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National Toxicology Program
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NTP Technical Report on the Toxicity Studies of Perfluoroalkyl Carboxylates (Perfluorohexanoic Acid, Perfluorooctanoic Acid, Perfluorononanoic Acid, and Perfluorodecanoic Acid) Administered by Gavage to Sprague Dawley (Hsd:Sprague Dawley SD) Rats

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Foreword

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Toxicity Study Report series began in 1991. The studies described in the Toxicity Study Report series are designed and conducted to characterize and evaluate the toxicologic potential of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in the Toxicity Study Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection per se is not an indicator of a substance's toxic potential.

The NTP conducts its studies in compliance with its laboratory health and safety guidelines and FDA Good Laboratory Practice Regulations and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. Studies are subjected to retrospective quality assurance audits before being presented for public review.

NTP Toxicity Study Reports are indexed in National Center for Biotechnology Information (NCBI) Bookshelf and are available free of charge electronically on the NTP website (http://ntp.niehs.nih.gov). Toxicity data are available through NTP's Chemical Effects in Biological Systems (CEBS) database.

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Peer Review

The draft *NTP Technical Report on the Toxicity Studies of Perfluoroalkyl Carboxylates (Perfluorohexanoic Acid, Perfluorooctanoic Acid, Perfluorononanoic Acid, and Perfluorodecanoic Acid) Administered by Gavage to Sprague Dawley (Hsd:Sprague Dawley SD) Rats* was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the design and conditions of these NTP studies were appropriate and ensured that this NTP Toxicity Study Report presented the experimental results and conclusions fully and clearly.

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Abstract

Widespread exposure to several per/polyfluorinated alkyl substances (PFAS) is associated with a wide array of toxicities. The National Toxicology Program (NTP) conducted 28-day toxicity studies in male and female Sprague Dawley (Hsd:Sprague Dawley SD) rats (n = 10/dose; five doses) to compare the toxicities of seven PFAS chemicals (three sulfonic acids or salt: perfluorobutane sulfonic acid, perfluorohexane sulfonate potassium salt, and perfluorooctane sulfonic acid; and four carboxylates: perfluorohexanoic acid [PFHxA], perfluorooctanoic acid [PFOA], perfluorononanoic acid [PFNA], and perfluorodecanoic acid [PFDA]) via gavage in deionized water with 20% Tween® 80. This report describes the studies of the four carboxylates (PFHxA, PFOA, PFNA, and PFDA); a companion report (NTP Toxicity Study Report 96) describes the studies of the three PFAS sulfonates. Doses were 0 to 1,000 mg/kg/day for PFHxA, 0 to 5 mg/kg/day for PFOA males, 0 to 100 mg/kg/day for PFOA females, 0 to 5 mg/kg/day for PFNA males, 0 to 25 mg/kg/day for PFNA females, and 0 to 2.5 mg/kg/day for PFDA.

A peroxisome proliferator-activated receptor alpha (PPARα) agonist (Wyeth-14,643) was used at a dose of 0 to 25 mg/kg/day for qualitative comparison to the PFAS evaluated. These studies evaluated clinical pathology, thyroid hormones, liver expression of PPARα- (*Cyp4a1*, *Acox1)* and constitutive androstane receptor (CAR)-related genes (*Cyp2b1*, *Cyp2b2*), liver acyl-CoA oxidase enzyme activity (males only), plasma and liver (males only) concentrations, and histopathology.

There was no effect on survival in animals administered PFHxA, PFOA, or PFDA, but treatment-related reduced survival was observed in males and females administered PFNA. Lower body weights were observed in male rats treated with PFHxA, PFOA, PFNA, and PFDA; lower body weights were also observed in females administered PFNA and PFDA. Plasma and liver concentrations normalized to dose were generally higher in animals administered the longer-chain PFAS. PFHxA and PFOA females had lower plasma concentrations than did males when normalized to dose. Common findings across two or more of the PFAS presented in this report included increased liver weights (absolute and relative to body weight); increased *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* expression; and increased acyl-CoA oxidase activity. Clinical chemistry endpoints were altered in several PFAS, including increased liver enzyme activities (aspartate aminotransferase, alkaline phosphatase, alanine aminotransferase, and sorbitol dehydrogenase); decreased globulin and cholesterol concentrations; and increased bile acids and direct bilirubin concentrations. PFHxA male and female rats had a dose-dependent hypochromic, macrocytic, regenerative decrease in the red blood cell mass characterized by a decrease in erythrocyte count, hemoglobin concentration, and hematocrit with an increase in reticulocyte counts. In most PFAS, total thyroxine (T4) and free T4 were decreased with no compensatory increase in thyroid stimulating hormone. Histopathologic findings observed in more than one PFAS were in the liver (hepatocyte hypertrophy, hepatocyte cytoplasmic alteration, and hepatocyte necrosis), nose (olfactory epithelium degeneration and hyperplasia), bone marrow (hypocellularity), thymus (atrophy), testes (germinal epithelium degeneration, interstitial cell atrophy, and spermatid retention), and epididymis (exfoliated germ cell).

PFHxA, PFNA, and PFDA were negative in bacterial mutagenicity tests; PFOA was equivocal in bacterial mutagenicity tests.[†] In vivo, no increases in micronucleated reticulocytes were seen in male or female rats exposed to PFHxA, PFNA, or PFDA; a small increase in micronucleated reticulocytes was observed in male rats exposed to PFOA.

In general, the effects in male and female rats administered PFHxA were of lower magnitude (e.g., liver or clinical pathology findings) or not apparent compared to the effects in animals exposed to PFNA or PFDA. Several of the effects observed in the liver were also observed in Wyeth-14,643 animals, but some extrahepatic effects were not observed with Wyeth-14,643, suggesting that PFAS chemicals induce toxicity by a variety of pathways apart from PPARα. These data provide a basis for comparisons across the PFAS class, either using external (e.g., mol/kg/day) or internal (e.g., plasma μM) dose.

[†]ERRATUM: An error was identified in the NTP Toxicity Report on Perfluoroalkyl Carboxylates (TOX 97). The text was changed from “negative in bacterial mutagenicity tests” to “equivocal in bacterial mutagenicity tests” for PFOA. [20 May 2020]

Summary of Findings Considered to Be Toxicologically Relevant in Sprague Dawley (Hsd:Sprague Dawley SD) Rats Administered Perfluorinated Carboxylates by Gavage for 28 Days

	PFHxA		PFOA		PFNA		PFDA	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Doses in Deionized Water with Tween® 80 (mg/kg/d)	0–1,000#	0–1,000#	0–10	0–50	0–5	0–5	0–25	0–25
Survival Rates	No effect	No effect	No effect	No effect	↓	↓	No effect	No effect
Body Weights	↓	No effect	↓	No effect	↓	↓	↓	↓
Organ Weights								
R. Adrenal Gland								
Absolute	No effect	No effect	No effect	No effect	↓	No effect	↓	↓
Relative	No effect	No effect	No effect	No effect	↑	No effect	↑	No effect
R. Kidney								
Absolute	No effect	↑	↑	↑	↓	↑	↓	↓
Relative	↑	↑	↑	↑	↑	↑	↑	↑
Liver								
Absolute	↑	↑	↑	↑	↑	↑	↑	↑
Relative	↑	↑	↑	↑	↑	↑	↑	↑
Spleen								
Absolute	No effect	No effect	↓	No effect	↓	↓	↓	↓
Relative	No effect	No effect	No effect	No effect	↓	↓	↓	↓
R. Testis								
Absolute	No effect	–	No effect	–	↓	–	↓	–
Thymus								
Absolute	↓	No effect	↓	No effect	↓	No effect	↓	↓
Relative	No effect	No effect	No effect	No effect	↓	No effect	↓	↓

	PFHxA		PFOA		PFNA		PFDA	
Thyroid Gland								
Absolute	No effect	No effect	↑	No effect	↓	No effect	No effect	↑
Relative	No effect	No effect	↑	No effect	↑	No effect	↑	↑
Hematology								
Hematocrit	↓	↓	↓	↓	No effect	No effect	No effect	↑[a]
Hemoglobin	↓	↓	↓	↓	No effect	No effect	No effect	↑[a]
Erythrocytes	↓	↓	↓	↓	No effect	No effect	↑[a]	↑[a]
Mean corpuscular hemoglobin concentration	↓	↓	No effect	No effect	↑	No effect	↑	↑
Mean corpuscular volume	↑	↑	↓	No effect	↓	No effect	↓	↓
Reticulocytes	↑	↑	↓	No effect	↓	No effect	↓	↓
Platelets	↑	↑	No effect	No effect	No effect	No effect	No effect	↓
Clinical Chemistry								
Total Protein	↓	↓	↓	No effect	↓	↑[a]	↓	No effect
Albumin	↓	No effect	No effect	↑[a]	↓	↑[a]	↓	No effect
Globulin	↓	↓	↓	↓	↓	↓	↓	↓
Albumin/Globulin Ratio	↑	↑	↑	↑	↑	↑	↑	↑
T Bilirubin	↓[a]	↓[a]	↓[a]	No effect	↑	No effect	↑	↑
Direct Bilirubin	No effect	No effect	↑	No effect	↑	↑	↑	↑
Indirect Bilirubin	↓[a]	No effect	↓[a]	No effect	↑	No effect	↑	↑
Cholesterol	↓	No effect	↓	↑	↓	No effect	↓	↓
Triglycerides	No effect	No effect	↓	↑	↓	No effect	↓	No effect
Alanine Aminotransferase	↑	↑	↑	↑	↑	↑	↑	↑
Alkaline Phosphatase	↑	↑	↑	↑	↑	↑	↑	↑
Aspartate Aminotransferase	↑	↑	↑	No effect	↑	No effect	↑	↑
Sorbitol Dehydrogenase	↑	No effect	No effect	No effect	↑	↑	No effect	No effect

	PFHxA		PFOA		PFNA		PFDA	
Creatinine Kinase	No effect	No effect	↓[a]	No effect	No effect	No effect	No effect	↑
Bile Acid	↑	↑	↑	No effect	↑	↑	↑	↑
Thyroid Stimulating Hormone	No effect	No effect	↓	↑	↓	No effect	No effect	No effect
Total Thyroxine	↓	No effect	↓	↓	↓	↓	No effect	No effect
Free Thyroxine	↓	No effect	↓	↓	↓	↓	↓	↓
Total Triiodothyronine	↓	No effect	↓	No effect	↑	No effect	No effect	↑
Testosterone	No effect	No effect	No effect	No effect	↓	↑	No effect	↑
Gene Expression								
Acox1	↑	↑	↑	↑	↑	↑	↑	↑
Cyp4a1	↑	↑	↑	↑	↑	↑	↑	↑
Cyp2b1	↑	↑	↑	↑	↑	↑	↑	↑
Cyp2b2	↑	↑	↑	↑	↑	↑	↑	↑
Acyl-CoA Oxidase	↑	NA	↑	NA	↑	NA	↑	NA
Reproductive Toxicity								
Altered Estrous Cyclicity	–	No call	–	Yes	–	No call	–	Yes
Altered Sperm Parameters	Yes	–	Yes	–	Yes	–	Yes	–
Nonneoplastic Effects								
Liver:								
Hepatocyte, Cytoplasmic Alteration	↑	↑	↑	↑	↑	↑	↑	↑
Hepatocyte, Hypertrophy	↑	↑	↑	↑	↑	↑	↑	↑
Hepatocyte, Vacuolization Cytoplasmic	No effect	No effect	No effect	No effect	↑	↑	↑	↑
Necrosis	No effect	No effect	No effect	No effect	↑	No effect	No effect	↑

	PFHxA		PFOA		PFNA		PFDA	
Nose:								
Olfactory Epithelium, Degeneration	↑	↑	No effect	↑	No effect	↑	No effect	No effect
Olfactory Epithelium, Hyperplasia	↑	↑	No effect	↑	No effect	No effect	No effect	No effect
Olfactory Epithelium, Inflammation, Suppurative	↑	↑	No effect	↑	No effect	No effect	No effect	No effect
Respiratory Epithelium, Hyperplasia	No effect	No effect	↑	↑	No effect	No effect	No effect	No effect
Respiratory Epithelium, Inflammation, Chronic Active	No effect	No effect	↑	↑	No effect	No effect	No effect	No effect
Bone Marrow:								
Hypocellularity	No effect	No effect	↑	No effect	↑	↑	↑	↑
Spleen:								
Extramedullary Hematopoiesis, Increased	↑	↑	No effect	No effect	No effect	No effect	No effect	No effect
Atrophy	No effect	No effect	No effect	No effect	↑	↑	No effect	No effect
Kidney:								
Nephropathy, Chronic Progressive	No effect	↑	No effect	No effect	No effect	No effect	No effect	No effect
Thymus:								
Atrophy	No effect	No effect	No effect	No effect	↑	↑	↑	↑
Lymphocyte, Apoptosis	No effect	No effect	No effect	No effect	No effect	No effect	↑	No effect
Thyroid Gland:								
Follicular Cell Hypertrophy	No effect	No effect	↑	↑	No effect	No effect	No effect	No effect

	PFHxA		PFOA		PFNA		PFDA	
Lymph Nodes:								
Mandibular, Atrophy	No effect	No effect	No effect	No effect	↑	↑	No effect	No effect
Mesenteric, Atrophy	No effect	No effect	No effect	No effect	↑	↑	No effect	No effect
Forestomach:								
Epithelium, Hyperplasia	No effect	No effect	No effect	No effect	↑	↑	No effect	No effect
Inflammation, Chronic Active	No effect	No effect	No effect	No effect	↑	–	No effect	No effect
Testes:								
Germinal Epithelium, Degeneration	No effect	–	No effect	–	↑	–	↑	–
Interstitial Cell, Atrophy	No effect	–	No effect	–	↑	–	↑	–
Seminiferous Tubule, Spermatid Retention	No effect	–	No effect	–	↑	–	↑	–
Epididymis:								
Hypospermia	No effect	–	No effect	–	↑	–	No effect	–
Duct, Exfoliated Germ Cell	No effect	–	No effect	–	↑	–	↑	–
Epithelium, Apoptosis	No effect	–	No effect	–	↑	–	No effect	–
Genetic Toxicology								
Bacterial Gene Mutations	Negative in TA98, TA100, and *E. coli* with or without S9		Equivocal in TA98 without S9 and negative in TA98 with S9;[b] negative in TA100, and *E. coli* with or without S9		Negative in TA98, TA100, and *E. coli* with or without S9		Negative in TA98, TA100, and *E. coli* with or without S9	
Micronucleated Erythrocytes								
Rat Peripheral Blood In Vivo	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative

PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid.

[#]One-half the dose was administered twice daily.

[a]Treatment-related effect not considered toxicologically relevant.

[b]ERRATUM: An error was identified in the NTP Toxicity Report on Perfluoroalkyl Carboxylates (TOX 97). The table was changed from negative in all bacterial mutagenicity tests for PFOA to equivocal in bacterial strain TA98 without S9 for PFOA. [20 May 2020]

Summary of Findings Considered to Be Toxicologically Relevant in Sprague Dawley (Hsd:Sprague Dawley SD) Rats Administered Wyeth-14,643 by Gavage for 28 Days

	Wyeth-14,643	
	Male	**Female**
Doses in Deionized Water with Tween® 80 (mg/kg/day)	0–25	0–25
Survival Rates	No effect	No effect
Body Weights	↓	No effect
Organ Weights		
R. Adrenal Gland		
Absolute	No effect	No effect
Relative	No effect	No effect
R. Kidney		
Absolute	↑	↑
Relative	↑	↑
Liver		
Absolute	↑	↑
Relative	↑	↑
Spleen		
Absolute	↓	No effect
Relative	No effect	No effect
R. Testis		
Absolute	No effect	–
Thymus		
Absolute	No effect	No effect
Relative	No effect	No effect
Thyroid Gland		
Absolute	No effect	No effect
Relative	↑	No effect
Hematology		
Hematocrit	No effect	No effect
Hemoglobin	No effect	No effect
Erythrocytes	No effect	No effect
Mean corpuscular hemoglobin concentration	No effect	No effect
Mean corpuscular volume	No effect	No effect
Reticulocytes	No effect	No effect
Platelets	No effect	No effect

	Wyeth-14,643	
Clinical Chemistry		
Total Protein	↓	↑[a]
Albumin	↑[a]	↑[a]
Globulin	↓	↓
Albumin/Globulin Ratio	↑	↑
T Bilirubin	No effect	No effect
Direct Bilirubin	↑	No effect
Indirect Bilirubin	No effect	No effect
Cholesterol	↓	No effect
Triglycerides	No effect	No effect
Alanine Aminotransferase	↑	↑
Alkaline Phosphatase	↑	↑
Aspartate Aminotransferase	↑	↑
Sorbitol Dehydrogenase	↑	↑
Creatinine Kinase	No effect	No effect
Bile Acid	↑	↑
Thyroid Stimulating Hormone	↓	↑
Total Thyroxine	↓	No effect
Free Thyroxine	↓	No effect
Total Triiodothyronine	No effect	↑
Testosterone	↓	NA
Gene Expression		
Acox1	↑	↑
Cyp4a1	↑	↑
Cyp2b1	↑	↑
Cyp2b2	↑	↑
Acyl-CoA Oxidase	↑	Not tested
Reproductive Toxicity		
Altered Estrous Cyclicity	–	Yes
Altered Sperm Parameters	Yes	–
Nonneoplastic Effects		
Liver:		
Hepatocyte, Cytoplasmic Alteration	↑	↑
Hepatocyte, Hypertrophy	↑	↑
Hepatocyte, Vacuolization Cytoplasmic	No effect	No effect
Necrosis	No effect	No effect

	Wyeth-14,643	
Nose:		
Olfactory Epithelium, Degeneration	No effect	No effect
Olfactory Epithelium, Hyperplasia	No effect	No effect
Olfactory Epithelium, Inflammation, Suppurative	No effect	No effect
Respiratory Epithelium, Hyperplasia	No effect	No effect
Respiratory Epithelium, Inflammation, Chronic Active	No effect	No effect
Bone Marrow		
Hypocellularity	No effect	No effect
Spleen:		
Extramedullary Hematopoiesis, Increased	No effect	No effect
Atrophy	No effect	No effect
Kidney:		
Nephropathy, Chronic Progressive	No effect	No effect
Thymus:		
Atrophy	No effect	No effect
Lymphocyte, Apoptosis	No effect	No effect
Thyroid Gland:		
Follicular Cell Hypertrophy	No effect	No effect
Lymph Nodes:		
Mandibular, Atrophy	No effect	No effect
Mesenteric, Atrophy	No effect	No effect
Forestomach:		
Epithelium, Hyperplasia	No effect	No effect
Inflammation, Chronic Active	No effect	No effect
Testes:		
Germinal Epithelium, Degeneration	No effect	–
Interstitial Cell, Atrophy	No effect	–
Seminiferous Tubule, Spermatid Retention	No effect	–
Epididymis:		
Hypospermia	No effect	–
Duct, Exfoliated Germ Cell	No effect	–
Epithelium, Apoptosis	No effect	–
Genetic Toxicology		
Bacterial Gene Mutations	–	
Micronucleated Erythrocytes Rat Peripheral Blood In Vivo	Negative	Negative

[a]Treatment-related effect not considered toxicologically relevant.

Overview

The U.S. Environmental Protection Agency nominated the per/polyfluorinated alkyl substances (PFAS) class to the National Toxicology Program (NTP) for a variety of toxicity assessments. A major component of this nomination was a class toxicity evaluation of PFAS through in vitro and in vivo studies. The studies presented in this Toxicity Study Report are part of the in vivo class evaluation, which evaluated a suite of seven PFAS chemicals consisting of three sulfonates and four carboxylates of varying chain lengths (see list below). Measurement of plasma and liver levels of each chemical was conducted to allow for the correlation of toxicities to internal exposure parameters. An agonist of peroxisome proliferator-activated receptor alpha (PPARα), Wyeth-14,643, was included for qualitative comparisons to the PFAS tested because several PFAS are known to activate the PPARα pathway, and inclusion of a positive control would allow for the identification of non-PPARα-mediated toxicities. These studies are divided into two reports: those focused on the carboxylate subclass are presented in this report and those focused on the sulfonate subclass are presented in NTP Toxicity Study Report 96.

Other PFAS studies that were conducted with this nomination included an assessment of the contribution of perinatal exposure to perfluorooctanoic acid (PFOA) chronic toxicity and carcinogenicity in rats; an evaluation of the toxicokinetics of seven PFAS chemicals after a single dose in rats; and in vitro class evaluations of potential neurotoxicity, mitochondrial toxicity, and immunotoxicity with a follow-up in vivo immune toxicity study on perfluorodecanoic acid. As the PFAS class continues to expand with new uses and replacements, NTP continues to assess the potential toxicity of these chemicals through a variety of methods, including in silico, in vitro, and in vivo studies.

