with the typical variability in age at sexual maturation reported by others (Ashby and LeFevre, 2000; Clark, 1999; Marty et al., 1999; Stoker et al., 2000b). Since non-specific effects on body weight can cause general delays in sexual maturation, interpreting delays in

sexual maturation can be problematic in studies where generalized delays in growth occur, such as those that were observed in the current study of APFO. Nevertheless, it is clear that effects on sexual maturation, if any, following exposure to APFO up to 30 mg/kg did not compromise reproductive success (i.e. mating and fertility) in rats.

It is noteworthy that APFO did not produce the perinatal mortality that has been observed with an analogous perfluorinated acid, perfluorooctanesulfonate (Butenhoff et al., 2002b; Lau et al., 2003). PFOS exposure to rats and mice in utero produces a dose-response for perinatal mortality that is quite steep, with mortality increasing sharply from a dose of 1–1.6 mg/kg. Body burden of PFOS in neonatal rats and mice at birth appears to be a controlling factor. It is not known if APFO would act by a similar mechanism; however, it is evident from the current study that, at doses much higher than those required to produce neonatal mortality in rats with PFOS, APFO does not produce neonatal mortality.

Consistent with previous studies with APFO, increased liver weight in male rats was a sensitive indicator of exposure. Increased liver weights occurred in males of both the P and F_1 generations at all dose levels of APFO. It is well established that APFO and other perfluoroalkanoic acids are peroxisome proliferators and inducers of hepatic CYP450 (Pastoor et al., 1987; Ikeda et al., 1989; Permadi et al., 1992). Microscopic changes in the liver of some PFOA-treated male rats, including hepatocellular hypertrophy and focal to multifocal necrosis, were also consistent with findings from previous studies in the rat. The effects that were observed with PFOA in the two-generation reproduction study are also consistent with those observed in studies with other peroxisome-proliferating compounds (Fitzgerald et al., 1987; Gibson et al., 1981; Malinverno et al., 1996).

Kidney weights relative to body weights were also statistically increased in all APFO-treated male rat groups of both the P and F_1 generations. The magnitude of the kidney weight increases did not demonstrate a clear dose-response. Increased kidney weight relative to body weight was also seen in male rats in 90-day and 2-year feeding studies with APFO (Kennedy et al., 2004). In these studies, kidney weight changes were not associated with renal cytotoxicity or decreased renal function, as there were no correlative effects on renal histopathology or clinical chemistry. Previous studies have demonstrated that APFO treatment in rats is associated with induction of peroxisomal and microsomal enzymes in the kidney, although generally not to the degree seen in the liver (Kawashima et al., 1989; Diaz et al., 1994). As in the liver, enzyme induction in the kidney is more pronounced in male than female rats. Thus, the kidney weight increases observed in APFO-treated male rats in the current study are likely the result of enzyme induction rather than renal cytotoxicity.

Pituitary weight parameters were statistically decreased in F_1-generation female rats administered 3 mg/kg and above. However, these decreases did not occur in a dose-related manner, and pituitary weights for individual animals in the 30 mg/kg groups were within the range of values for the control group. Furthermore, a similar pattern of pituitary weight change was not observed in P-generation female rats or in either generation of male rats (which are consistently more sensitive than female rats to APFO-induced effects). Therefore, the decreased pituitary weights observed in F_1-generation female rats was likely a spurious finding. Consistent with this conclusion is the absence of APFO-related microscopic findings in the pituitary in either sex in the current study or in previous studies with APFO. Also, there are no known effects of APFO that are biologically consistent with decreased function of pituitary cell types.

In addition to organ weight changes noted above, statistically significant changes (increases or decreases) in weight parameters for a number of other organs occurred in P- and F_1-generation male rats, especially in the 30 mg/kg dose group. Since these organ weight effects occurred in association with APFO-induced decrements in body weights, primary compound-induced organ weight effects must be distinguished from those occurring secondary to body weight changes. Most organ weights are, to some extent, body weight dependent. That is, they tend to decrease with decreases in body weight so that organ-to-body weight ratios are unaffected or only slightly affected (Oishi et al., 1979; Feron et al., 1973). Exceptions include the testes and brain. In APFO-treated male rats, a number of organs including spleen, thymus, adrenal glands and pituitary gland, had weight changes that occurred in a pattern typically associated with decrements in body weight. For these

organs, absolute weight and/or organ-to-brain weight ratio were decreased while organ-to-body weight ratio was normal or increased. This pattern of change in weight parameters for these organs indicates that such weight effects were most likely a consequence of the body weight decrements and are not indicative of primary target organ toxicity.

Microscopic hypertrophy and vacuolation of the zona glomerulosa of the adrenal gland was present in male rats of both the P and F_1 generations exposed to 30 mg/kg APFO. A low incidence of this change was also present in P-generation male rats in the 10 mg/kg groups. No adrenal effects were observed following exposure of male rats to lower doses of APFO. In addition, treatment-related microscopic changes were not observed in the adrenal glands of APFO-treated females at any of the dose levels tested. There are no previous reports of histopathological changes in the adrenal glands of rats or other species following exposure to APFO. Hypertrophy of the adrenal zona glomerulosa has been produced in rats by the hypolipidemic drug, nafenopin, which like APFO, is a peroxisome proliferator (Robba et al., 1986). Nafenopin-induced hypertrophy was not associated with changes in plasma aldosterone (the primary secretory product of the zona glomerulosa) and ultrastructurally was characterized by proliferation of smooth endoplasmic reticulum. The mechanism underlying the microscopic effects on the zona glomerulosa following administration of high doses of APFO in the current study is not known.

In summary, in the two-generation reproduction study with APFO, paternal toxicity (P and F_1 generations) was observed at all dose levels (1, 3, 10, and 30 mg/kg) and minimal maternal toxicity was observed at 30 mg/kg. All indices of reproductive success were normal in the APFO-exposed rats. Decreased pup weights, increased pup mortality, and delayed sexual maturation in F_1-generation offspring, were seen at 30 mg/kg but not at 10 mg/kg. The overall results of the first and second generation appear to be similar in that there was no apparent increase in adverse outcome(s) in the second generation. The no-observed-adverse-effect-level (NOAEL) for functional reproduction in the two-generation reproduction study was 30 mg/kg and the NOAEL for pup mortality, weight, and sexual maturity was 10 mg/kg. Consistent with other studies, the NOAEL for general toxicity was less than 1 mg/kg for the males and 10 mg/kg for the females.

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