Perfluoroalkyl Substances Studied in Toxicity Reports 96 and 97

Chemical	CAS No.	Abbreviation	Toxicity Study Report
Perfluorobutane Sulfonic Acid	375-73-5	PFBS	96
Perfluorohexane Sulfonate Potassium Salt	3871-99-6	PFHSK	96
Perfluorooctane Sulfonic Acid	1763-23-1	PFOS	96
Perfluorohexanoic Acid	307-24-4	PFHxA	97
Perfluorooctanoic Acid	335-67-1	PFOA	97
Perfluorononanoic Acid	375-95-1	PFNA	97
Perfluorodecanoic Acid	335-76-2	PFDA	97
Wyeth-14,643	50892-23-4	WY	96/97

Due to the comparative nature of the studies and concerns of variable kinetics across the class, NTP conducted toxicokinetic studies in the Sprague Dawley rat to understand the relationship of external dose to internal dose. A maximum tolerated daily dose was identified and four lower doses were selected on the basis of the resulting data and supporting kinetic and toxicity information from the literature. PFAS that displayed rapid elimination due to chain length or sex differences were administered at higher doses compared to PFAS with longer half-lives. Doses were administered twice daily for PFHxA and PFBS. The following table lists doses in mg/kg/day and µmol/kg/day for comparison for the chemicals discussed in both Toxicity Study Reports.

Doses Administered to Sprague Dawley Rats in the Gavage Studies of Perfluoroalkyl Substances and Wyeth-14,643

PFAS	Sex	Dose (mg/kg/day)	Micromolar Dose (µmol/kg/day)
PFBS	M/F	0, 62.6, 125, 250, 500, 1,000*	0, 208.6, 416.5, 833.1, 1,666, 3,332*
PFHxSK	M	0, 0.625, 1.25, 2.5, 5, 10	0, 1.4, 2.9, 5.7, 11.4, 22.8
	F	0, 3.12, 6.25, 12.5, 25, 50	0, 7.1, 14.3, 28.5, 57.1, 114.1
PFOS	M/F	0, 0.312, 0.625, 1.25, 2.5, 5	0, 0.6, 1.2, 2.5, 5.0, 10.0
PFHxA	M/F	0, 62.6, 125, 250, 500, 1,000*	0, 199.3, 398.0, 796.0, 1,592, 3,184*
PFOA	M	0, 0.625, 1.25, 2.5, 5, 10	0, 1.5, 3.0, 6.0, 12.1, 24.2
	F	0, 6.25, 12.5, 25, 50, 100	0, 15.1, 30.2, 60.4, 120.8, 241.5
PFNA	M	0, 0.625, 1.25, 2.5, 5, 10	0, 1.3, 2.7, 5.4, 10.8, 21.5
	F	0, 1.56, 3.12, 6.25, 12.5, 25	0, 3.4, 6.7, 13.5, 26.9, 53.9
PFDA	M/F	0, 0.156, 0.312, 0.625, 1.25, 2.5	0, 0.3, 0.6, 1.2, 2.4, 4.9
WY	M/F	0, 6.25, 12.5, 25	0, 19.3, 38.6, 77.2

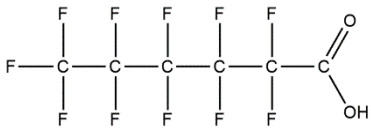
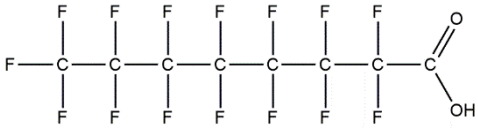
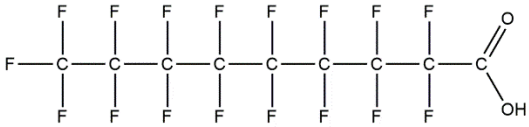
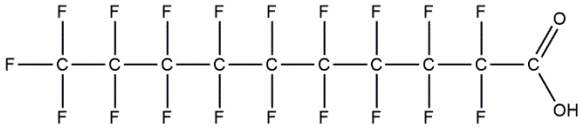
PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonate potassium salt; PFOS = perfluorooctane sulfonic acid; PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; WY = Wyeth-14,643.
*These are the total doses per day; one-half the dose was administered twice daily.

Introduction

Chemical and Physical Properties

The per/polyfluorinated alkyl substances (PFAS) are a class of chemicals that consist of alkyl chains in which hydrogen is substituted with fluorines. This Toxicity Study Report focuses on straight-chain PFAS with a carboxylic acid end group, including perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) (Table 1). The hydrophobic and lipophilic qualities of these chemicals make them useful in surfactants and polymers[1].

Table 1. Perfluoroalkyl Carboxylates

Chemical Chemical Formula	CAS Number	Chemical Structure	Molecular Weight
Perfluorohexanoic Acid $C_6HF_{11}O_2$	307-24-4		314.054
Perfluorooctanoic Acid $C_8HF_{15}O_2$	335-67-1		414.07
Perfluorononanoic Acid $C_9HF_{17}O_2$	375-95-1		464.078
Perfluorodecanoic Acid $C_{10}HF_{19}O_2$	335-76-2		514.086

PFHxA is a colorless liquid with a boiling point of 157°C and a vapor pressure of 1.98 mm Hg at 25°C[2]. PFOA is a white, pungent powder with a boiling point of 192°C and a vapor pressure of 0.525 mm Hg (25°C)[3]. PFNA is a white, crystalline powder with a boiling point of 218°C at 740 mm Hg and a vapor pressure of 0.083 mm Hg (25°C)[4]. PFDA is a liquid with a boiling point of 189°C and a vapor pressure of 10 mm Hg (0°C)[5].

Production, Use, and Human Exposure

PFAS are widely used in the manufacturing of a variety of consumer products and are used in food-contact substances such as nonstick coatings for cookware[6]. Indirect human exposure to perfluoroalkyl carboxylates, such as PFOA, can also occur as a degradation by-product or from internal metabolism of other PFAS, such as polyfluoroalkyl phosphate surfactants, which are often applied to food-contact paper products[7].

Levels of PFAS can persist in the human population for a prolonged period, contributing to a lifetime of exposure to a variety of PFAS species. The four carboxylates evaluated in this report display a wide range of human internal exposure.

In the U.S. National Health and Nutrition Examination Survey (NHANES), the national geometric means measured in human plasma for one year from 2013 to 2014 were 1.94 ng/mL PFOA, 0.68 ng/mL PFNA, and 0.19 ng/mL PFDA[8]. All children, ages 3 to 11, surveyed in that year had detectable levels of PFOA (1.92 ng/mL) and PFNA (0.794 ng/mL) but not PFDA[9]. A more recent survey year (2015 to 2016) reported decreases in the geometric means measured in the total population for each PFAS: 1.56 ng/mL PFOA, 0.58 ng/mL PFNA, and 0.15 ng/mL PFDA[8]. In a different 2015 survey, 13 PFAS were analyzed in human plasma collected from American Red Cross donors[10]. PFOA (1.09 ng/mL), PFNA (0.43 ng/mL), and PFDA (0.15 ng/mL) were all detected. PFHxA was below the lower limit of quantitation (0.04 ng/mL) for this analysis[10]. These values were compared to the concentrations in 2001 samples, which indicated a 77% decline in PFOA, a 33% decline in PFNA, and a 50% decline in PFDA between 2001 and 2015[10]. Nevertheless, PFAS persist in surface soil, water, and wildlife providing a continuous source of exposure and environmental concern[11; 12].

Areas near sites of PFAS manufacturing or use are often associated with higher plasma levels due to contamination of water supplies. A 2015 survey of a New Hampshire community close to a former military base was conducted when the drinking water supply was found to be contaminated with PFAS. The survey revealed that more than 94% of individuals were found to have PFOA detectable in serum, with a geometric mean of 3.1 ng/mL[13]. A 20-year survey of PFAS exposure in the mid-Ohio River Valley from 1991 to 2012 revealed that PFOA was detected in 99.9% of sera collected from test subjects, 47% of which were in the 95th percentile of PFOA sera levels in the United States, with a peak median concentration of 7.6 ng/mL[14].

Regulatory Status

The PFOA Stewardship Program, started in 2006, is an agreement with industry manufacturers to enact a decade-long plan to phase out the use of PFOA, PFOA precursors, and higher homologues, the goals of which have been met (PFOA Stewardship Program, 2014 https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/20102015-pfoa-stewardship-program-2014-annual-progress). Although PFOA is no longer used in the United States, some fluoropolymers containing PFOA may be imported in other consumer products, and it can be produced by the breakdown of some fluorinated telomers. The U.S. Environmental Protection Agency (USEPA) established a drinking water level of 70 ppt in 2016[15].

PFNA and PFOA, as with several other PFAS chemicals, are currently listed under USEPA's UCMR3 (Third Unregulated Contaminant Monitoring Rule), which considers suspected contaminants. PFHxA and PFDA are not covered under this monitoring rule. As of November 2018, the Agency for Toxic Substances and Disease Registry set minimal risk levels for PFOA and PFNA to be 78 parts per trillion (ppt) for adults, and 21 ppt for children[16]. Recently, the European Food Safety Authority (EFSA) developed tolerable weekly intakes of 6 ng/kg/week for PFOA based on human endpoints[17].

Absorption, Distribution, Metabolism, Excretion, and Toxicokinetics

Several studies have evaluated the absorption, distribution, metabolism, excretion, and toxicokinetics of these PFAS; results of these studies indicate large differences in excretion rates and are summarized in this section. Generally, PFAS are water soluble and most of their elimination occurs via urine. The kinetics across the class is variable depending on species and sex in some cases. Reuptake via renal absorption has been proposed to explain the differential elimination across species and sexes. Sex differences in PFOA elimination in rats is mediated by sex hormone-dependent regulation of organic anion transporters (OATs) OAT2 and OAT3[18]. This mechanism is generally assumed to apply to the other PFAS displaying similar sex differences. Physiologically-based pharmacokinetic (PBPK) modeling of PFAS has included a saturable renal resorption function[19-24]. The pharmacokinetic properties were evaluated for several PFAS in the Hsd:Sprague Dawley rat, the animal model used in these 28-day toxicity studies[25; 26].

PFHxA: PFHxA was evaluated in Sprague Dawley rats administered either a bolus intravenous dose of 10 mg/kg or repeated oral gavage doses of 50, 150, or 300 mg/kg/day for 26 days[27]. In the repeated administration gavage study, serum half-lives were reported to be 2 to 3 hours, with no apparent difference observed between males and females. In the single-dose intravenous studies, the terminal half-life was estimated to be 1 hour in males and 0.42 hours in females. Similar half-lives were observed in male and female Wistar rats administered 15 mg/kg intravenously[28]. Terminal half-lives of 5 to 13 hours were observed in male and female Sprague Dawley rats administered 40, 80, or 160 mg PFHxA/kg orally; half-lives increased with dose[25].

PFOA: The half-life of PFOA in male and female Sprague Dawley rats administered a single intraperitoneal dose of 4 mg/kg was found to be from four hours (females) to 15 days (males)[29]. This is longer than the results of a study done in Wistar rats administered PFOA intravenously (20 mg/kg), where male rats exhibited a half-life of 5 days. A full toxicokinetic evaluation of PFOA in males (6, 12, or 48 mg/kg/day) and females (40, 80, or 320 mg/kg/day) exhibited a k_{10} halflife- in females of 3 to 14 hours, but in males it ranged from 8 to 12 days, decreasing with dose[25]. An observational study of PFAS in retired fluorochemical production workers estimated the half-life of PFOA to be approximately 3.5 years[30]. A small study of ski waxers (n = 6) estimated a half-life average of 2.4 years[31]. A half-life average of 2.7 years was estimated from a community (n = 106) exposed to drinking water contaminated with PFOA[32].

PFNA: Sprague Dawley rats were administered a single gavage dose of 1, 3, or 10 mg/kg PFNA, and blood samples were collected over the course of 50 days[33]. Serum half-lives ranged from 24 to 42 days with no sex differences. Male and female Wistar rats administered PFNA intravenously (22 mg/kg) exhibited halflives- of 30 days (males) and 2 days (females).

PFDA: In male and female Wistar rats, evaluation of distribution, metabolism, and excretion after a single 20 mg/kg intraperitoneal dose showed PFDA was eliminated slowly over the course of 14 days with little to no urinary excretion[34]. This is consistent with a report of male and female Wistar rats administered PFDA intravenously (24 mg/kg) exhibiting half-lives of 40 days (males) and 60 days (females). An evaluation of PFDA toxicokinetics in male and female rats (2, 10, or 20 mg/kg) also exhibited half-lives greater than 50 days[25].

Toxicity

Experimental Animals

The literature on potential PFAS toxicity is extensive, and the following summaries focus mainly on guideline studies in rats for comparison to the results within this report. Liver toxicity is a common finding across this class, ranging from liver hypertrophy or necrosis to alterations in liver gene expression and clinical chemistry changes. Peroxisome proliferator-activated receptor alpha (PPARα) activation—leading to increased related gene expression and enzyme activity (e.g., cytochrome P450 4a1 and acyl-CoA oxidase 1 [*Acox1*] along with related enzyme activity of acyl-CoA oxidase) by PFAS in rodents—is a mechanistic pathway with varying potency across the class, depending on chain length[35-39]. Generally, longer-chain PFAS display a greater toxicity than shorter-chain PFAS; and rodent species display a greater PPARα sensitivity than humans[38-40]. Gene expression profiling of the liver suggests that constitutive androstane receptor (CAR) induction is another factor in liver toxicity and enzyme alterations[41; 42].

PFHxA: Several evaluations of PFHxA toxicity have been performed in rodents. A subchronic evaluation of PFHxA toxicity revealed nasal lesions and induction of hepatic peroxisomal β-oxidation in SD rats[43]. In reproductive and developmental studies, no effects on reproductive parameters were observed; however, PFHxA-treated rats had lower fetal body weights compared to controls[43]. In a separate subchronic study, PFHxA induced centrilobular hepatocellular hypertrophy that correlated with higher liver weights[44]. In a perinatal study in CD-1 mice, PFHxA exposure at levels as low as 7 mg/kg/day led to lower F_1 body weights at birth[45].

PFOA: Subchronic exposure to PFOA in BR rats caused increased liver weights and induced hepatocyte hypertrophy[46]. A two-generation study on ammonium perfluorooctanoate in Sprague Dawley rats revealed no changes in mating, fertility, or delivery endpoints in the parent or F_1 generations[47]. Parent and F_1 generation male rats had decreased body weight and increased liver and kidney weights, and F_1 generation pups had a lower birth weight and decreased viability at 30 mg/kg. PFOA significantly decreased fetal weight, live birth, and postnatal growth and survival and caused delays in eye opening in the offspring of dosed pregnant CD-1 mice[48].

PFNA: In a subchronic study, Sprague Dawley rats were administered S-111-S-WB, a mixture of PFAS, once daily for 90 days; the predominant species in the mixture was PFNA[49]. All rats survived to the end of the study, but recorded toxicities included lethargy, decreased body weight, lower serum protein concentrations, higher bilirubin, increased liver weights, and hepatocellular hypertrophy, degeneration, and necrosis. The no-observed-effect levels (NOELs) were 0.025 mg/kg/day for males and 0.125 mg/kg/day for females.

A two-generation reproductive toxicity study of S-111-S-WB was also conducted in Crl:CD(SD) rats[50]. There were no test article-related reproductive effects at the doses tested; however, there were significant increases in liver and kidney weights in the top doses (0.125 and 0.6 mg/kg/day). In the liver, hepatocellular hypertrophy, centrilobular vacuolation, and clear cell foci were also observed and deemed to be related to dose, with male rats being more sensitive than female rats. Hypertrophy of renal tubule cells was also observed at the top dose in males and females.

PFNA was evaluated in a perinatal study in CD-1 mice[51]. There were no differences observed in pregnancy parameters, but neonates exhibited dose-dependent delays in eye opening and onset of puberty, as well as increased liver weight that persisted into adulthood.

PFDA: In a teratology study in C57BL/6N mice, PFDA led to significantly reduced maternal body weights but no fetal toxicity in the absence of maternal toxicity[52].

Humans

A systematic review identified several human health categories of concern in children with respect to exposure to PFAS, including immunity, cardiometabolic, neurodevelopment, thyroid, renal, and puberty onset[53; 54]. Additionally, PFOA and PFOS levels in serum of women are correlated with thyroid disease[55]. PFOA exposure in an occupational population has been associated with cerebrovascular disease, ischemic heart disease, ulcerative colitis, and diabetes[56-58]. Dyslipidemia in adolescents has also been associated with exposure to PFOA and PFOS[59].

PFAS exposure in women has been linked to irregular and long menstrual cycles and to delayed onset of menarche[60; 61]. A meta-analysis of several human studies revealed an association between serum or plasma PFOA and a difference in birth weight[62]. Levels of PFOA in cord serum was not found to be associated with low birth weight or gestational age at birth in a Baltimore cohort[63]. Modest associations between PFOA and preeclampsia and birth defects were found in a PFOA-exposed population in mid-Ohio[64]. Elevated serum PFOA levels during development has been associated with a reduced antibody response (postvaccination) as observed in the Norwegian and Faroe Island birth cohort studies[65-67] and a cross-sectional study of adolescents using NHANES data[68]. NTP[69] conducted a systematic review of immune effects and concluded that PFOA is presumed to be an immune hazard due to evidence from human and animal studies. ATSDR[70] and the C8 panel reports[71-74] found associations between exposure and reproductive parameters.

Carcinogenicity

Experimental Animals

Chronic exposure to PFHxA in SD rats did not reveal carcinogenic activity but did lead to a dose-dependent decrease in survival[75]. A study was conducted with male and female Sprague Dawley rats exposed to 0, 30, or 300 ppm of ammonium perfluorooctonoate (APFO)[76]. An increase in Leydig cell adenomas of the testis was observed, as were increased female mammary fibroadenomas in the 300 ppm group. Further review of the mammary glands from this study found that they were not in fact increased, as more were observed in the control group than initially reported[77]. Review of the pancreas found increased incidence of acinar cell hyperplasia and a single incidence of an acinar cell carcinoma[78]. In a follow-up study with male Sprague Dawley rats exposed to 300 ppm, increased hepatocellular adenomas, Leydig cell adenomas, and pancreatic acinar cell tumors were observed[79].

Humans

Serum concentrations of various PFAS were measured in the blood of patients with hereditary prostate cancer in Sweden[80]. PFDA, but not PFOA or PFNA, was found to have a positive correlation between concentration and prostate cancer incidence. A cohort mortality study in

workers exposed to PFOA found an association between exposure and malignant and nonmalignant renal disease[81]. In another occupational exposure study, PFOA was found to be associated with prostate cancer but not with liver, pancreatic, or testicular cancers[56]. However, in a population of adults living near a chemical plant in Ohio, an association was found between PFOA exposure and kidney and testicular cancers[82]. These cancers were also implicated in a similar association study conducted in West Virginia[83]. In this analysis, PFOA serum levels were associated with not only kidney and testicular cancers but also prostate and ovarian cancers and non-Hodgkin's lymphoma. A general population study in Denmark concluded that plasma levels of PFOA and PFOS were not linked to prostate, bladder, pancreatic, or liver cancer[84]. Finally, PFAS were found, along with polychlorinated biphenyls, to be related to breast cancer risk in a Greenlandic Inuit population[85].

The carcinogenic activity of PFOA in humans was recently reviewed by the International Agency for Research on Cancer (IARC)[86]. Approximately a dozen epidemiological studies have evaluated the possible cancer effects of PFOA in different exposure scenarios (e.g., occupational, community). IARC's evaluation of these studies led to its conclusion that PFOA was possibly carcinogenic to humans (Group 2B) on the basis of limited evidence found in humans of associations with cancer of the testis and kidney and limited evidence in animals[86].

Genetic Toxicity

The genetic toxicity of PFHxA, PFOA, PFNA, and PFDA has been evaluated in bacterial mutagenicity assays and in vitro tests using human and rodent cells. PFOA and PFDA have also been evaluated in a limited number of in vivo genotoxicity tests. All four chemicals were negative in tests for bacterial mutagenicity. PFOA, PFNA, and PFDA showed mixed, primarily positive or primarily negative results, respectively, when tested in vitro using mammalian cell assays. Little is known about the genetic toxicity of PFHxA in mammalian cells. With regard to in vivo studies, a weak response was observed for PFOA and negative responses were observed for PFDA.

PFHxA, PFOA, PFNA, and PFDA have been shown to be negative in bacterial mutagenicity assays. PFHxA, PFOA, PFNA, and PFDA were not mutagenic when tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 in the absence or presence of rat liver metabolic enzymes (S9 mix) at concentrations up to 20 µmol/plate (PFHxA) or 5 µmol/plate (PFOA, PFNA, and PFDA)[87]. Sodium perfluorohexanoate, at concentrations ranging from 333 to 5,000 µg/plate, was negative when tested in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 *uvr*A in the absence or presence of S9 mix[43]. PFOA tested at concentrations up to 500 µM/plate was negative in *S. typhimurium* strains TA98, TA100, TA102, and TA104 in the absence or presence of S9 mix[88]. PFDA was negative when tested in *S. typhimurium* strains TA98, TA100, TA1537, and TA1538 at concentrations ranging from 333 to 10,000 µg/plate (without S9 mix) or 33.3 to 10,000 µg/plate (with S9 mix)[89]. PFDA was also negative when tested in *S. typhimurium* strains TA98 and TA1537 in the presence of S9 mix at notably high concentrations of 1, 10, or 100 g/plate[90].

In comet assay experiments using human hepatocellular carcinoma (HepG2) cells, PFOA and PFNA, but not PFDA, induced significant increases in DNA damage at concentrations ranging from 0.2 to 20 µM for 24 hours[91], and PFOA induced significant, dose-dependent increases in DNA damage at concentrations ranging from 50 to 400 µM[92]. In contrast, negative results were

reported with PFOA (5 to 400 µM for 1 or 24 hours) in a comet assay in HepG2 cells[93]; however, these authors reported using a relatively high final concentration of dimethyl sulfoxide (DMSO) (2.5%) for their experiments, which may have been a factor in the lack of observed response. DMSO is a known scavenger of free radicals and can protect DNA from oxidative damage[94]. In human lymphoblastoid TK6 cells, exposure to 0.25 or 0.5 mg/mL PFOA for 2 hours produced small but significant increases in DNA damage in the comet assay and in 8-hydroxy-2′-deoxyguanosine DNA adducts (per 10^5 dG) as detected using high-performance liquid chromatography (HPLC)-mass spectrometry (MS)[95]. Similar results were obtained for PFNA when tested at 0.125 or 0.25 mg/mL[95]. Exposure to PFOA at concentrations up to 300 µM for 5 or 24 hours did not induce DNA damage in Syrian hamster embryo cells as measured using the comet assay[96]. Negative results were also reported for the comet assay when freshly isolated Wistar rat testicular cells were exposed to 100 or 300 µM PFOA for 24 hours; however, a significant increase in DNA damage was observed when testicular cells were exposed to 300 µM PFNA for 24 hours[97]. PFHxA has not been tested in the in vitro comet assay.

Significant, dose-dependent increases in micronuclei were observed in HepG2 cells exposed to PFOA for 24 hours at concentrations ranging from 50 to 400 µM[92]. Micronuclei were not significantly increased in HepG2 cells exposed to PFOA for 24 hours at concentrations ranging from 5 to 400 µM[93]; however, Florentin et al.[93] reported a high baseline for micronuclei and use of a relatively high final concentration of DMSO (2.5%) for their experiments. PFHxA, PFOA, PFNA, and PFDA did not significantly increase micronuclei in Chinese hamster lung fibroblast V79 cells when exposed to concentrations of 100 µM (PFHxA) or 10 µM (PFOA, PFNA, and PFDA) for 3 hours in the absence or presence of S9 mix[87].

Several additional studies assessed PFDA for mutagenicity and chromosomal damage in rodent cells. PFDA was not mutagenic in the Chinese hamster ovary (CHO)/HGPRT forward mutation assay when cells were exposed to concentrations ranging from 0.01 to 0.16 mg/mL in the absence of S9 mix or from 0.005 to 0.1 mg/mL in the presence of S9 mix[89]. PFDA was also negative in the mouse lymphoma L5178Y/$tk^{+/-}$ forward mutation assay after 24 hours of exposure at concentrations ranging from 0.01 to 100 µg/mL in the absence of S9 mix[98]. Sister chromatid exchanges were not induced in CHO-WBL cells exposed to PFDA for 2.5 hours at concentrations ranging from 1.67 to 50 µg/mL in the absence of S9 mix or when exposed to PFDA for 2 hours at concentrations ranging from 5 to 167 µg/mL with S9 mix[89]. No significant increases in chromosomal aberrations were observed in CHO-WBL cells after 7.25 hours of exposure to concentrations of PFDA ranging from 75 to 200 µg/mL in the absence of S9 mix. Chromosomal aberrations were also not induced in CHO-WBL cells after 2 hours of exposure to concentrations of PFDA ranging from 10 to 100 µg/mL in the presence of S9 mix and harvested approximately 8 hours after exposure; however, chromosomal aberrations were induced after 2 hours of exposure to 151 or 201 µg/mL PFDA in the presence of S9 mix when harvested approximately 18 hours after exposure[89].

In a review of unpublished genetic toxicity test data generated in industry-sponsored studies, PFOA, tested as either an ammonium or sodium salt, was found to be negative in bacterial mutagenicity assays, in the CHO/HGPRT forward mutation assay, in the chromosomal aberration assay when performed using CHO cells or primary human lymphocytes, and in the mouse bone marrow micronucleus assay[99].

One study in the published literature examined the genotoxicity of PFOA in vivo. Using HPLC and an electrochemical detector system, small increases in 8-hydroxy-2′-deoxyguanosine DNA adducts (per 10^5 dG) were detected in DNA obtained from the livers, but not the kidneys, of male F344 rats exposed to 0.02% PFOA in feed for 2 weeks or at 3, 5, or 8 days after a single intraperitoneal injection of 100 mg/kg[100]. Increases in 8-hydroxy-2′-deoxyguanosine (per 10^5 dG) in DNA obtained from liver or kidney were not observed at one day after injection. Considering that oxidative damage to DNA typically undergoes rapid repair, the small increases in 8-hydroxy-2′-deoxyguanosine that were detected by Takagi et al.[100] several days after injection may not necessarily have been due to exposure to PFOA.

Two studies examined the genotoxicity of PFDA in vivo. Female Sprague Dawley rats were given single intraperitoneal injections of 10 mg/kg PFDA once a week for 2 or 8 weeks and liver tissue was analyzed for fatty acylCoA- oxidase (FAO) activity, catalase activity, and 8-oxo-7,8-dihydroguanine DNA adducts (per 10^5 dG)[90]. FAO activity was significantly increased at both time points; catalase activity was significantly increased at the 8-week time point, and liver weights were increased at both time points; however, 8-oxo-7,8-dihydroguanine DNA adducts were not increased at either time point. Unscheduled DNA synthesis was not induced in hepatocytes isolated from male Fischer 344 rats 16 hours after administration of a single dose of 5.5, 11, 22, or 44 mg/kg PFDA by oral gavage; however, S-phase replicative DNA synthesis was induced by PFDA in hepatocytes isolated at 16 and 48 hours[89].

Study Rationale

USEPA nominated the PFAS class for comparative toxicity evaluation to understand the relative toxicity of individual members of the class. Chemicals were selected by comparing newer replacement chemicals (PFHxA and perfluorobutane sulfonic acid) that are shorter in chain length to the older longer-chained PFAS (PFOA and PFOS) in addition to other environmental PFAS of varying exposure levels (perfluorohexane sulfonate, PFNA, and PFDA). The 28-day toxicity study design was used to evaluate multiple endpoints for each of these chemicals for comparison. Plasma concentrations of each PFAS were measured due to the wide range in kinetics of the individual chemicals across species and sexes. A PPARα positive control, Wyeth-14,643 (WY), was included to understand which PFAS toxicological findings were consistent with this mechanism of toxicity and which appear to be independent of this mechanism.

This study is part of a larger NTP program to evaluate PFAS chemicals, which has included an assessment of the contribution of perinatal exposure to PFOA chronic toxicity and carcinogenicity in rats[101]; evaluation of the toxicokinetics of seven PFAS chemicals after a single dose in rats[25]; and in vitro class evaluations of potential neurotoxicity, mitochondrial toxicity[102], and immunotoxicity[103; 104] with a follow-up in vivo immune toxicity study on perfluorodecanoic acid[105].

Materials and Methods

Procurement and Characterization

Perfluoroalkyl Carboxylates

Perfluorohexanoic acid (PFHxA) was obtained from Matrix Scientific (Columbia, SC) in one lot (Q02G); perfluorooctanoic acid (PFOA) was obtained from Sigma-Aldrich (St. Louis, MO) in one lot (03427TH); perfluorononanoic acid (PFNA) was obtained from Oakwood Products, Inc. (West Columbia, SC), in one lot (D11G); and perfluorodecanoic acid (PFDA) was obtained from Sigma-Aldrich in one lot (01820LE). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory and the study laboratory at Battelle (Columbus, OH) (Appendix C). Reports on analyses performed in support of the perfluoroalkyl carboxylate studies are on file at the National Institute of Environmental Health Sciences.

Lot Q02G (PFHxA), a clear, colorless liquid; lot 03427TH (PFOA), white crystals; lot D11G (PFNA), a white, crystalline powder; and lot 01820LE (PFDA), white, transparent wet crystals, were identified using Fourier transform infrared (FTIR) spectroscopy, mass spectrometry (MS), and carbon-13 and fluorine-19 nuclear magnetic resonance (NMR) spectroscopy.

The purity of each perfluoroalkyl carboxylate lot was determined by high-performance liquid chromatography (HPLC)/ion chromatography (IC) with suppressed conductivity (SC) detection (all lots except 01820LE), and gas chromatography (GC) with flame ionization detection (FID) and electron capture detection (ECD); in addition, GC/MS was used to confirm the identity of each perfluoroalkyl carboxylate test article and identify impurities within each lot. Additional GC/FID, GC/ECD, and GC/MS analyses were performed on lot D11G to screen it for selected volatile solvents. All GC analyses were performed on derivatized (methylated) aliquots of each perfluoroalkyl carboxylate. Differential scanning calorimetry (DSC) was used to determine the purities of lots 03427TH and D11G. In addition, Karl Fischer titration for lots Q02G and 03427TH was performed by Prevalere Life Sciences, Inc. (Whitesboro, NY), and similar analysis for lot D11G was performed by Galbraith Laboratories, Inc. (Knoxville, TN).

For lot Q02G (PFHxA), Karl Fischer titration indicated 0.47% water. HPLC/IC/SC analysis indicated one major peak that was 99.6% of the total peak area and two reportable (areas ≥0.1% of the total peak area) impurities with a combined area of 0.37% of the total peak area. GC/FID analysis indicated one major peak (99.1%) and five reportable impurities with a combined area of 0.95% of the total peak area. GC/ECD analysis showed that the impurities were likely fluorinated compounds. GC/MS analysis confirmed the identity of the test article and indicated that one of the impurities representing 0.33% of the total was an isomer of PFHxA. The overall purity of lot Q02G was determined to be greater than 99% (Table 2).

For lot 03427TH (PFOA), Karl Fischer titration indicated 0.24% water. DSC indicated an average purity of 98.96%. HPLC/IC/SC analysis indicated one major peak that was 98.8% of the total peak area and three reportable impurities with a combined area of 1.2% of the total peak area. GC/FID analysis indicated one major peak (98.3%) and four reportable impurities with a combined area of 1.66% of the total peak area. GC/ECD analysis showed that the impurities were likely fluorinated compounds. GC/MS analysis confirmed the identity of the test article and

indicated that two of the impurities representing 1.04% of the total were isomers of PFOA. The overall purity of lot 03427TH was determined to be greater than 98% (Table 2).

For lot D11G (PFNA), Karl Fischer titration indicated 0.29% water. DSC indicated an average purity of 99.5%. HPLC/IC/SC analysis indicated one major peak that was 98.5% of the total peak area and two reportable impurities with a combined area of 1.5% of the total peak area. GC/FID analysis by one system indicated a purity of 100%. GC/ECD by one system analysis indicated a purity of 99.0% with six reportable impurities. GC/MS analysis confirmed the identity of the test article and indicated the presence of perfluoroheptanoic acid as an impurity. GC/FID analysis by a second system showed the presence of low concentrations of the nonhalogenated solvents (≤0.007%) hexane, diethyl ether, and acetone. GC/ECD analysis by a second system showed the presence of low concentrations of the halogenated solvents (≤0.0009%) chloroform and carbon tetrachloride. The overall purity of lot D11G was determined to be greater than 98% (Table 2).

For lot 01820LE (PFDA), GC/FID analysis indicated one major peak (97.2%) and 10 reportable impurities with a combined area of 2.8% of the total peak area. GC/ECD analysis showed that the impurities were likely fluorinated compounds. GC/MS analysis confirmed the identity of the test article and indicated that one of the impurities representing 0.59% of the total was an isomer of PFDA. The overall purity of lot 01820LE was determined to be greater than 97% (Table 2).

Table 2. Purity of Chemicals in the 28-day Gavage Studies of Perfluoroalkyl Carboxylates

Chemical Name	Lot Number	Percent Purity	Number of Impurities[a]	Percent Impurity[a]
PFHxA	Q02G	>99	≤5	≤0.95
PFOA	03427TH	>98	≤5	≤2.0
PFNA	D11G	>98	≤6	≤1.5
PFDA	01820LE	>97	10	≤3.0

PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid.
[a]Value depends on analytical method applied.

Stability studies of lots Q02G (PFHxA) and D11G (PFNA) of the bulk chemicals were performed using HPLC/IC/SC and GC/ECD, respectively. Stability was confirmed for bulk chemical samples stored in sealed amber glass bottles at temperatures up to 60°C for at least 14 days. To ensure stability, all the bulk chemicals were stored at room temperature in sealed opaque plastic containers (lots Q02G and D11G) or sealed amber glass bottles (lots 03427TH and 01820LE). Reanalyses of the bulk chemicals were performed by the study laboratory; no degradation was detected.

Wyeth-14,643

Wyeth-14,643 (WY) was obtained from Chem Syn Laboratories (Lenexa, KS) in one lot (91-314-72-07/91-314-100-33A). Identity and purity analyses were conducted by the analytical chemistry laboratory and the study laboratory at Battelle (Columbus, OH) (Appendix C).

Lot 91-314-72-07/91-314-100-33A, a white powder, was identified using FTIR spectroscopy and proton and carbon-13 NMR spectroscopy. The purity of WY was determined by HPLC with ultraviolet light (UV) detection and DSC.

For lot 91-314-72-07/91-314-100-33A, HPLC/UV analysis indicated one major peak (99.4%) and two reportable impurities with a combined area of 0.64% of the total peak area. DSC indicated an average purity of 94.3%; this lower calculated purity compared to that shown by HPLC was considered an indication of thermal decomposition rather than impurity of the bulk chemical. The overall purity of lot 91-314-72-07/91-314-100-33A was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored at room temperature in sealed amber glass bottles. Reanalyses of the bulk chemical were performed by the study laboratory, and no degradation was detected.

Tween® 80

Tween 80 (polysorbate 80) was obtained from Spectrum Laboratory Products, Inc. (Gardena, CA), and was used at a 2% concentration as the vehicle in the 28-day gavage studies. The vehicle was prepared by mixing the appropriate amount of Tween 80 with deionized water in a calibrated carboy and stirring with an overhead stirrer until all the Tween 80 was dissolved.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing PFHxA, PFOA, PFNA, PFDA, or WY with 2% Tween 80 in deionized water to give the required concentrations. Each formulation pH was adjusted to between 6 and 8. The dose formulations were stored at room (PFHxA, PFOA, PFNA, and PFDA) or refrigerated (WY) temperatures in amber glass bottles sealed with Teflon®-lined lids for no more than 43 days.

Homogeneity studies of the 6.26 and 100 mg/mL dose formulations of PFHxA and a stability study of the 6.26 mg/mL dose formulation were performed by the analytical chemistry laboratory using GC/ECD. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.05 mg/mL formulation and the 20 mg/mL dose formulation of PFOA and a stability study of a 0.05 mg/mL formulation were performed by the analytical chemistry laboratory using the same GC/ECD system. These studies were performed on lot 02702EE (Sigma-Aldrich) which was not used in the animal studies. In addition, homogeneity and stability of a 0.05 mg/mL formulation of lot 03427TH were assessed by the analytical chemistry laboratory using GC/ECD. Homogeneity was confirmed, and stability was confirmed for at least 42 days for formulations stored in amber glass bottles with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.12 mg/mL formulation and the 5 mg/mL dose formulation of PFNA and a stability study of a 0.12 mg/mL formulation were performed by the analytical chemistry laboratory using GC/ECD. Homogeneity was confirmed, and stability was confirmed for at least

43 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.04 mg/mL formulation and the 0.5 mg/mL dose formulation of PFDA and a stability study of a 0.04 mg/mL formulation were performed by the analytical chemistry laboratory using GC/ECD. An additional stability study of the 0.0312 mg/mL dose formulation was performed by the study laboratory using the same GC/ECD system. Homogeneity was confirmed, and stability was confirmed for at least 42 (analytical chemistry laboratory) or 45 (study laboratory) days for formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of the 1.26 and 5 mg/mL dose formulations of WY and a stability study of the 1.26 mg/mL dose formulation were performed by the analytical chemistry laboratory using HPLC/UV. Homogeneity was confirmed, and stability was confirmed for at least 42 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at refrigerated temperature and for at least 3 hours under simulated animal room conditions.

Analyses of the dose formulations for the 28-day studies of PFHxA, PFOA, PFNA, and PFDA were conducted once for each study by the study laboratory using GC/ECD. HPLC/UV was used to conduct similar analyses for the 28-day study of WY. All dose formulations for all five chemicals were within 10% of the target concentrations (Table C-4). Animal room samples of these dose formulations were also analyzed; with the exception of two of 10 samples for the PFOA study, all animal room samples for all five chemicals were within 10% of the target concentrations.

Animal Source

Male and female Sprague Dawley (Hsd:Sprague Dawley SD) rats were obtained from Envigo (formerly Harlan Laboratories, Inc., Indianapolis, IN).

Animal Welfare

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by AAALAC International. Studies were approved by the Battelle Columbus Operations Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

28-day Studies

On receipt, rats were approximately 7 to 9 weeks old. Animals were quarantined for 12 days for PFHxA, 11 (males) or 12 (females) days for PFOA, 14 (males) or 15 (females) days for PFNA, 19 (males) or 20 (females) days for PFDA, and 20 (males) or 21 (females) days for WY. The rats were 10 to 11 weeks old on the first day of each study. Before the studies began, five male and five female rats were randomly selected for parasite evaluation and gross observation for evidence of disease. The health of the animals was monitored during the studies according to the NTP Sentinel Animal Program (Appendix E). All results were negative.

All dose groups consisted of 10 male and 10 female rats. All test compounds were administered in deionized water with 2% Tween 80 by gavage, 7 days per week for 28 days; control animals received the vehicle only. Doses were selected based on a maximum tolerated daily dose and kinetic information obtained from toxicokinetics studies. PFHxA was administered twice daily at one-half the dose for total daily doses of 0, 62.6, 125, 250, 500, or 1,000 mg/kg body weight. PFOA was administered once daily at 0, 0.625, 1.25, 2.5, 5, or 10 mg/kg (males) or 0, 6.25, 12.5, 25, 50, or 100 mg/kg (females). PFNA was administered once daily at 0, 0.625, 1.25, 2.5, 5, or 10 mg/kg (males) or 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/kg (females). PFDA was administered once daily at 0, 0.156, 0.312, 0.625, 1.25, or 2.5 mg/kg. WY was administered once daily at 0, 6.25, 12.5, or 25 mg/kg. All formulations were administered at a volume of 5 mL/kg. NTP-2000 feed and water were available ad libitum. Rats were housed five per cage and were observed twice daily. The animals were weighed, and clinical observations were recorded initially, weekly thereafter, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 3. Information on feed composition and contaminants is provided in Appendix D.

Animals were anesthetized with a 70%:30% CO_2:O_2 mixture and blood was collected from the abdominal aorta at the end of the studies for hematology, clinical chemistry, thyroid hormone and testosterone analyses, parent compound concentration, and micronucleus assays. For each animal, blood was collected into two tubes containing K_3 EDTA and one tube devoid of an anticoagulant. An aliquot of whole blood was removed from one K_3 EDTA tube and centrifuged, and the plasma was harvested and shipped to Battelle (Columbus, OH) for internal dose analyses[25; 26]; the remaining whole blood was used for hematology analysis. The other K_3 EDTA tube was shipped on wet ice to ILS, Inc. (Durham, NC), for micronuclei analysis the day it was collected. Any remaining plasma was frozen and shipped to NTP Frozen Tissue Bank (Durham, NC). Blood collected in tubes devoid of anticoagulant was allowed to clot, and the serum harvested for clinical chemistry and hormonal analysis, except aspartate aminotransferase activity and total and direct bilirubin concentrations, which were analyzed at a later date using an aliquot of the stored frozen plasma. The hematology analyses were conducted using an Advia 120 (Bayer Diagnostics Division, Tarrytown, NY) and reagents supplied by the manufacturer. A manual hematocrit was determined with the use of a microcentrifuge. Blood smears were stained with a Romanowsky-type stain and the morphology of the leukocytes, erythrocytes, and platelets reviewed. Clinical chemistry analyses (including total thyroxine) were completed using a Cobas c501 Chemistry Analyzer (Roche, Indianapolis, IN); aspartate aminotransferase activity and bilirubin concentrations were analyzed on an Olympus AU400 Analyzer (Irving, TX). The remaining thyroid hormones were determined by radioimmunoassay (free thyroxine and total triiodothyronine [MP Biomedicals, Irvine, CA]; thyroid stimulating hormone [Institute of Isotopes, Ltd., Budapest, Hungary]). Testosterone was determined by enzyme-linked immunosorbent assay (Calbiotech, Inc., Spring Valley, CA). The parameters measured are listed in Table 3.

Necropsies were performed on all rats. The right adrenal gland, heart, right kidney, liver, lung, spleen, right testis, thymus, thyroid gland, and uterus/cervix/vagina from each animal were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except eyes, testes, epididymides, and vaginal tunics were first fixed in Davidson's solution or modified Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of approximately 5 µm, and stained with hematoxylin and eosin.

Histopathologic examinations were performed on all rats. Table 3 lists the tissues and organs routinely examined.

At the end of the 28-day studies, samples were collected for sperm motility evaluations on male rats administered 0, 250, 500, or 1,000 mg/kg/day (PFHxA); 0, 2.5, 5, or 10 mg/kg/day (PFOA); 0, 0.625, 1.25, and 2.5 mg/kg/day (PFNA, PFDA); and 0, 6.25, 12.5, or 25 mg/kg/day (WY). It was recognized that the 28-day dosing duration would not cover the full spermatogenic cycle. The parameters evaluated are listed in Table 3. Male rats were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65°C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer. For vaginal cytology, samples were taken from females in the 0, 125, 250, and 500 mg/kg/day (PFHxA); 0, 25, 50, and 100 mg/kg/day (PFOA); 0, 1.56, 3.12, and 6.25 mg/kg/day (PFNA); 0, 0.625, 1.25, and 2.5 mg/kg/day (PFDA); and 0, 6.25, 12.5, and 25 mg/kg/day (WY) groups. For 16 consecutive days prior to scheduled terminal euthanasia, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus).

At the end of the PFHxA, PFOA, PFNA, and PFDA studies, 500 mg liver samples from the right anterior and posterior lobes of male rats were collected for determination of parent compound concentrations, given that male rat liver responses to PFAS are generally greater compared to females. At the end of all studies, approximately 1 g of liver tissue was collected from the median lobe of male rats, homogenized, frozen, and stored until analyzed for acyl-CoA oxidase activity. From the left liver lobe, tissue samples were placed in *RNAlater*® (Ambion, Inc., Austin, TX) and stored at 2° to 8°C overnight. The *RNAlater* supernatant was then removed from the samples, and the samples were frozen at −70°C ± 10°C. Samples weighing between 22.0 and 30.0 mg were added to lysis buffer, homogenized, and stored at −80°C ± 10°C until RNA was isolated. RNA was extracted from the supernatant, subjected to DNase digestion, and isolated using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Each RNA sample was analyzed for quantity and purity by ultraviolet analysis using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). All samples were evaluated for RNA integrity using an RNA 6000 Nano Chip kit with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and then stored at −80°C ± 10°C until further processing. Total RNA (1 µg) isolated from rat tissue samples was reverse transcribed into cDNA using Qiagen RT HT First Strand Kits (Qiagen, Valencia, CA). The cDNA samples were analyzed using Qiagen RT^2 Custom Arrays in a 96-well plate format. Relative fold change was calculated within the analysis software based on

the ΔΔCt method for relative quantitation. Twelve samples were run on each array plate with a single sample in each column of the plate. The eight wells in each column of the array plates contained the four genes of interest: PPARα-related genes acyl-CoA oxidase (*Acox1)* and cytochrome P450 4a1 (*Cyp4a1)*, constitutive androstane receptor (CAR)-related genes cytochrome P450 2b1 (*Cyp2b1)* and cytochrome P450 2b2 (*Cyp2b2*), a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and three array plate quality controls. The parameters measured are listed in Table 3.

Quality control measurements for each sample on each qPCR array plate were evaluated to determine if the data generated from the sample were of sufficient quality prior to analysis using the web-based RT Profiler PCR Array Data Analysis software (version 3.5, SABiosciences, Valencia, CA). The controls on the qPCR array were analyzed for each sample and included a PCR positive/array reproducibility control (PPC), a reverse transcription control (RTC), and a genomic DNA contamination (GDC) control. A sample successfully passed all of these control parameters when the cycle threshold (Ct) value for the PPC was 20 ± 2, the Ct value for the GDC was greater than or equal to 35, and the Ct value of the PPC subtracted from the Ct value of the RTC was less than or equal to 5. If a sample failed the acceptance criteria for the GDC and/or RTC controls, the sample was repeated starting with the reverse transcription (cDNA synthesis) process followed by analysis on a new qPCR array plate. If a sample failed the PPC control, the cDNA sample was used to repeat the sample on a new qPCR array plate. Once all the data from a single experiment were analyzed and collected (male or female, control animals, and all dosages), the quality control data were assessed a second time after the total experimental data were uploaded into the web-based RT Array Data Analysis software (version 3.5, SABiosciences). This provided additional assurance that the data for the entire experiment passed the quality control criteria.

The relative fold change for each gene of interest (*Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2*) was calculated within the analysis software for relative quantitation. All the samples were normalized using the housekeeping gene *GAPDH*, and the geometric mean of all samples in a particular group was used for normalization. The p values (based on the Student's t-test of the replicate values for each gene in the vehicle control group and dose groups) and 95% confidence intervals for each fold change value were also calculated during analysis. The threshold for determining significant differential expression of each gene was a fold change of ≥2 or ≤−2 and a p value ≤0.05.

After a review of the laboratory reports, selected histopathology slides were submitted to quality assessment (QA) pathologists for review. After the review, diagnostic discrepancies/inconsistencies were discussed and resolved with the NTP pathologists. The QA pathologist also served as the Pathology Working Group (PWG) pathologist and coordinator. Any remaining discrepancies/inconsistencies were resolved by the PWG. Final diagnoses for reviewed lesions represent a consensus of the PWG or a consensus between the study laboratory pathologist, NTP pathologist, and the QA/PWG pathologist. Details of these review procedures have been described, in part, by Maronpot and Boorman[106] and Boorman et al.[107].

Table 3. Experimental Design and Materials and Methods in the 28-day Gavage Studies of Perfluoroalkyl Carboxylates[a]

28-day Studies
Study Laboratory
Battelle Columbus Operations (Columbus, OH)
Strain and Species
Sprague Dawley (Hsd:Sprague Dawley SD) rats
Animal Source
Envigo (formerly Harlan Laboratories, Inc., Indianapolis, IN)
Time Held Before Studies
PFHxA: 12 days
PFOA: 11 or 12 days
PFNA: 14 or 15 days
PFDA: 19 (males) or 20 (females) days
WY: 20 (males) or 21 (females) days
Average Age When Studies Began
10 to 11 weeks
Date of First Dose
PFHxA: January 17 (males) or 18 (females), 2012
PFOA: January 31 (males) or February 1 (females), 2012
PFNA: February 2 (males) or 3 (females), 2012
PFDA: February 7 (males) or 8 (females), 2012
WY: January 26 (males) or 27 (females), 2012
Duration of Dosing
7 days/week for 28 days (gavage)
Date of Last Dose
PFHxA: February 13 (males) or 14 (females), 2012
PFOA: February 27 (males) or 28 (females), 2012
PFNA: February 29 (males) or March 1 (females), 2012
PFDA: March 5 (males) or 6 (females), 2012
WY: February 22 (males) or 23 (females), 2012
Necropsy Dates
PFHxA: February 14 (males) or 15 (females), 2012
PFOA: February 28 (males) or 29 (females), 2012
PFNA: March 1 (males) or 2 (females), 2012
PFDA: March 6 (males) or 7 (females), 2012
WY: February 23 (males) or 24 (females), 2012

28-day Studies

Average Age at Necropsy

PFHxA: 14 to 16 weeks
PFOA, PFNA, PFDA, WY: 14 to 15 weeks

Size of Study Groups

10 males and 10 females

Method of Distribution

Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage

5

Method of Animal Identification

Tail tattoo

Diet

Irradiated NTP-2000 wafer feed (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed weekly

Water

Tap water (Columbus municipal supply) via automatic watering system (Edstrom, Inc., Waterford, WI), available ad libitum

Cages

Solid, polycarbonate (Lab Products, Inc., Seaford, DE), changed twice weekly

Bedding

Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ), changed twice weekly with cages

Rack Filters

Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks

Racks

Stainless steel (Lab Products, Inc., Seaford, DE), changed at least every 2 weeks

Animal Room Environment

Temperature: 72° ± 3°F

Relative humidity: 50% ± 15%

Room fluorescent light: 12 hours/day

Room air changes: 10/hour

Doses

PFHxA: 0, 62.6, 125, 250, 500, or 1,000 mg/kg per day; onehalf the dose was administered twice daily approximately 6 hours apart (males and females)

PFOA: males: 0, 0.625, 1.25, 2.5, 5, or 10 mg/kg per day; females: 0, 6.25, 12.5, 25, 50, or 100 mg/kg per day

PFNA: males: 0, 0.625, 1.25, 2.5, 5, or 10 mg/kg per day; females: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/kg per day

PFDA: 0, 0.156, 0.312, 0.625, 1.25, or 2.5 mg/kg per day (males and females)

WY: 0, 6.25, 12.5, or 25 mg/kg per day

All doses were administered in deionized water with 2% Tween® 80 by gavage (dosing volume 5 mL/kg).

28-day Studies

Type and Frequency of Observation

Observed twice daily; animals were weighed and clinical findings were recorded on day 1, weekly thereafter, and at the end of the study.

Method of Euthanasia

Anesthetization with CO_2:O_2 (70%:30%) followed by exsanguination by severing the abdominal aorta

Necropsy

Necropsies were performed on all rats. Organs weighed were right adrenal gland, heart, right kidney, liver, lungs, spleen, right testis, thymus, thyroid gland, and uterus/cervix/vagina.

Clinical Pathology

Blood was collected from the abdominal aorta of all rats at the end of the studies for hematology and clinical chemistry.

Hematology: hematocrit; manual hematocrit, hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials, blood smear morphological evaluation

Clinical chemistry: urea nitrogen; creatinine; glucose; total protein; albumin; globulin; total, direct, and indirect bilirubin; aspartate aminotransferase; alanine aminotransferase; alkaline phosphatase; creatine kinase; sorbitol dehydrogenase; cholesterol; triglycerides; bile acids; total triiodothyronine; thyroid stimulating hormone; total and free thyroxine; and testosterone.

Histopathology

PFHxA: Complete histopathology was performed on 0 and 1,000 mg/kg per day rats.

PFOA: Complete histopathology was performed on 0, 10 (males), and 100 (females) mg/kg per day rats.

PFNA: Complete histopathology was performed on 0, 2.5, 5, and 10 mg/kg per day (males) and 0, 6.25, 12.5, and 25 mg/kg per day (females) rats.

PFDA: Complete histopathology was performed on rats in the 0 and 2.5 mg/kg per day rats.

WY: Complete histopathology was performed on rats in the 0 and 25 mg/kg per day rats.

In addition to gross lesions and tissue masses, the following tissues were examined to a noeffect level: adrenal gland, bone with marrow, brain, clitoral gland, epididymis esophagus, eyes, Harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine, (duodenum, jejunum, ileum), lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, spleen, stomach (forestomach and glandular), thymus, trachea, urinary bladder, and uterus. The kidney, liver, testis, and thyroid gland were examined in all dose groups.

Reproductive Parameters

PFHxA: Blood was collected from the abdominal aorta of all rats at the end of the study and analyzed for testosterone levels. Sperm samples were collected from 0, 250, 500, and 1,000 mg/kg per day males at study termination. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal motility and concentration. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for 16 consecutive days prior to the end of the study from females in the 0, 125, 250, and 500 mg/kg per day groups for vaginal cytology evaluations. The percentage of time spent in the various estrus cycle stages and estrus cycle length were evaluated.

PFOA: Same as the PFHxA study except sperm samples were collected from 0, 2.5, 5, and 10 mg/kg per day males, and vaginal samples were collected from 0, 25, 50, and 100 mg/kg per day females.

PFNA: Same as the PFHxA study except sperm samples were collected from 0, 0.625, 1.25, and 2.5 mg/kg per day males, and vaginal samples were collected from 0, 1.56, 3.12, and 6.25 mg/kg per day females.

28-day Studies
PFDA: Same as the PFHxA study except sperm samples were collected from 0, 0.625, 1.25, and 2.5 mg/kg per day males, and vaginal samples were collected from 0, 0.625, 1.25, and 2.5 mg/kg per day females.
WY: Same as the PFHxA study except sperm and vaginal samples were collected from rats in all dose groups.
Internal Dose Assessment
Approximately 24 hours after the last dose in the PFHxA, PFOA, PFNA, PFDA studies, parent compound concentrations were determined in plasma from blood collected from the abdominal aorta of all rats and in liver samples from the right anterior and posterior lobes of male rats.
Liver Enzyme Levels and Gene Expression
Samples were collected from the median liver lobe of surviving male rats at study termination for determination of acyl-CoA oxidase activity. Samples were collected from the left liver lobe of all surviving rats at study termination for determination of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2*.

[a]PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; WY = Wyeth-14,643.

Statistical Methods

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in this report and in the CEBS database (https://manticore.niehs.nih.gov/cebssearch) as the numbers of animals bearing such lesions at a specific anatomic site and as the numbers of animals with that site examined microscopically. The Fisher exact test[108], a procedure based on the overall proportion of affected animals, was used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and vehicle control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett[109] and Williams[110; 111]. Hematology, clinical chemistry, hormones, parent compound, spermatid, epididymal spermatozoal, hepatic enzymes, and hepatic gene expression data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley[112] (as modified by Williams[113]) and Dunn[114]. Jonckheere's test[115] was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey[116] were examined by NTP personnel, and implausible values were eliminated from the analysis. Proportions of regular cycling females in each dosed group were compared to the vehicle control group using the Dunn's or Shirley's test on the cycle length and number of cycles[108]. Tests for extended periods of estrus, diestrus, metestrus, and proestrus, as well as skipped estrus and skipped diestrus, were constructed based on a Markov chain model proposed by Girard and Sager[117]. The "Overall" test checks for any of the extended or skipped states. For each dose group, a transition probability matrix was estimated for transitions among the proestrus, estrus, metestrus, and diestrus stages, with provision for extended stays within each stage as well as for skipping estrus or diestrus within a cycle.

Equality of transition matrices among dose groups and between the vehicle control group and each dosed group was tested using chi-square statistics.

Quality Assurance Methods

The 28-day studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations[118]. In addition, as records from the 28-day studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent QA contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Study Report. Audit procedures and findings are presented in the reports and are on file at the National Institute of Environmental Health Sciences. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this report.

Genetic Toxicology

Bacterial Mutagenicity Test Protocol

The test articles were coded prior to testing. Testing was conducted as reported by Zeiger et al.[119], with some modifications. Compounds were incubated with the bacterial tester strains (*Salmonella typhimurium* strains TA98 and TA100 and *Escherichia coli* strain WP2*uvr*ApKM101) either in buffer or S9 mix (metabolic activation enzymes and cofactors from livers of phenobarbital/benzoflavone-induced male Sprague Dawley rats) for 20 minutes at 37°C. Top agar supplemented with L-histidine or L-tryptophan (for the *E. coli* strain only) and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine- or tryptophan-independent mutant colonies arising on these plates were counted using an automatic colony counter following incubation for 2 days at 37°C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of the test compound. The highest concentration tested was limited by toxicity in the absence of S9; with S9, concentrations were either limited by toxicity or achieved the limit concentration of 10,000 µg/plate. All trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, not reproducible, or not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. No minimum percentage or fold increase is required for a chemical to be judged positive or weakly positive.

Rat Peripheral Blood Micronucleus Test Protocol

Blood samples (200 µL per animal) were collected at the end of the studies, stabilized in EDTA tubes, and packaged on ice packs for shipment later that same day by overnight courier to ILS, Inc. (Durham, NC). Samples were kept chilled upon arrival at the laboratory the following day and were fixed within 4 hours of receipt. For each sample, 50 µL of blood were dispensed into a microcentrifuge tube containing heparin and mixed by inverting several times. A fixation tube containing ultra-cold methanol was then removed from a −80°C freezer and 180 µL of the heparinized blood sample was forcefully dispensed into the tube, rapidly mixed, and quickly

transferred back to the −80°C freezer. The fixed blood samples were stored in the −80°C freezer for at least 3 days prior to flow cytometry analysis of micronucleated red blood cells.

Flow cytometry analysis was performed using MicroFlow^{PLUS} Kit reagents (Litron Laboratories, Rochester, NY) and a Becton-Dickinson FACSCalibur™ dual-laser bench top system (BD Biosciences, San Jose, CA). The analysis was performed according to the kit's instruction manual with minimal modification[120; 121]. For up to five peripheral blood samples per dose group, 20,000 (±2,000) immature CD71-positive erythrocytes (also referred to as polychromatic erythrocytes [PCE]) were analyzed by flow cytometry to determine the frequency of normal and micronucleated PCE (MN-PCE). Aggregates were excluded on the basis of forward and side scatter; platelets were excluded on the basis of staining with an anti-CD61 antibody; and nucleated leukocytes were excluded on the basis of intense propidium iodide staining. Normal and micronucleated mature erythrocytes (also referred to as normochromatic erythrocytes [NCE]) were enumerated concurrently during MN-PCE analysis, allowing for calculation of the percentage of PCE among total erythrocytes as a measure of bone marrow toxicity.

In rats, although both immature and mature erythrocytes are evaluated for presence of micronuclei concurrently, the appropriate cell population for determining the frequency of micronucleated cells is the youngest portion of the immature erythrocyte population, i.e., the fraction of immature erythrocytes that is newly emerged from the bone marrow, due to the efficiency with which the rat spleen removes micronucleated red blood cells from circulation.

Prior experience with the large number of cells scored using flow cytometric scoring techniques[122; 123] suggested it was reasonable to assume that the proportion of micronucleated reticulocytes was approximately normally distributed. Mean MN-PCE/1,000 PCE and MN-NCE/1,000 erythrocytes, as well as % PCE, were calculated for each animal. These data are summarized in the tables as mean ± standard error of the mean. Levene's test was used to determine if variances among dose groups were equal at $\alpha = 0.05$. When variances were equal, linear regression analysis was used to test for linear trend and Williams' test was used to evaluate pairwise differences between each dosed group and the vehicle control group. When variances were unequal, nonparametric methods were used to analyze the data; Jonckheere's test was used to evaluate linear trend; and Dunn's test was used to assess the significance of pairwise differences between each dosed group and the vehicle control group. Trend tests and pairwise comparisons with the controls are considered statistically significant for PCEs and NCEs when the onesided- p value is less than 0.025 and for percent PCEs when the two-sided p value is less than 0.05. A result was considered positive if the trend test was significant and if at least one dose group was significantly elevated over the vehicle control group or if two or more dose groups were significantly increased over the corresponding vehicle control group. A response was considered equivocal if only the trend test was significant or if only a single dosed group was significantly increased over the vehicle control group.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple samples of a chemical were tested in the same assay, and different results were obtained among these samples and/or among

laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the in vitro assays have another variable that must be considered in arriving at an overall test result. In vitro assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The results presented in the Abstract of this toxicity study report represent a scientific judgment of the overall evidence for activity of the chemical in an assay.

Results

NTP evaluated all study data. Data relevant for evaluating toxicological findings are presented here. All study data are available in the NTP Chemical Effects in Biological Systems (CEBS) database: http://doi.org/10.22427/NTP-OTHER-2.

28-day Studies

Perfluorohexanoic Acid (PFHxA)

All rats survived until the end of the study (Table 4). No clinical observations related to PFHxA administration were observed. Body weights of 1,000 mg/kg/day males were 12% and 13% less than those of the vehicle controls on days 22 and 29, respectively; body weights of female dose groups remained within 10% of the vehicle control body weights throughout the study (Table 4; Figure 1).

Plasma concentrations of PFHxA increased with increasing dose in both males and females (Table 5). Males generally had a higher (1.6- to threefold) plasma concentration compared to females across the dose groups. When normalized to dose administered (µM/mmol/kg), male plasma concentrations were generally similar (<twofold) across the 62.6 to 500 mg/kg/day dose groups, with a near twofold increase from the 62.6 mg/kg/day dose group to the 1,000 mg/kg/day dose group, which was greater than dose-proportional. Females exhibited a similar pattern with comparable values at the lower doses (<twofold) and a threefold increase from the 62.6 mg/kg/day dose group to the 1,000 mg/kg/day dose group.

Liver concentrations of PFHxA were measured in males only and were only quantifiable in 250, 500, and 1,000 mg/kg/day rats (Table 5). When normalized to dose administered, these values marginally increased with dose (2.6 to 4.8 µM/mmol/kg). The liver/plasma ratios in males were less than 1 across the dose groups with quantifiable PFHxA.

Dose-related increases were observed in the absolute and relative liver weights of male and female rats (Table 6). The mean absolute and relative liver weights of 500 and 1,000 mg/kg/day males and females and the mean relative liver weight of 250 mg/kg/day males were significantly greater than those of the respective vehicle control groups. The mean relative kidney weights of 500 and 1,000 mg/kg/day males and the mean absolute and relative kidney weights of 1,000 mg/kg/day females were significantly greater than those of the respective vehicle control groups. The mean absolute thymus weight was significantly decreased in male rats in the 1,000 mg/kg/day group.

Table 4. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

Day	Vehicle Control		62.6 mg/kg/day			125 mg/kg/day			250 mg/kg/day			500 mg/kg/day			1,000 mg/kg/day		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
Male																	
1	253	10	246	97	10	252	100	10	252	99	10	256	101	10	253	100	10
8	276	10	275	100	10	270	98	10	273	99	10	274	99	10	260	94	10
15	296	10	301	102	10	292	99	10	293	99	10	291	99	10	267	90	10
22	316	10	327	104	10	310	98	10	313	99	10	307	97	10	278	88	10
29	331	10	340	103	10	326	99	10	327	99	10	319	96	10	287	87	10
Female																	
1	198	10	195	99	10	196	99	10	197	100	10	195	99	10	195	98	10
8	203	10	205	101	10	209	103	10	209	103	10	207	102	10	202	100	10
15	219	10	216	99	10	214	98	10	218	100	10	214	98	10	212	97	10
22	230	10	225	98	10	225	98	10	228	99	10	222	97	10	219	95	10
29	225	10	227	101	10	229	102	10	228	102	10	223	99	10	220	98	10

[a]One-half the dose was administered twice daily.

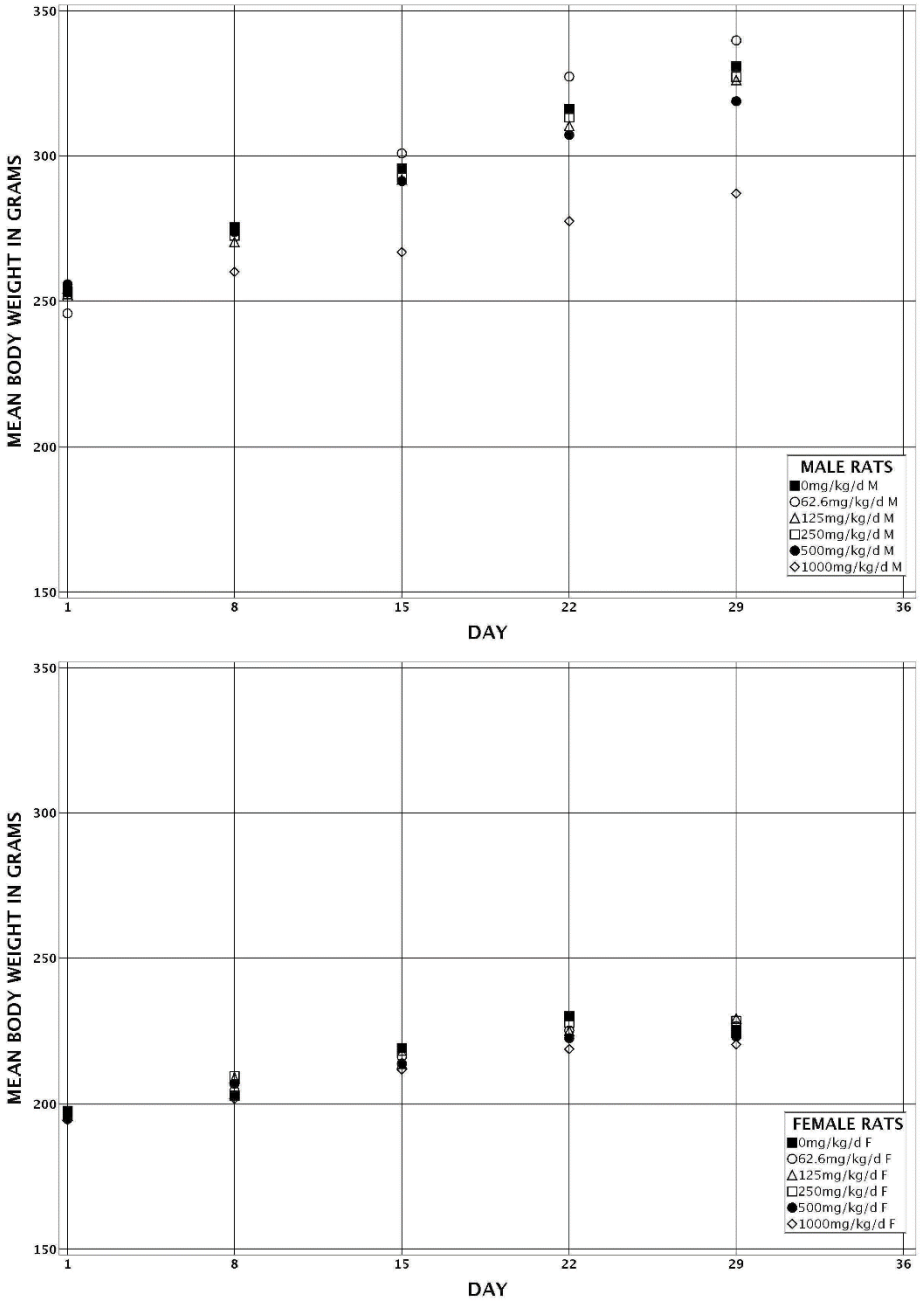


Figure 1. Growth Curves for Rats Administered Perfluorohexanoic Acid by Gavage for 28 Days

Table 5. Perfluorohexanoic Acid Concentrations in Tissues of Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Millimolar Dose (mmol/kg/day)	0	0.199	0.398	0.796	1.592	3.184
n	10	10	10	10	10	10
Male						
Plasma						
Plasma Concentration (ng/mL)	BD	378.2 ± 177.8	503.2 ± 66.2	1,296.8 ± 264.7	3,339.0 ± 497.2	10,898.6 ± 2,516.1
Plasma Concentration (µM)	BD	1.2 ± 0.6	1.6 ± 0.2	4.1 ± 0.8	10.6 ± 1.6	34.7 ± 8.0
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	6.0 ± 2.8	4.0 ± 0.5	5.2 ± 1.1	6.7 ± 1.0	10.9 ± 2.5
Liver						
Liver Concentration (ng/g)	BD	BD	BD	654.6 ± 148.0	1,552.1 ± 221.6	4,844.9 ± 1,055.8
Liver Concentration (µM)[b]	BD	BD	BD	2.1 ± 0.5	4.9 ± 0.7	15.4 ± 3.4
Normalized Liver Concentration (µM/mmol/kg/day)	NA	BD	BD	2.6 ± 0.6	3.1 ± 0.4	4.8 ± 1.1
Liver/Plasma Ratio	NA	BD	BD	0.52 ± 0.06	0.47 ± 0.01	0.44 ± 0.02
Female						
Plasma						
Plasma Concentration (ng/mL)	BD	128.9 ± 16.1	291.5 ± 57.6	475.4 ± 76.9	1,667.6 ± 373.2	6,712.0 ± 840.5
Plasma Concentration (µM)	BD	0.4 ± 0.1	0.9 ± 0.2	1.5 ± 0.2	5.3 ± 1.2	21.4 ± 2.7
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	2.1 ± 0.3	2.3 ± 0.5	1.9 ± 0.3	3.3 ± 0.7	6.7 ± 0.8

BD = below detection; group did not have over 20% of its values above the limit of quantification and no statistical analyses were conducted for the endpoint; NA = not applicable; value could not be calculated when dose value was 0.

[a]Tissue concentration data are presented as mean ± standard error. If over 20% of the values in a group were above the limit of quantification (plasma LOQ = 25 ng/mL; liver LOQ = 500 ng/g), then values that were below the limit of quantification were substituted with one-half the limit of quantification value. One-half the dose was administered twice daily.

[b]Density is assumed to be 1.0 g/mL.

Table 6. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
n	10	10	10	10	10	10
Male						
Necropsy body wt.	331 ± 5	340 ± 5	326 ± 9	327 ± 5	319 ± 7	287 ± 8**
R. Kidney						
Absolute	1.01 ± 0.01	1.03 ± 0.03	1.01 ± 0.04	1.02 ± 0.03	1.09 ± 0.04	1.04 ± 0.03
Relative	3.04 ± 0.04	3.04 ± 0.07	3.10 ± 0.03	3.11 ± 0.07	3.40 ± 0.09**	3.61 ± 0.04**
Liver						
Absolute	11.41 ± 0.28	12.63 ± 0.85	12.01 ± 0.48	12.88 ± 0.22	14.49 ± 0.56**	16.19 ± 0.60**
Relative	34.44 ± 0.41	37.26 ± 2.62	36.70 ± 0.50	39.35 ± 0.29**	45.33 ± 0.85**	56.35 ± 1.07**
Thymus						
Absolute	0.41 ± 0.03	0.46 ± 0.03	0.41 ± 0.03	0.42 ± 0.02	0.41 ± 0.02	0.30 ± 0.01**
Relative	1.25 ± 0.06	1.36 ± 0.09	1.24 ± 0.05	1.27 ± 0.07	1.28 ± 0.06	1.04 ± 0.02
Female						
Necropsy body wt.	225 ± 3	227 ± 2	229 ± 4	228 ± 5	223 ± 3	220 ± 4
R. Kidney						
Absolute	0.68 ± 0.02	0.67 ± 0.01	0.69 ± 0.01	0.69 ± 0.01	0.69 ± 0.02	0.74 ± 0.01**
Relative	3.02 ± 0.07	2.95 ± 0.05	3.01 ± 0.06	3.02 ± 0.06	3.10 ± 0.08	3.37 ± 0.05**
Liver						
Absolute	7.24 ± 0.15	7.40 ± 0.19	7.53 ± 0.19	7.84 ± 0.21	8.26 ± 0.20**	10.44 ± 0.29**
Relative	32.18 ± 0.74	32.63 ± 0.67	32.82 ± 0.46	34.37 ± 0.94	37.06 ± 0.73**	47.45 ± 0.99**

**Significantly different ($p \leq 0.01$) from the vehicle control group by Williams' or Dunnett's test.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error). One-half the dose was administered twice daily.

Dose-dependent significant decreases in the erythron—characterized by decreases in erythrocyte counts, hematocrit values, and hemoglobin concentrations—were seen in all male dose groups and in 250 mg/kg/day and greater females (Table 7). Reticulocytes were significantly increased in the most affected groups (i.e., high-dose groups), indicating a bone marrow response to the decreased erythron (regenerative response). Mean cell volumes were significantly increased in 250 mg/kg/day and greater males and 500 and 1,000 mg/kg/day females. Also, the mean cell hemoglobin concentrations were significantly decreased in 1,000 mg/kg/day males and females. Platelets were significantly increased in 500 mg/kg/day males and 1,000 mg/kg/day males and females; this is a common secondary finding to a highly regenerative bone marrow response. Blood smear findings in the high-dose groups included mild to moderate hypochromasia and anisocytosis, which were expected findings in light of the decreased erythron with regenerative response.

Table 7. Selected Hematology and Clinical Chemistry Data for Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Male						
n	10	10	9	10	10	10
Hematology						
Hematocrit (%)	51.9 ± 0.5	49.6 ± 0.7*	48.6 ± 0.7**	48.7 ± 0.6**	43.3 ± 0.9**	34.2 ± 1.8**
Manual Hematocrit (%)	47 ± 0	45 ± 1	44 ± 1*	44 ± 0**	39 ± 1**	33 ± 2**
Hemoglobin (g/dL)	15.5 ± 0.2	15.0 ± 0.2*	14.7 ± 0.2**	14.6 ± 0.2**	12.6 ± 0.3**	9.3 ± 0.6**
Erythrocytes (10^6/µL)	8.69 ± 0.08	8.28 ± 0.11*	8.24 ± 0.12**	7.91 ± 0.10**	6.68 ± 0.22**	4.56 ± 0.32**
Reticulocytes (10^3/µL)	207.9 ± 6.0	196.5 ± 8.7	176.3 ± 7.6	238.6 ± 9.6	523.7 ± 71.7**	947.7 ± 43.0**
Mean Cell Volume (fL)	59.7 ± 0.4	59.9 ± 0.4	59.0 ± 0.7	61.6 ± 0.5*	65.2 ± 1.1**	75.8 ± 1.9**
Mean Cell Hemoglobin Concentration (g/dL)	29.9 ± 0.1	30.2 ± 0.1	30.2 ± 0.1	30.0 ± 0.1	29.1 ± 0.3	27.0 ± 0.3**
Platelets (10^3/µL)	927 ± 26	913 ± 28	979 ± 40	939 ± 36	1,115 ± 44**	1,381 ± 69**
Clinical Chemistry						
Total Protein (g/dL)	6.5 ± 0.1	6.2 ± 0.1	6.1 ± 0.1**	6.1 ± 0.1**	5.9 ± 0.1**[b]	5.2 ± 0.1**
Albumin (g/dL)	4.0 ± 0.1	3.9 ± 0.1	3.8 ± 0.0*	3.9 ± 0.0	4.0 ± 0.0[b]	3.7 ± 0.1**
Globulin (g/dL)	2.5 ± 0.1	2.3 ± 0.1	2.3 ± 0.1*	2.1 ± 0.1**	1.9 ± 0.1**[b]	1.5 ± 0.1**
Albumin/Globulin Ratio	1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.9 ± 0.1**	2.1 ± 0.1**[b]	2.6 ± 0.2**
Cholesterol (mg/dL)	126 ± 4	101 ± 4**	105 ± 4**	105 ± 3**	103 ± 5**	98 ± 5**
Alanine Aminotransferase (IU/L)	48 ± 1[b]	50 ± 2	50 ± 2	52 ± 2	61 ± 1**	79 ± 3**
Alkaline Phosphatase (IU/L)	200 ± 8	191 ± 6	195 ± 6	203 ± 11	245 ± 10**	301 ± 19**[b]
Aspartate Aminotransferase (IU/L)	57 ± 2[b]	59 ± 2	58 ± 2[c]	61 ± 1	67 ± 2**	78 ± 3**
Sorbitol Dehydrogenase (IU/L)	3 ± 0[b]	4 ± 1	4 ± 1	4 ± 1	3 ± 1	5 ± 1*
Bile Salts/Acids (µmol/L)	11.1 ± 3.5	9.9 ± 2.6	8.4 ± 1.9	13.8 ± 3.188	16.8 ± 2.5	24.2 ± 2.6**
Testosterone (ng/mL)	3.82 ± 0.81	3.53 ± 0.53	3.63 ± 1.02	3.61 ± 0.46	3.56 ± 0.59	2.93 ± 0.71
Thyroid Stimulating Hormone (ng/mL)	23.08 ± 2.63	25.16 ± 3.38[b]	24.28 ± 3.04	24.47 ± 3.00	20.95 ± 2.85	18.29 ± 3.56
Triiodothyronine (ng/dL)	84.17 ± 5.25	68.88 ± 3.76*	62.16 ± 3.51**	71.59 ± 3.90*	71.03 ± 4.09*	60.07 ± 4.74**
Free Thyroxine (ng/dL)	2.88 ± 0.09	2.16 ± 0.17**	1.78 ± 0.12**	1.74 ± 0.09**	1.30 ± 0.07**	0.77 ± 0.10**
Total Thyroxine (µg/dL)	4.26 ± 0.15	3.40 ± 0.23**	2.93 ± 0.16**	2.90 ± 0.17**	2.37 ± 0.10**	1.77 ± 0.17**

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Female						
n	10	10	10	10	10	10
Hematology						
Hematocrit (%)	45.1 ± 0.5	44.8 ± 0.3	45.1 ± 0.4	42.1 ± 0.3**	41.7 ± 0.7**	37.6 ± 1.2**
Manual Hematocrit (%)	41 ± 1	41 ± 1	41 ± 0	38 ± 0**	38 ± 1**	35 ± 1**
Hemoglobin (g/dL)	14.0 ± 0.2	14.0 ± 0.1	13.9 ± 0.1	13.1 ± 0.1**	12.9 ± 0.2**	11.3 ± 0.4**
Erythrocytes (10^6/µL)	7.66 ± 0.09	7.61 ± 0.07	7.78 ± 0.11	7.10 ± 0.09**	6.88 ± 0.15**	5.64 ± 0.18**
Reticulocytes (10^3/µL)	177.9 ± 17.2	177.9 ± 11.5	173.7 ± 14.5	213.3 ± 17.5	335.8 ± 28.1**	574.2 ± 38.2**
Mean Cell Volume (fL)	58.8 ± 0.2	58.8 ± 0.5	57.9 ± 0.6	59.4 ± 0.6	60.7 ± 0.6*	66.6 ± 0.7**
Mean Cell Hemoglobin Concentration (g/dL)	31.2 ± 0.1	31.2 ± 0.1	30.9 ± 0.1	31.1 ± 0.1	31.0 ± 0.1	30.1 ± 0.1**
Platelets (10^3/µL)	976 ± 20	998 ± 39	1,049 ± 28	985 ± 21	1,021 ± 45	1,151 ± 55*
Clinical Chemistry						
Total Protein (g/dL)	6.4 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	6.3 ± 0.1	6.3 ± 0.1	5.9 ± 0.1**
Albumin (g/dL)	4.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	4.6 ± 0.1
Globulin (g/dL)	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.0	1.8 ± 0.1	1.4 ± 0.1**
Albumin/Globulin Ratio	2.4 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	3.3 ± 0.2**
Alanine Aminotransferase (IU/L)	40 ± 1	44 ± 2	46 ± 2	44 ± 3	54 ± 2**	57 ± 3**
Alkaline Phosphatase (IU/L)	138 ± 4	149 ± 8	163 ± 7	141 ± 5	147 ± 7	190 ± 10**
Aspartate Aminotransferase (IU/L)	56 ± 3	55 ± 2	56 ± 1	56 ± 1	62 ± 1**	66 ± 3**
Bile Salts/Acids (µmol/L)	10.1 ± 2.5	13.0 ± 3.3	10.9 ± 2.5	12.4 ± 2.6	11.8 ± 2.2	26.5 ± 3.3**
Thyroid Stimulating Hormone (ng/mL)	17.32 ± 1.40	14.75 ± 1.17	15.8 ± 0.90[b]	15.73 ± 1.06	24.18 ± 9.46	15.69 ± 0.77
Triiodothyronine (ng/dL)	98.19 ± 8.94	97.12 ± 6.10	92.57 ± 6.16	101.26 ± 7.50	111.58 ± 6.37	94.95 ± 7.91
Free Thyroxine (ng/dL)	1.97 ± 0.13	1.96 ± 0.13	1.89 ± 0.13	2.14 ± 0.19	1.90 ± 0.13	1.61 ± 0.15
Total Thyroxine (µg/dL)	3.62 ± 0.30	3.38 ± 0.23	3.210 ± 0.180	3.45 ± 0.28	3.30 ± 0.16	2.95 ± 0.21

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. One-half the dose was administered twice daily.
[b]n = 9.
[c]n = 10.

Significant, dose-dependent decreases in total protein and globulin concentrations were observed in 125 mg/kg/day and greater males compared to the vehicle control group; decreases in these two endpoints were also observed in the 1,000 mg/kg/day females (Table 7). Albumin concentrations decreased inconsistently in males. The overall effect of these changes resulted in significant increases in the albumin/globulin ratios in 250 mg/kg/day and greater males and in 1,000 mg/kg/day females. Cholesterol concentrations were significantly decreased in all male dose groups compared to the vehicle control group.

The enzymatic activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were significantly elevated in 500 and 1,000 mg/kg/day males compared to the vehicle control group; sorbitol dehydrogenase (SDH) activity and bile acid concentrations were also significantly elevated in the 1,000 mg/kg/day group (Table 7). In female rats, ALT and AST activities were significantly elevated at 500 and 1,000 mg/kg/day, and ALP activity and bile acid concentration were elevated at 1,000 mg/kg/day.

Total thyroxine (T4), free T4, and triiodothyronine (T3) concentrations were significantly decreased in all male dose groups (Table 7). There was no change in the female thyroid hormone concentrations.

Male and female rats administered PFHxA exhibited significant increases in gene expression for *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* compared to controls, suggesting significant increases in PPARα constitutive androstane receptor (CAR) activities (Table 8). Except for *Acox1* in males and females and *Cyp4a1* in females, the expression of genes of interest were increased compared to controls in all dose groups examined. Females exhibited a greater fold increase in CAR-related genes (*Cyp2b1* and *Cyp2b2*) compared to controls than did males compared to controls. Acyl-CoA oxidase activity was increased compared to controls in the 250, 500, and 1,000 mg/kg/day males.

Table 8. Hepatic Parameters for Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Male						
n	10	10	10	10	10	10
Enzyme Activity						
Acyl-CoA Oxidase (nmol/min/mg)	2.204 ± 0.073	2.166 ± 0.092	2.596 ± 0.155	5.303 ± 0.174**	8.436 ± 0.367**	16.080 ± 0.797**
Gene Expression						
Acox1	1.02 ± 0.06	1.09 ± 0.05	1.09 ± 0.06	1.22 ± 0.08	1.53 ± 0.15**	2.01 ± 0.16**
Cyp4a1	1.03 ± 0.09	2.81 ± 0.33**	4.76 ± 0.33**	6.81 ± 0.66**	10.08 ± 1.15**	12.54 ± 1.12**
Cyp2b1	1.29 ± 0.35	2.65 ± 0.32**	2.88 ± 0.39**	3.71 ± 0.51**	7.41 ± 1.63**	7.01 ± 1.54**
Cyp2b2	1.16 ± 0.20	2.22 ± 0.25**	2.64 ± 0.30**	2.79 ± 0.25**	3.29 ± 0.25**	3.01 ± 0.29**

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Female						
n	10	10	10	10	9	10
Gene Expression						
Acox1	1.01 ± 0.06	1.11 ± 0.17	1.04 ± 0.09	1.19 ± 0.10	1.54 ± 0.17**	1.58 ± 0.11**
Cyp4a1	1.04 ± 0.09	1.10 ± 0.20	1.00 ± 0.12	1.17 ± 0.09	1.84 ± 0.32*	2.58 ± 0.23**
Cyp2b1	1.83 ± 0.62	4.66 ± 1.22*	9.95 ± 3.36*	23.28 ± 4.99**	53.48 ± 8.17**	124.02 ± 29.12**
Cyp2b2	1.95 ± 0.72	6.32 ± 1.32*	13.06 ± 3.61**	29.78 ± 4.62**	68.07 ± 7.23**	81.98 ± 8.60**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's or Dunn's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values. One-half the dose was administered twice daily.

Cauda epididymal sperm counts in male rats administered 1,000 mg/kg/day were significantly lower (25%) than vehicle controls and occurred in the presence of a slight decrease in epididymal weight (5%) (

Table 9). When normalized to total sperm count/g of cauda epididymis, the counts/g were 18% lower than vehicle controls. An epididymal histopathologic finding (exfoliated germ cells) was noted in one rat administered 1,000 mg/kg/day (Table 11). Testis weights and spermatid counts in the PFHxA-treated rats were similar to vehicle controls. Seminiferous tubule spermatid retention of the testis was observed in two rats administered 1,000 mg/kg/day and in one vehicle control rat (Table 11). Males administered PFHxA had testosterone levels similar to those of the vehicle controls (Table 7).

Table 9. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

	Vehicle Control	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
n	10	10	10	10
Weights (g)				
Necropsy Body Wt.	331 ± 5	327 ± 5	319 ± 7	287 ± 8**
L. Cauda Epididymis	0.190 ± 0.005	0.195 ± 0.005	0.186 ± 0.005	0.176 ± 0.005
L. Epididymis	0.527 ± 0.010	0.523 ± 0.013	0.524 ± 0.011	0.496 ± 0.014
L. Testis	1.814 ± 0.035	1.821 ± 0.028	1.851 ± 0.057	1.798 ± 0.043
Spermatid Measurements				
Spermatid Heads (10^6/testis)	377.0 ± 7.9	356.5 ± 11.0	376.0 ± 11.6	352.3 ± 12.4
Spermatid Heads (10^6/g testis)	208.3 ± 4.8	195.9 ± 5.4	203.9 ± 6.1	196.0 ± 5.0
Epididymal Spermatozoal Measurements				
Sperm Motility (%)	87.9 ± 0.4	87.8 ± 0.3	87.2 ± 0.4	87.4 ± 0.4
Sperm (10^6/cauda epididymis)	111.9 ± 8.4	102.5 ± 5.2	105.1 ± 4.3	83.7 ± 4.7**
Sperm (10^6/g cauda epididymis)	586.4 ± 34.9	526.7 ± 25.8	566.4 ± 20.2	478.3 ± 28.2

**Significantly different ($p \leq 0.01$) from the vehicle control group by Williams' or Dunnett's test (weights) or Shirley's or Dunn's test (epididymal spermatozoal measurements).

[a]Data are presented as mean ± standard error. One-half the dose was administered twice daily.

Inspection of the daily vaginal cytology data indicated that the vehicle control rats did not cycle as expected (e.g., mean length of 7.2 days versus normal length of ~4.5 days; the vehicle control females showed disproportionately more time spent in diestrus and less time in estrus than expected); the PFHxA-treated females cycled as would generally be expected (Table 10). Given this limitation, the summary statistics and Markov analyses are not used for drawing conclusions (Table A-1; Figure A-1). There were no apparent PFHxA-related changes in female testosterone levels.

Table 10. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

	Vehicle Control	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day
Number Weighed at Necropsy	10	10	10	10
Necropsy Body Wt. (g)	225 ± 3	229 ± 4	228 ± 5	223 ± 3
Proportion of Regular Cycling Females[b]	6/9	9/10	10/10	10/10
Estrous Cycle Length (days)	7.2 ± 1.45	5.0 ± 0.12	5.0 ± 0.13	4.8 ± 0.20
Estrous Stages (% of cycle)				
Diestrus	61.8	53.1	55.0	52.5
Proestrus	3.5	8.1	4.4	6.9
Estrus	29.2	36.9	37.5	36.9
Metestrus	2.8	0.6	2.5	3.8
Uncertain	2.8	1.3	0.6	0.0

[a]Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated that 125, 250, and 500 mg/kg/day females had extended estrus and 125 and 250 mg/kg/day females had extended diestrus. One-half the dose was administered twice daily.
[b]Number of females with a regular cycle/number of females cycling.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following the Wyeth-14,643 results.Histopathologic_Descriptions

Liver: The incidences of hepatocyte cytoplasmic alteration in 1,000 mg/kg/day males and females were significantly increased compared to those of the vehicle control groups (Table 11). The incidences of hepatocyte hypertrophy were significantly increased in 500 mg/kg/day males and 1,000 mg/kg/day males and females. Hepatocyte cytoplasmic alteration and hepatocyte hypertrophy were generally of minimal to mild severity.

Nose: The incidences of olfactory epithelium degeneration were significantly increased in males and females administered 250 mg/kg/day or greater compared to the vehicle controls (Table 11). The incidences of olfactory epithelium hyperplasia were significantly increased in males administered 250 mg/kg/day or greater and females administered 250 or 500 mg/kg/day. The incidences of olfactory epithelium suppurative inflammation were significantly increased in

1,000 mg/kg/day males and in females administered 500 or 1,000 mg/kg/day. The olfactory epithelial lesions ranged from minimal to moderate severity.

Spleen: The incidences of increased extramedullary hematopoiesis were significantly increased in 500 mg/kg/day males and 1,000 mg/kg/day males and females compared to the vehicle control groups (Table 11). Increased extramedullary hematopoiesis was generally of minimal to mild severity.

Kidney: The incidence of minimal chronic progressive nephropathy in 1,000 mg/kg/day females was significantly increased compared to the vehicle control group (Table 11).

Table 11. Incidences of Selected Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Perfluorohexanoic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Male						
Liver[b]	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration[c]	0	0	0	0	2 (1.5)[d]	10** (1.8)
Hepatocyte, Hypertrophy	0	0	0	0	9** (1.2)	10** (1.8)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory Epithelium, Degeneration	0	0	1 (1.0)	6** (1.0)	6** (1.7)	6** (2.7)
Olfactory Epithelium, Hyperplasia	0	0	0	6** (1.0)	5* (1.0)	6** (1.2)
Olfactory Epithelium, Inflammation, Suppurative	0	0	0	0	3 (1.0)	6** (2.2)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Increased Extramedullary Hematopoiesis	0	0	0	0	6** (1.3)	10** (1.6)
Epididymis	(10)	(0)	(0)	(0)	(10)	(10)
Duct, Exfoliated Germ Cell	0	0	0	0	0	1 (1.0)
Testes	(10)	(10)	(10)	(10)	(10)	(10)
Seminiferous Tubule, Spermatid Retention	1 (1.0)	0	0	0	0	2 (1.5)
Female						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	0	0	0	0	9** (1.1)
Hepatocyte, Hypertrophy	0	0	0	0	0	9** (1.1)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory Epithelium, Degeneration	0	1 (1.0)	3 (1.0)	9** (1.1)	9** (1.7)	6** (2.5)
Olfactory Epithelium, Hyperplasia	0	0	3 (1.0)	4* (1.0)	7** (1.0)	3 (1.0)
Olfactory Epithelium, Inflammation, Suppurative	0	0	0	1 (1.0)	5* (1.0)	8** (1.5)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Increased Extramedullary Hematopoiesis	0	0	0	0	2 (1.0)	9** (1.4)
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Nephropathy, Chronic Progressive	2 (1.0)	4 (1.0)	4 (1.0)	1 (1.0)	3 (1.0)	8* (1.1)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]One-half the dose was administered twice daily.
[b]Number of animals with tissue examined microscopically.
[c]Number of animals with lesion.
[d]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Perfluorooctanoic Acid (PFOA)

All rats survived to the end of the study with the exception of one female in the 100 mg/kg/day group (Table 12). The one female that died was ataxic, lethargic, and appeared thin immediately prior to death. There were no significant treatment-related clinical observations in male or female rats. The mean body weights of 0.625, 1.25, and 2.5 mg/kg/day males and all treated females were within 10% of the respective vehicle control groups throughout the study (Table 12; Figure 2). Mean body weights of the 5 and 10 mg/kg/day males were 12% to 19% lower, respectively, than those of the vehicle control group by the end of the study.

Plasma concentrations of PFOA increased with increasing dose in both males and females (Table 13). Although females were administered a 10-fold higher dose of PFOA, males had a higher plasma concentration compared to females across the dose groups. When normalized to dose administered (µM/mmol/kg), males had a 1,000-fold higher level at the lowest dose compared, but this difference decreased with increasing dose. The difference at the highest dose was 63-fold. Males exhibited a decreasing normalized plasma concentration with dose, whereas females exhibited the opposite response.

Liver concentrations of PFOA were measured in males only and increased with dose, but when normalized to dose administered, these values (µM/mol/kg) decreased with increasing dose (Table 13). The liver/plasma ratios ranged from 0.87 to 1.08.

There were dose-related increases in the absolute and relative liver weights in male and female rats (Table 14). The absolute and relative liver weights of all male dose groups were significantly greater than those of the vehicle control group; the absolute and relative liver weights of 25, 50, and 100 mg/kg/day females were significantly greater than those of the vehicle control group. The mean absolute and relative kidney weights of males administered 1.25, 2.5, or 5 mg/kg/day and the mean relative kidney weights of males administered 0.625 or 10 mg/kg/day were significantly greater than those of the vehicle controls. The mean relative kidney weights of 50 mg/kg/day females and the absolute and relative kidney weights of 100 mg/kg/day females were significantly greater than those of the vehicle control group. The mean absolute spleen weights of males administered 2.5 mg/kg/day or greater were significantly less than those of the vehicle control group. Absolute thymus weights were decreased in 10 mg/kg/day males, and relative thyroid gland weights were increased in male groups administered 1.25 mg/kg/day and greater. Absolute thyroid weight was only significantly increased in the 2.5 mg/kg/day group.

Table 12. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Perfluorooctanoic Acid

Day	Vehicle Control		0.625 mg/kg/day			1.25 mg/kg/day			2.5 mg/kg/day			5 mg/kg/day			10 mg/kg/day		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
Male																	
1	257	10	254	99	10	255	99	10	258	101	10	254	99	10	255	99	10
8	286	10	283	99	10	284	99	10	284	99	10	276	97	10	261	91	10
15	309	10	310	100	10	307	99	10	296	96	10	279	90	10	268	87	10
22	329	10	327	100	10	320	97	10	305	93	10	292	89	10	270	82	10
29	347	10	344	99	10	331	95	10	317	91	10	304	88	10	280	81	10

Day	Vehicle Control		6.25 mg/kg/day			12.5 mg/kg/day			25 mg/kg/day			50 mg/kg/day			100 mg/kg/day		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
Female																	
1	194	10	194	100	10	193	99	10	195	100	10	196	101	10	193	99	10
8	205	10	205	100	10	202	99	10	200	98	10	202	99	10	196	96	9
15	218	10	213	98	10	213	98	10	210	96	10	211	97	10	208	96	9
22	222	10	219	99	10	216	97	10	214	96	10	214	96	10	207	93	9
29	227	10	226	100	10	226	100	10	219	97	10	221	98	10	215	95	9

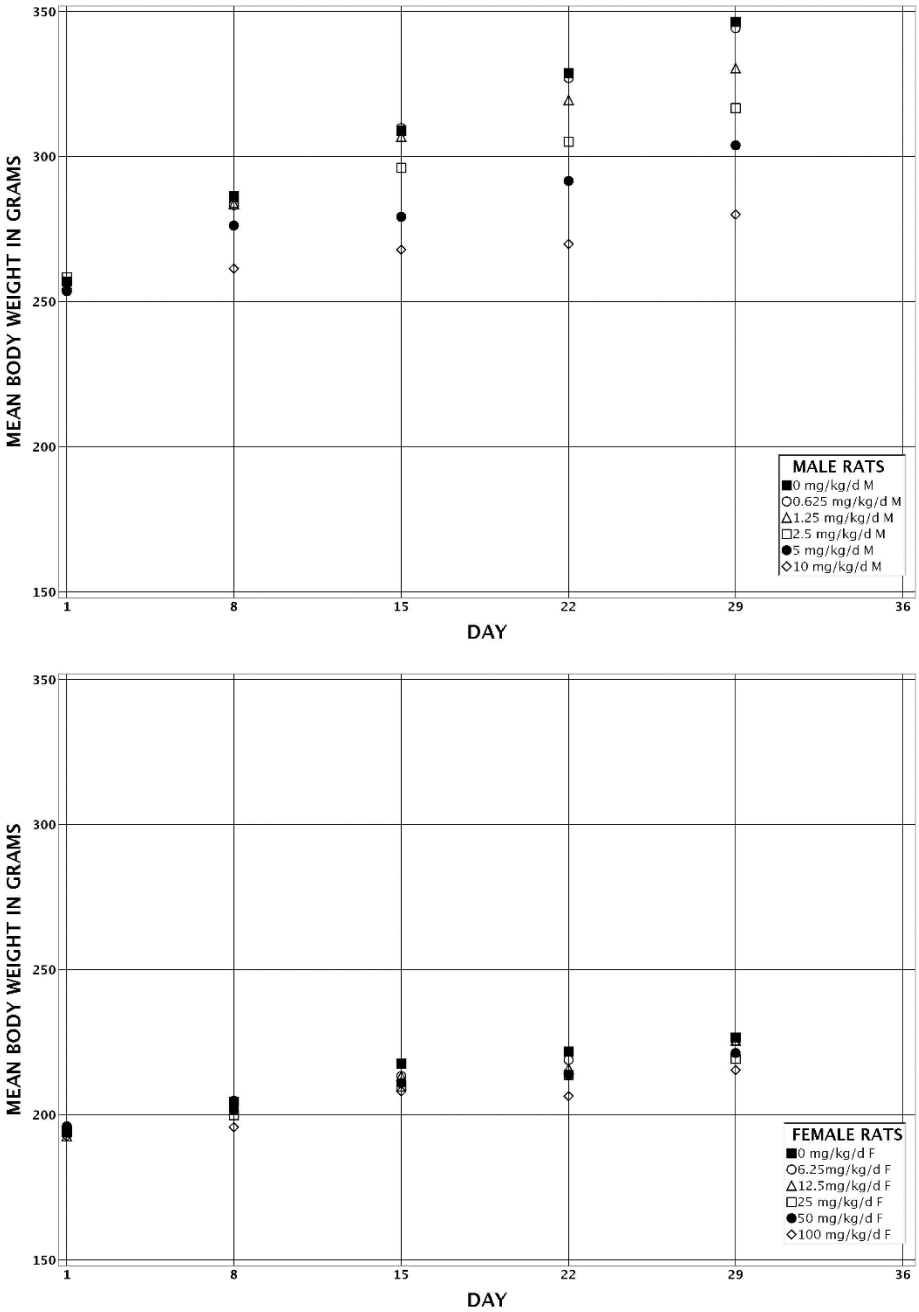


Figure 2. Growth Curves for Rats Administered Perfluorooctanoic Acid by Gavage for 28 Days

Table 13. Perfluorooctanoic Acid Concentrations in Tissues of Rats in the 28-day Gavage Study of Perfluorooctanoic Acid[a]

	Vehicle Control	**0.625 mg/kg/day**	**1.25 mg/kg/day**	**2.5 mg/kg/day**	**5 mg/kg/day**	**10 mg/kg/day**
Millimolar Dose (mmol/kg/day)	0	0.0015	0.003	0.006	0.0121	0.0242
Male						
n	10	10	10	10	10	10
Plasma						
Plasma Concentration (ng/mL)	97.6 ± 6.0	50,690.0 ± 2,207.2**	73,480.0 ± 3,205.9**	95,430.0 ± 4,036.0**	110,720.0 ± 3,891.2**	148,570.0 ± 15,405.4**
Plasma Concentration (µM)	0.2 ± 0.0	122.4 ± 5.3**	177.5 ± 7.7**	230.5 ± 9.7**	267.4 ± 9.4**	358.8 ± 37.2**
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	81,104.0 ± 3,531.6	58,784.0 ± 2,564.7	38,172.0 ± 1,614.4	22,144.0 ± 778.2	14,857.0 ± 1,540.5
Liver						
Liver Concentration (ng/mL)	BD	54,610.0 ± 2,232.5	85,220.0 ± 3,185.5	110,740.0 ± 4,466.8	109,030.0 ± 3,557.2	124,470.0 ± 9,250.6
Liver Concentration (µM)[b]	BD	131.9 ± 5.4	205.8 ± 7.7	267.4 ± 10.8	263.3 ± 8.6	300.6 ± 22.3
Normalized Liver Concentration (µM/mmol/kg/day)	NA	87,376.0 ± 3,572.0	68,176.0 ± 2,548.4	44,296.0 ± 1,786.7	21,806.0 ± 711.4	12,447.0 ± 925.1
Liver/Plasma Ratio	NA	1.08 ± 0.02	1.16 ± 0.02	1.17 ± 0.03	0.99 ± 0.03	0.87 ± 0.05
	Vehicle Control	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**	**100 mg/kg/day**
Millimolar Dose (mmol/kg/day)	0	0.0151	0.0302	0.0604	0.121	0.242
Female						
n	10	10	10	10	10	9
Plasma						
Plasma Concentration (ng/mL)	BD	490.5 ± 72.1	1,152.8 ± 187.2	2,960.1 ± 480.7	9,326.0 ± 1,821.4	23,444.4 ± 3,247.2
Plasma Concentration (µM)	BD	1.2 ± 0.2	2.8 ± 0.5	7.1 ± 1.2	22.5 ± 4.4	56.6 ± 7.8
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	78.5 ± 11.5	92.2 ± 15.0	118.4 ± 19.2	186.5 ± 36.4	234.4 ± 32.5

BD=below detection; group did not have over 20% of its values above the limit of quantification and no statistical analyses were conducted for the endpoint. NA=not applicable; value could not be calculated when dose value was 0.

[a]Tissue concentration data are presented as mean ± standard error. If over 20% of the values in a group were above the limit of quantification (plasma LOQ = 25 ng/mL; liver LOQ = 500 ng/g), values that were below the limit of quantification were substituted with one-half the limit of quantification value. One-half the dose was administered twice daily.

[b]Density is assumed to be 1.0 g/mL.

Table 14. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Perfluorooctanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
Male						
n	10	10	10	10	10	10
Necropsy Body Wt.	347 ± 5	344 ± 7	331 ± 4	317 ± 7**	304 ± 8**	280 ± 10**
R. Kidney						
Absolute	0.97 ± 0.03	1.07 ± 0.02	1.13 ± 0.03**	1.14 ± 0.03**	1.09 ± 0.02*	1.06 ± 0.03
Relative	2.80 ± 0.06	3.10 ± 0.02**	3.41 ± 0.06**	3.59 ± 0.06**	3.59 ± 0.06**	3.81 ± 0.10**
Liver						
Absolute	12.96 ± 0.41	14.94 ± 0.32**	15.80 ± 0.29**	16.44 ± 0.64**	16.70 ± 0.46**	17.22 ± 0.38**
Relative	37.34 ± 0.72	43.41 ± 0.55**	47.80 ± 0.54**	51.75 ± 1.09**	55.01 ± 0.89**	62.05 ± 2.23**
Spleen						
Absolute	0.704 ± 0.020	0.656 ± 0.037	0.645 ± 0.020	0.619 ± 0.040*	0.581 ± 0.022**	0.533 ± 0.024**
Relative	2.03 ± 0.04	1.90 ± 0.08	1.95 ± 0.06	1.94 ± 0.09	1.91 ± 0.05	1.90 ± 0.04
Thymus						
Absolute	0.429 ± 0.022	0.419 ± 0.015	0.420 ± 0.019	0.415 ± 0.031	0.385 ± 0.031	0.318 ± 0.035*
Relative	1.24 ± 0.06	1.21 ± 0.03	1.27 ± 0.06	1.30 ± 0.08	1.26 ± 0.08	1.11 ± 0.10
Thyroid Gland						
Absolute	0.0209 ± 0.0010	0.0231 ± 0.0013	0.0245 ± 0.0017	0.0274 ± 0.0016**	0.0208 ± 0.0010	0.0214 ± 0.0014
Relative	0.06 ± 0.00	0.07 ± 0.00	0.07 ± 0.01**	0.09 ± 0.00**	0.07 ± 0.00**	0.08 ± 0.00**
	Vehicle Control	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**	**100 mg/kg/day**
Female						
n	10	10	10	10	10	9
Necropsy Body Wt.	227 ± 5	226 ± 3	226 ± 4	219 ± 2	221 ± 3	215 ± 4
R. Kidney						
Absolute	0.71 ± 0.03	0.71 ± 0.02	0.70 ± 0.01	0.72 ± 0.01	0.74 ± 0.01	0.79 ± 0.02**
Relative	3.12 ± 0.10	3.13 ± 0.06	3.09 ± 0.05	3.31 ± 0.07	3.35 ± 0.05*	3.66 ± 0.10**
Liver						
Absolute	7.77 ± 0.34	8.13 ± 0.19	8.21 ± 0.20	8.58 ± 0.14*	9.79 ± 0.27**	12.07 ± 0.40**
Relative	34.20 ± 0.98	36.06 ± 0.65	36.44 ± 0.58	39.12 ± 0.54**	44.22 ± 0.88**	56.00 ± 1.09**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

Erythrocyte count, hematocrit, manual hematocrit, and hemoglobin concentrations were significantly decreased in 1.25 mg/kg/day and higher males compared to the vehicle control group (Table 15). The reticulocyte count was significantly decreased in all male dose groups compared to the vehicle control group. The combination of these changes resulted in a small but significant decrease in the mean cell volume in the 10 mg/kg/day males. Significant decreases in the female rat erythron were observed, most consistently starting at 12.5 mg/kg/day, characterized by decreases in the hematocrit, hemoglobin concentrations, and erythrocyte counts compared to the vehicle control group.

In general, urea nitrogen concentrations were significantly increased and creatinine concentrations significantly decreased in 1.25 mg/kg/day and higher males compared to the vehicle control group (Table 15). Urea nitrogen concentration was significantly increased in 100 mg/kg/day females. Glucose concentrations were decreased in 2.5 mg/kg/day and greater males compared to the vehicle control group. The decreases in creatinine and glucose concentrations in male rats were likely related to the decreased food intake and body weight. In male rats, cholesterol and triglyceride concentrations were decreased in most dose groups compared to the vehicle control group. Cholesterol and triglyceride concentrations were increased in 50 and 100 mg/kg/day females compared to the vehicle control group.

Total protein and globulin concentrations were decreased in all male dose groups compared to the vehicle control group, which resulted in significant increases in the albumin/globulin ratio in all male dose groups (Table 15). Globulin concentrations were significantly decreased in 12.5 mg/kg/day and greater females and albumin concentrations significantly increased in 25 mg/kg/day and greater females; these changes resulted in increases in the albumin/globulin ratios in the 12.5 mg/kg/day and greater females.

ALT and ALP activities were significantly increased in all male dose groups, and AST activity was increased in 2.5 mg/kg/day and greater males compared to the vehicle control group (Table 15). Bile acid concentrations were increased in 5 and 10 mg/kg/day males, and the direct bilirubin concentration was increased in 10 mg/kg/day males compared to the vehicle control group. Creatine kinase activity was significantly decreased in 2.5 mg/kg/day and greater males. ALT activity was significantly increased in 50 and 100 mg/kg/day females, and ALP activity was significantly increased in all female dose groups compared to the vehicle control group.

T4, free T4, and T3 concentrations were significantly decreased in all male dose groups (except T3 at 10 mg/kg/day) compared to the vehicle control group, with thyroid stimulating hormone (TSH) being significantly decreased in 5 and 10 mg/kg/day males (Table 15). TSH concentrations were significantly increased in all female dose groups compared to the vehicle control group.

Table 15. Selected Hematology and Clinical Chemistry Data for Rats in the 28-day Gavage Study of Perfluorooctanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
Male						
n	10	10	10	10	10	10
Hematology						
Hematocrit (%)	53.3 ± 0.6	52.1 ± 0.6	50.5 ± 0.5**	50.9 ± 0.5**	49.3 ± 0.6**	48.3 ± 0.5**
Manual Hematocrit (%)	48 ± 1	47 ± 1	46 ± 0*	46 ± 0*	45 ± 1**	44 ± 0**
Hemoglobin (g/dL)	16.0 ± 0.1	15.6 ± 0.1	15.2 ± 0.2**	15.3 ± 0.1**	14.9 ± 0.2**	14.5 ± 0.1**
Erythrocytes (10^6/µL)	8.80 ± 0.13	8.50 ± 0.10	8.27 ± 0.09**	8.51 ± 0.12*	8.28 ± 0.13*	8.28 ± 0.08**
Reticulocytes (10^3/µL)	188.8 ± 8.2	161.6 ± 2.9**	148.7 ± 6.8**	144.5 ± 7.3**	161.6 ± 11.1**	131.0 ± 10.9**
Mean Cell Volume (fL)	60.6 ± 0.5	61.3 ± 0.3	61.2 ± 0.4	59.9 ± 0.5	59.6 ± 0.4	58.3 ± 0.6**
Clinical Chemistry						
Urea Nitrogen (mg/dL)	16.6 ± 0.4	15.9 ± 0.7	20.3 ± 0.5**	19.6 ± 1.0**	18.1 ± 0.9*	20.1 ± 1.2*
Creatinine (mg/dL)	0.77 ± 0.04	0.70 ± 0.00*	0.69 ± 0.01**	0.70 ± 0.01*	0.65 ± 0.02**	0.65 ± 0.02**
Glucose (mg/dL)	196 ± 8	183 ± 9	187 ± 8	168 ± 8*	156 ± 4**	141 ± 5**
Total Protein (g/dL)	6.6 ± 0.1	6.0 ± 0.1**	5.9 ± 0.1**	6.0 ± 0.1**	5.8 ± 0.1**	5.8 ± 0.2**
Albumin (g/dL)	4.3 ± 0.0	4.3 ± 0.1	4.4 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.1
Globulin (g/dL)	2.3 ± 0.1	1.7 ± 0.1**	1.6 ± 0.1**	1.5 ± 0.0**	1.3 ± 0.1**	1.4 ± 0.1**
Albumin/Globulin Ratio	1.9 ± 0.0	2.5 ± 0.1**	2.8 ± 0.1**	3.0 ± 0.1**	3.4 ± 0.1**	3.3 ± 0.1**
Direct Bilirubin (mg/dL)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00*
Cholesterol (mg/dL)	114 ± 6	72 ± 2**	79 ± 4**	76 ± 3**	80 ± 5**	100 ± 5
Triglycerides (mg/dL)	138 ± 12	101 ± 8*	83 ± 7**	82 ± 6**	82 ± 5**	78 ± 5**
Alanine Aminotransferase (IU/L)	57 ± 3	68 ± 3*	65 ± 3*	73 ± 5**	70 ± 2**	84 ± 11**
Alkaline Phosphatase (IU/L)	207 ± 9	233 ± 8*	238 ± 12*	316 ± 11**	356 ± 12**	392 ± 40**
Aspartate Aminotransferase (IU/L)	67 ± 3	71 ± 2	68 ± 2	82 ± 5**	74 ± 3*	93 ± 14**
Bile Salts/Acids (µmol/L)	12.3 ± 2.8	9.6 ± 2.4	22.5 ± 3.5	22.0 ± 3.6	28.0 ± 3.6**	55.4 ± 16.1**
Testosterone (ng/mL)	4.03 ± 1.45	4.59 ± 1.14	4.99 ± 1.14	2.79 ± 0.67	4.79 ± 1.49	3.12 ± 1.21
Thyroid Stimulating Hormone (ng/mL)	21.76 ± 2.66	23.74 ± 2.34	15.50 ± 1.94	15.85 ± 2.43	12.63 ± 1.20**	15.07 ± 2.04*
Triiodothyronine (ng/dL)	88.55 ± 5.58	53.52 ± 1.45**	56.93 ± 2.48**	61.47 ± 3.64*	58.98 ± 3.52**	85.33 ± 6.72
Free Thyroxine (ng/dL)	2.14 ± 0.13	0.44 ± 0.04**	0.36 ± 0.02**	0.32 ± 0.01**	0.34 ± 0.02**	0.33 ± 0.02**
Total Thyroxine (µg/dL)	2.34 ± 0.24	0.21 ± 0.05**	0.17 ± 0.06**	0.07 ± 0.05**	0.11 ± 0.06**	0.39 ± 0.15**

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Female						
n	10	10	10	10	10	9
Hematology						
Hematocrit (%)	47.2 ± 0.5	45.8 ± 0.3*	45.7 ± 0.3*	45.4 ± 0.4**	45.8 ± 0.5*	43.2 ± 0.6**
Manual Hematocrit (%)	42 ± 1	42 ± 1	41 ± 0	41 ± 1	42 ± 1	40 ± 1*
Hemoglobin (g/dL)	14.4 ± 0.1	14.0 ± 0.1	14.0 ± 0.1*	13.9 ± 0.1**	13.9 ± 0.1**	13.1 ± 0.2**
Erythrocytes (10^6/μL)	7.78 ± 0.08	7.63 ± 0.10	7.67 ± 0.07	7.53 ± 0.07	7.63 ± 0.10	7.20 ± 0.11**
Clinical Chemistry						
Urea Nitrogen (mg/dL)	16.5 ± 0.9	17.3 ± 0.6	16.5 ± 0.5	18.4 ± 0.6	19.7 ± 1.3	23.3 ± 2.1**
Albumin (g/dL)	4.5 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	4.8 ± 0.1**	5.0 ± 0.1**	5.0 ± 0.1**
Globulin (g/dL)	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1*	1.7 ± 0.0**	1.7 ± 0.1*	1.5 ± 0.1**
Albumin/Globulin Ratio	2.4 ± 0.1	2.6 ± 0.1	2.8 ± 0.1**	2.9 ± 0.1**	3.1 ± 0.1**	3.5 ± 0.1**
Cholesterol (mg/dL)	96 ± 5	108 ± 5	96 ± 5	100 ± 3	112 ± 3*	114 ± 3**
Triglycerides (mg/dL)	66 ± 7	61 ± 3	74 ± 6	74 ± 6	84 ± 7*	106 ± 10**
Alanine Aminotransferase (IU/L)	43 ± 3	47 ± 2	46 ± 2	47 ± 2	48 ± 1*	52 ± 2**
Alkaline Phosphatase (IU/L)	132 ± 8	154 ± 7*	161 ± 8**	143 ± 4*	148 ± 7*	176 ± 6**
Thyroid Stimulating Hormone (ng/mL)	10.05 ± 0.81	14.08 ± 1.17**	12.99 ± 1.27*	14.00 ± 1.49*[b]	17.81 ± 1.72**	15.83 ± 2.20**[c]
Triiodothyronine (ng/dL)	109.10 ± 6.72	115.25 ± 6.41	94.87 ± 5.94	116.46 ± 6.33	114.41 ± 5.31	104.70 ± 4.14
Free Thyroxine (ng/dL)	1.63 ± 0.18	1.95 ± 0.15	1.48 ± 0.11	1.56 ± 0.2.0[b]	1.51 ± 0.13	1.13 ± 0.14*
Total Thyroxine (μg/dL)	2.10 ± 0.36	2.43 ± 0.28	1.56 ± 0.24	2.03 ± 0.33	1.56 ± 0.23	1.11 ± 0.24*

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error.
[b]n = 9.
[c]n = 8.

Male and female rats administered PFOA exhibited significant increases in gene expression of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* compared to controls, suggesting increases in PPARα and CAR activities (Table 16). Of the four genes evaluated, female rats displayed the greatest fold increase for the CAR-related genes *Cyp2b1* and *Cyp2b2* compared to respective controls. Males exhibited the greatest fold increase for *Cyp4a1* and *Cyp2b1* compared to respective controls. Acyl-CoA oxidase activity was increased in all male dose groups.

Table 16. Hepatic Parameters for Rats in the 28-day Gavage Study of Perfluorooctanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
Male						
n	10	10	10	10	10	9
Enzyme Activity						
Acetyl-CoA (nmol/min/mg)	1.843 ± 0.060	5.797 ± 0.362**	10.489 ± 0.535**	12.701 ± 0.456**	14.970 ± 0.568**	16.010 ± 0.670**[b]
Gene Expression						
Acox1	1.09 ± 0.15	3.78 ± 0.41**	5.69 ± 0.35**	6.20 ± 0.53**	5.01 ± 0.28**	5.94 ± 0.63**
Cyp4a1	1.07 ± 0.13	22.17 ± 2.17**	32.54 ± 2.10**	29.08 ± 1.99**	22.21 ± 1.26**	25.31 ± 2.68**
Cyp2b1	1.26 ± 0.27	5.75 ± 1.39**	11.81 ± 2.21**	10.38 ± 2.33**	10.45 ± 2.19**	22.11 ± 7.13**
Cyp2b2	1.09 ± 0.15	3.88 ± 0.50**	4.94 ± 0.40**	4.14 ± 0.31**	3.16 ± 0.27	4.33 ± 0.52**
	Vehicle Control	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**	**100 mg/kg/day**
Female						
n	10	10	10	10	10	9
Gene Expression						
Acox1	1.02 ± 0.06	1.03 ± 0.05	1.29 ± 0.08*	1.66 ± 0.16**	2.69 ± 0.25**	3.94 ± 0.28**
Cyp4a1	1.03 ± 0.08	1.13 ± 0.08	1.21 ± 0.09	1.41 ± 0.14*	3.19 ± 0.44**	7.81 ± 0.78**
Cyp2b1	1.29 ± 0.21	6.18 ± 1.76*	9.58 ± 1.90**	18.64 ± 5.59**	39.99 ± 5.85**	91.67 ± 15.04**
Cyp2b2	1.42 ± 0.35	5.87 ± 1.68*	12.40 ± 2.53**	16.56 ± 3.94**	34.57 ± 5.07**	47.83 ± 2.59**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's or Dunn's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values.

Male rats assessed for reproductive toxicity were administered 0, 2.5, 5, or 10 mg PFOA/kg/day for 28 days, which is much less than the time needed to make a full assessment of the effect on spermatogenesis. Epididymal weights were lower in the rats administered 5 or 10 mg/kg/day compared to the vehicle control group (6% and 9% respectively; statistically significant at 10 mg/kg); caudal epididymal weight was also lower in these groups (11%; both statistically significant) (Table 17). Cauda epididymal sperm count in the 10 mg/kg/day group was lower compared to the vehicle control group (24%; statistically significant). When normalized to total sperm count/g cauda epididymis, the count in the 10 mg/kg/day group was 15% lower compared to the vehicle control group. Histopathologic findings in the epididymis were noted in one rat each in the 5 and 10 mg/kg/day groups (hypospermia, exfoliated germ cells) (Table 18). Testis weights and spermatid counts in the PFOA-dosed animals were similar to the vehicle controls. Males administered 10 mg/kg/day displayed lower serum testosterone levels (22% lower; did not attain statistical significance) (Table 15).

Table 17. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Perfluorooctanoic Acid[a]

	Vehicle Control	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
n	10	10	10	10
Weights (g)				
Necropsy Body Wt.	347 ± 5	317 ± 7**	304 ± 8**	280 ± 10**
L. Cauda Epididymis	0.192 ± 0.006	0.187 ± 0.005	0.170 ± 0.003**	0.171 ± 0.005**
L. Epididymis	0.523 ± 0.012	0.506 ± 0.012	0.492 ± 0.012	0.474 ± 0.012**
L. Testis	1.866 ± 0.033	1.798 ± 0.049	1.834 ± 0.051	1.755 ± 0.036
Spermatid Measurements				
Spermatid Heads (10^6/testis)	234.2 ± 9.9	229.6 ± 7.0	240.5 ± 8.2	223.4 ± 8.2
Spermatid Heads (10^6/g testis)	125.5 ± 4.6	128.5 ± 5.2	131.1 ± 2.2	127.6 ± 4.9
Epididymal Spermatozoal Measurements				
Sperm Motility (%)	86.6 ± 0.8	86.1 ± 0.9	85.5 ± 0.7	85.2 ± 0.6
Sperm (10^6/cauda epididymis)	109.0 ± 6.9	98.1 ± 6.2	107.4 ± 7.3	83.0 ± 5.5*
Sperm (10^6/g cauda epididymis)	572.0 ± 41.4	524.3 ± 31.0	630.8 ± 41.1	486.6 ± 29.8

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test (weights) or Shirley's or Dunn's test (epididymal spermatozoal measurements).
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error.

Female rats assessed for reproductive toxicity were administered 0, 25, 50, or 100 mg/kg/day for 28 days. Inspection of the daily vaginal cytology data indicated that PFOA-dosed rats were generally cycling similar to the vehicle controls but with the 100 mg/kg/day dose group spending ~20% more time in diestrus (Table A-2). Markov analyses demonstrated that rats administered 100 mg/kg/day of PFOA also had higher probability than the control group of transitioning from normal cycling to extended diestrus (Table A-2 and Table A-3; Figure A-2). There were no apparent PFOA-related changes in female testosterone levels.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following the Wyeth-14,643 results.

Liver: The incidences of hepatocyte cytoplasmic alteration were significantly increased in all male dose groups and 25 mg/kg/day or greater females compared to the vehicle control groups (Table 18). The incidences of hepatocyte hypertrophy were significantly increased in 1.25 mg/kg/day or greater males and in 100 mg/kg/day females. The severities of hepatocyte cytoplasmic alteration and hepatocyte hypertrophy increased with increasing dose. Hepatocyte cytoplasmic alteration and hepatocyte hypertrophy were of minimal to marked severity.

Nose: The incidences of respiratory epithelium, hyperplasia were significantly increased in males administered 0.625, 5, or 10 mg/kg/day and in females administered 12.5 or 100 mg/kg/day compared to the vehicle control groups (Table 18). The incidences of respiratory epithelium, inflammation chronic active were significantly increased in 0.625, 5, and 10 mg/kg/day males and 12.5, 50, and 100 mg/kg/day females compared to the respective vehicle control groups. Respiratory epithelium, hyperplasia and respiratory epithelium, inflammation chronic active were primarily of minimal to mild severity. The incidences of olfactory epithelium degeneration were significantly increased in 50 and 100 mg/kg/day females compared to the vehicle control group; two incidences of this lesion occurred in 25 mg/kg/day females (Table 18). The incidence of olfactory epithelium hyperplasia was significantly increased in 50 mg/kg/day females compared to the vehicle control group, and two incidences occurred in 25 mg/kg/day females. The incidence of olfactory epithelium suppurative inflammation was significantly increased in 100 mg/kg/day females compared to the vehicle control group. Olfactory epithelium degeneration, hyperplasia, and suppurative inflammation were primarily of minimal to mild severity.

Bone Marrow: The incidence of bone marrow hypocellularity was significantly increased in 10 mg/kg/day males compared to the vehicle control group (Table 18). Bone marrow hypocellularity was minimal to mild in severity. There were two incidences in females.

Thyroid Gland: The incidence of thyroid gland follicular cell hypertrophy was increased, but not significantly in 10 mg/kg/day males and significantly increased in 100 mg/kg/day females compared to the vehicle control groups (Table 18). Follicle cell hypertrophy was of minimal severity.

Table 18. Incidences of Selected Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Perfluorooctanoic Acid

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
Male						
Liver[a]	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration[b]	0	4* (1.0)[c]	9** (1.0)	10** (1.3)	10** (2.2)	10** (2.6)
Hepatocyte, Hypertrophy	0	0*	6** (1.0)	10** (1.8)	10** (2.5)	10** (3.2)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Respiratory Epithelium, Hyperplasia	0	4* (1.3)	2 (1.0)	3** (1.3)	5** (1.4)	4* (1.3)
Respiratory Epithelium, Inflammation Chronic Active	0	5* (1.0)	3 (1.0)	2** (1.0)	5* (1.0)	4* (1.0)
Bone Marrow	(10)	(10)	(10)	(9)	(10)	(10)
Hypocellularity	1 (1.0)	0	0	0	2 (1.0)	6* (1.0)
Thyroid Gland	(10)	(10)	(10)	(10)	(10)	(10)
Follicular Cell Hypertrophy	0	0	0	0	0	3 (1.0)
Epididymis	(10)	(0)	(0)	(10)	(10)	(10)
Hypospermia	0	0	0	0	1 (2.0)	1 (2.0)
Duct, Exfoliated Germ Cell	0	0	0	0	1 (2.0)	1 (2.0)
	Vehicle Control	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**	**100 mg/kg/day**
Female						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	0	0	10** (1.0)	10** (1.0)	10** (1.9)
Hepatocyte, Hypertrophy	0	0	0	0	2 (1.5)	10** (2.0)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Respiratory Epithelium, Hyperplasia	0	0	4* (1.0)	0	3 (1.0)	8** (1.6)

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Female (continued)						
Respiratory Epithelium, Inflammation Chronic Active	0	0	4* (1.0)	1** (1.0)	6** (1.0)	9** (1.4)
Olfactory Epithelium, Degeneration	0	0	0	2** (1.0)	8** (1.4)	9** (1.8)
Olfactory Epithelium, Hyperplasia	0	0	0	2** (1.0)	8** (1.1)	0
Olfactory Epithelium, Suppurative Inflammation	0	0	0	0	1 (1.0)	6** (1.5)
Thyroid Gland	(10)	(10)	(10)	(10)	(10)	(10)
Follicular Cell Hypertrophy	0	0	0	0	1 (1.0)	8** (1.0)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Perfluorononanoic Acid (PFNA)

Survival of 5 and 10 mg/kg/day males and 12.5 and 25 mg/kg/day females was significantly less than that of the respective vehicle control groups (Table 19). Eight 5 mg/kg/day males, all 10 mg/kg/day males, nine 12.5 mg/kg/day females, and all 25 mg/kg/day females died before the end of the study. The rats in these dose groups were described as thin. The final mean body weights of the 1.25, 2.5, and 5 mg/kg/day males were 17%, 44%, and 55% less than that of the vehicle control group, respectively (Table 19; Figure 3). The final mean body weights of the 6.25 and the 12.5 mg/kg/day females were 10% and 23% less than that of the vehicle control group, respectively.

Table 19. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Perfluorononanoic Acid

	Vehicle Control		0.625 mg/kg/day			1.25 mg/kg/day			2.5 mg/kg/day			5 mg/kg/day			10 mg/kg/day		
Day	**Av. Wt. (g)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**
Male																	
1	267	10	269	101	10	268	100	10	266	100	10	268	100	10	266	100	10
8	289	10	293	101	10	292	101	10	288	99	10	260	90	10	220	76	10
15	307	10	314	102	10	305	99	10	267	87	10	210	69	10	155	51	8
22	329	10	331	101	10	299	91	10	233	71	10	156	48	10	–	–	0
29	344	10	332	97	10	286	83	10	193	56	10	155	45	2	–	–	0
	Vehicle Control		**1.56 mg/kg/day**			**3.12 mg/kg/day**			**6.25 mg/kg/day**			**12.5 mg/kg/day**			**25 mg/kg/day**		
	Av. Wt. (g)	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**
Female																	
1	195	10	200	102	10	196	100	10	196	101	10	196	100	10	200	102	10
8	204	10	210	103	10	201	98	10	196	96	10	161	79	10	150	73	10
15	215	10	218	101	10	210	97	10	196	91	10	146	68	6	–	–	0
22	222	10	226	102	10	212	96	10	200	90	10	130	59	4	–	–	0
29	230	10	231	100	10	217	94	10	207	90	10	178	77	1	–	–	0

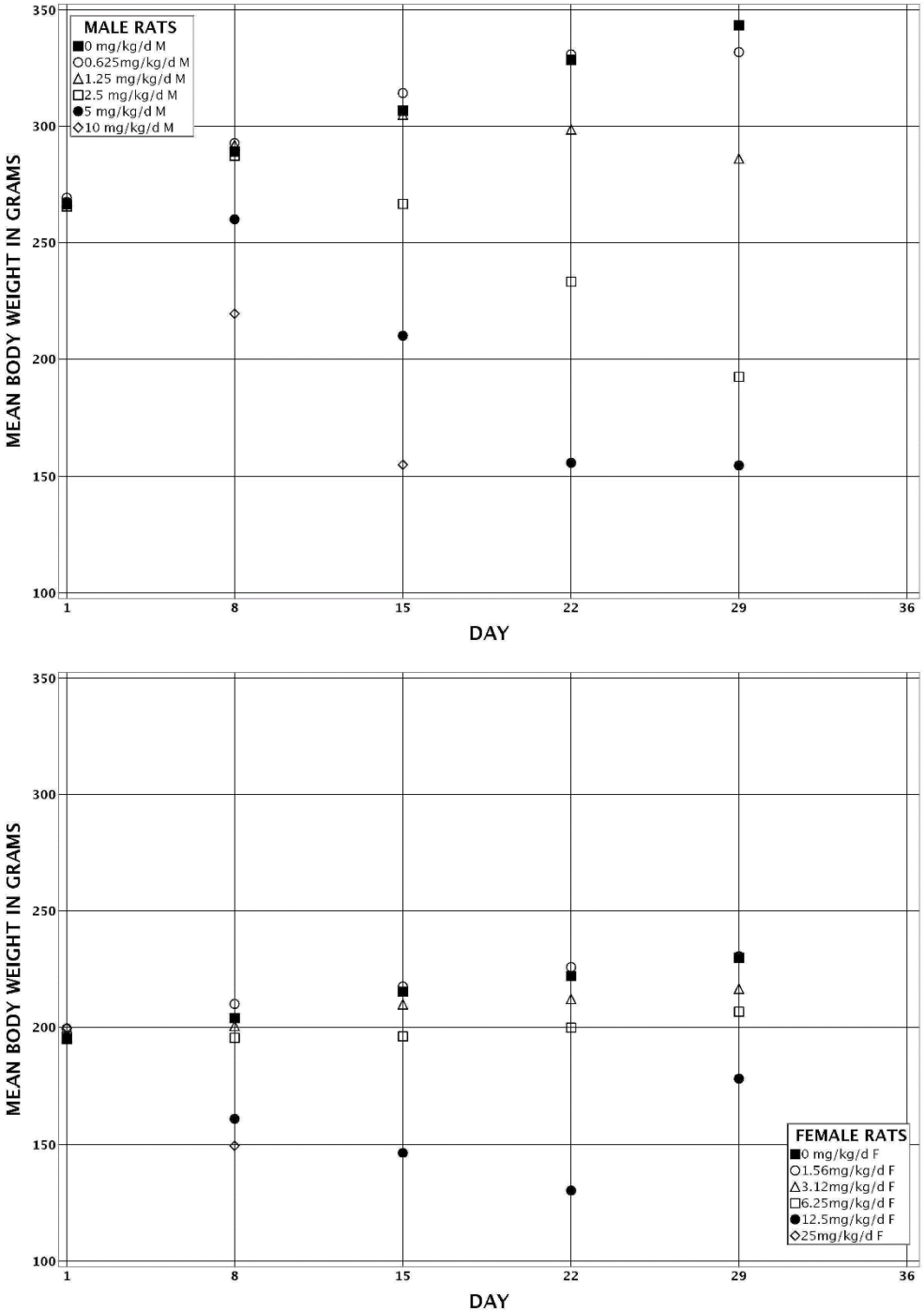


Figure 3. Growth Curves for Rats Administered Perfluorononanoic Acid by Gavage for 28 Days

Plasma concentrations of PFNA were higher with increasing dose in both males and females (Table 20). Plasma concentrations normalized to dose generally increased with dose in males except for the 5.0 mg/kg/day group, which might be due to only two animals available to sample. Normalized concentrations in females were similar across the available doses (1.56 to 6.25 mg/kg/day). Normalized plasma concentrations were generally five- to ninefold higher in males compared to females (adjusting for the 2.5-fold higher dose compared to males).

Liver concentrations (measured in males only) increased with dose, and when normalized to dose administered (µM/mmol/kg), these values decreased with dose (Table 20). The liver/plasma ratio ranged from 0.87 to 2.59.

The mean absolute and relative liver weights of 0.625 and 1.25 mg/kg/day males and the mean relative liver weight of 2.5 mg/kg/day males were significantly greater than those of the vehicle control group (Table 21). The mean absolute and relative liver weights of 1.56, 3.12, and 6.25 mg/kg/day females were significantly greater than those of the vehicle control group.

The mean absolute adrenal gland weights of 0.625, 1.25, and 2.5 mg/kg/day males were significantly less than that of the vehicle control group (Table 21). The mean relative adrenal gland weights of 2.5 mg/kg/day males were significantly greater than that of the vehicle control group.

The mean absolute kidney weight of 2.5 mg/kg/day males was significantly less than that of the vehicle control group (Table 21). The mean relative kidney weights of 0.625, 1.25, and 2.5 mg/kg/day males were significantly greater than that of the vehicle control group. The mean absolute and relative kidney weights of 1.56, 3.12, and 6.25 mg/kg/day females were significantly greater than those of the vehicle control group.

The mean absolute and relative spleen and thymus weights of 1.25 and 2.5 mg/kg/day males were significantly less than those of the vehicle control group (Table 21). The mean absolute spleen weight of 3.12 mg/kg/day females and the absolute and relative spleen weights of 6.25 mg/kg/day females were significantly less than those of the vehicle control group (Table 21). The decreases in the spleen and thymus weights had histologic correlates of lymphocyte atrophy and were attributed to stress.

The mean absolute thyroid gland weights of 2.5 and 5 mg/kg/day males were significantly less than that of the vehicle control group (Table 21). The mean relative thyroid gland weights of the 2.5 and 5 mg/kg/day males were significantly greater than that of the vehicle control group.

The mean absolute testis weight of 2.5 mg/kg/day males was significantly less than that of the vehicle control group (Table 21).

Table 20. Perfluorononanoic Acid Concentrations in Tissues of Rats in the 28-day Gavage Study of Perfluorononanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Millimolar Dose (mmol/kg/day)	0	0.0013	0.0027	0.0054	0.0108
Male					
n	10	10	10	10	2
Plasma					
Plasma Concentration (ng/mL)	54.5 ± 11.6	56,730.0 ± 1,878.4**	161,000.0 ± 4,928.4**	380,000.0 ± 15,639.0**	358,000.0 ± 54,000.0**
Plasma Concentration (µM)	0.1 ± 0.0	122.2 ± 4.0**	346.9 ± 10.6**	818.8 ± 33.7**	771.4 ± 116.4**
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	90,768.0 ± 3,005.4	128,800.0 ± 3,942.7	152,000.0 ± 6,255.6	71,600.0 ± 10,800.0
Liver					
Liver Concentration (ng/mL)	761.8 ± 32.7	145,500.0 ± 2,684.3**	249,200.0 ± 4,692.3**	311,400.0 ± 7,449.4**	313,000.0 ± 59,000.0**
Liver Concentration (µM)[b]	1.6 ± 0.1	313.5 ± 5.8**	537.0 ± 10.1**	671.0 ± 16.1**	674.5 ± 127.1**
Normalized Liver Concentration (µM/mmol/kg/day)	NA	232,800.0 ± 4,294.9	199,360.0 ± 3,753.8	124,560.0 ± 2,979.8	62,600.0 ± 11,800.0
Liver/Plasma Ratio	16.36 ± 1.53	2.59 ± 0.10**	1.56 ± 0.06**	0.83 ± 0.04**	0.87 ± 0.03**
	Vehicle Control	**1.56 mg/kg/day**	**3.12 mg/kg/day**	**6.25 mg/kg/day**	**12.5 mg/kg/day**
Millimolar Dose (mmol/kg/day)	0	0.0034	0.0067	0.0135	0.0269
Female					
n	10	10	10	10	1[c]
Plasma					
Plasma Concentration (ng/mL)	97.9 ± 11.5	26,400.0 ± 1,085.5**	54,360.0 ± 2,486.5**	112,200.0 ± 9,772.4**	–
Plasma Concentration (µM)	0.2 ± 0.0	56.9 ± 2.3**	117.1 ± 5.4**	241.8 ± 21.1**	–
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	16,923.1 ± 695.8	17,423.1 ± 796.9	17,952.0 ± 1,563.6	–

**Significantly different ($p \leq 0.01$) from the vehicle control group by Shirley's or Dunn's test.
[a]Tissue concentration data are presented as mean ± standard error. If over 20% of the values in a group were above the limit of quantification (plasma LOQ = 25 ng/mL; liver LOQ = 500 ng/g), then values that were below the limit of quantification were substituted with one-half the limit of quantification value. BD=below detection; group did not have over 20% of its values above the limit of quantification and no statistical analyses were conducted for the endpoint. NA=not applicable; value could not be calculated when dose value was 0. One-half the dose was administered twice daily.
[b]Density is assumed to be 1.0 g/mL.
[c]Statistical tests were not performed due to there being only one survivor.

Table 21. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Perfluorononanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Male				
n	10	10	10	10
Necropsy Body Wt.	344 ± 6	332 ± 6	286 ± 5**	193 ± 9**
R. Adrenal Gland				
Absolute	0.0220 ± 0.0009	0.0187 ± 0.0006**	0.0179 ± 0.0010**	0.0175 ± 0.0006**
Relative	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.09 ± 0.00**
R. Kidney				
Absolute	1.02 ± 0.02	1.14 ± 0.03	1.04 ± 0.02	0.82 ± 0.03**
Relative	2.97 ± 0.05	3.43 ± 0.05**	3.64 ± 0.07**	4.26 ± 0.06**
Liver				
Absolute	11.73 ± 0.23	13.99 ± 0.33*	15.58 ± 0.27**	12.33 ± 0.92
Relative	34.14 ± 0.3	42.12 ± 0.58**	54.47 ± 0.59**	63.37 ± 1.86**
Spleen				
Absolute	0.674 ± 0.015	0.615 ± 0.035	0.496 ± 0.017**	0.286 ± 0.021**
Relative	1.96 ± 0.03	1.85 ± 0.09	1.73 ± 0.05*	1.48 ± 0.06**
R. Testis				
Absolute	1.870 ± 0.045	1.793 ± 0.035	1.757 ± 0.032	1.493 ± 0.055**
Thymus				
Absolute	0.389 ± 0.021	0.427 ± 0.022	0.268 ± 0.012**	0.087 ± 0.017**
Relative	1.13 ± 0.06	1.29 ± 0.07	0.93 ± 0.03*	0.43 ± 0.06**
Thyroid Gland				
Absolute	0.0226 ± 0.0010	0.0251 ± 0.0016	0.0210 ± 0.0009	0.0161 ± 0.0009**
Relative	0.07 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	0.09 ± 0.01**
	Vehicle Control	**1.56 mg/kg/day**	**3.12 mg/kg/day**	**6.25 mg/kg/day**
Female				
n	10	10	10	10
Necropsy Body Wt.	230 ± 5	231 ± 3	217 ± 3*	207 ± 4**
R. Kidney				
Absolute	0.66 ± 0.01	0.71 ± 0.02*	0.73 ± 0.01**	0.73 ± 0.02**
Relative	2.89 ± 0.06	3.10 ± 0.04*	3.38 ± 0.04**	3.53 ± 0.08**
Liver				
Absolute	7.67 ± 0.29	9.30 ± 0.30**	9.74 ± 0.22**	10.12 ± 0.28**
Relative	33.29 ± 0.70	40.30 ± 0.91**	44.95 ± 0.74**	48.92 ± 0.68**
Spleen				
Absolute	0.598 ± 0.018	0.609 ± 0.022	0.533 ± 0.013*	0.481 ± 0.019**
Relative	2.60 ± 0.05	2.64 ± 0.07	2.46 ± 0.04	2.32 ± 0.06**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error). No data presented for the 5 or 10 (males) or 12.5 or 25 (females) mg/kg/day groups due to high mortality.

There was no clinical pathology interpretation for 5 or 10 mg/kg/day male rats or 12.5 or 25 mg/kg/day female rats due to high mortality in these groups. A significant, dose-dependent decrease in the reticulocyte count was observed in male rats, with the 2.5 mg/kg/day group decreased by 95% of the vehicle controls (Table 22). As a result of these decreased reticulocyte counts, the mean cell volume was significantly decreased, and the mean cell hemoglobin concentration significantly increased in the 2.5 mg/kg/day males. The leukocyte count was significantly decreased in the 2.5 mg/kg males compared to the vehicle control group; the decrease was mostly a result of a significantly decreased lymphocyte count.

Urea nitrogen concentrations were significantly increased in all male dose groups (except 5 mg/kg/day) and in 3.12 and 6.25 mg/kg/day females compared to the vehicle control groups (Table 22). In males, the creatinine and glucose concentrations were significantly decreased at 2.5 mg/kg/day compared to the vehicle control group; these decreases were likely related to the decreased food intake and body weight. Cholesterol and triglyceride concentrations were significantly decreased in the majority of the dose groups compared to the vehicle control group.

Total protein and globulin concentrations were significantly decreased in all male dose groups and the albumin concentrations were significantly decreased in 1.25 and 2.5 mg/kg/day males compared to the vehicle control group (Table 22). These changes resulted in an increase in the albumin/globulin ratio in all the dose groups. Total protein and globulin concentrations were significantly decreased and albumin concentrations significantly increased in all female dose groups (except 12.5 mg/kg/day) compared to the vehicle control group; the combination of these changes resulted in significant increases in the albumin/globulin ratio of all the female dose groups.

Table 22. Hematology and Clinical Chemistry Data for Rats in the 28-day Gavage Study of Perfluorononanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Male				
Hematology				
n	10	10	10	10
Reticulocytes (10^3/µL)	168.3 ± 6.5	135.5 ± 8.1*	85.3 ± 6.7**	8.2 ± 1.7**
Mean Cell Volume (fL)	60.2 ± 0.4	60.5 ± 0.6	59.7 ± 0.3	56.9 ± 0.4**
Mean Cell Hemoglobin Concentration (g/dL)	30.0 ± 0.1	30.1 ± 0.1	30.1 ± 0.1	30.7 ± 0.1**
Leukocytes (10^3/µL)	10.92 ± 0.36	11.01 ± 0.76	9.74 ± 0.57	5.66 ± 0.74**
Lymphocytes (10^3/µL)	9.17 ± 0.29	9.53 ± 0.70	8.53 ± 0.52	4.22 ± 0.62**
Clinical Chemistry				
n	10	10	10	10
Urea Nitrogen (mg/dL)	18.5 ± 0.9	21.6 ± 0.6*	24.8 ± 0.7**	27.6 ± 1.5**
Creatinine (mg/dL)	0.73 ± 0.02	0.72 ± 0.01	0.69 ± 0.02	0.62 ± 0.02**
Glucose (mg/dL)	188 ± 8	202 ± 7	168 ± 6.0	104 ± 5**
Total Protein (g/dL)	6.6 ± 0.0	6.0 ± 0.1**	5.5 ± 0.1**	4.3 ± 0.1**
Albumin (g/dL)	4.4 ± 0.0	4.4 ± 0.1	4.1 ± 0.1**	3.2 ± 0.1**
Globulin (g/dL)	2.2 ± 0.0	1.5 ± 0.0**	1.4 ± 0.1**	1.1 ± 0.0**
Albumin/Globulin Ratio	2.0 ± 0.0	2.9 ± 0.1**	3.1 ± 0.1**	3.1 ± 0.1**
Total Bilirubin (mg/dL)	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0*	0.7 ± 0.1**
Direct Bilirubin (mg/dL)	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00**	0.32 ± 0.04**
Indirect Bilirubin (mg/dL)	0.132 ± 0.009	0.114 ± 0.009	0.150 ± 0.009	0.398 ± 0.040**
Cholesterol (mg/dL)	117 ± 5	86 ± 2**	91 ± 4**	108 ± 9
Triglycerides (mg/dL)	120 ± 9	59 ± 9**	81 ± 6**	59 ± 4**
Alanine Aminotransferase (IU/L)	58 ± 2[c]	75 ± 5	86 ± 4**	59 ± 4
Alkaline Phosphatase (IU/L)	207 ± 5	247 ± 15	397 ± 25**	204 ± 31
Aspartate Aminotransferase (IU/L)	85 ± 23	74 ± 3	96 ± 3**	119 ± 11**
Sorbitol Dehydrogenase (IU/L)	9 ± 1c	12 ± 1	13 ± 1*	13 ± 1*
Bile Salts/Acids (µmol/L)	13.1 ± 2.4	27.6 ± 3.1**	71.0 ± 7.8**	221.9 ± 16.1**
Thyroid Stimulating Hormone (ng/mL)	20.33 ± 2.31	13.70 ± 1.27	10.97 ± 1.22**[c]	10.16 ± 3.35**[d]
Triiodothyronine (ng/dL)	78.21 ± 4.54	58.54 ± 2.11	84.93 ± 2.94	111.79 ± 10.16[c]
Free Thyroxine (ng/dL)	2.16 ± 0.15	0.55 ± 0.02**	0.33 ± 0.01**	0.30 ± 0.00**[e]
Total Thyroxine (µg/dL)	2.36 ± 0.27	0.21 ± 0.07**	0.38 ± 0.07**	1.49 ± 0.13
Testosterone (ng/mL)	4.48 ± 1.31	4.86 ± 1.33	3.23 ± 1.38	0.85 ± 0.51**[c]

	Vehicle Control	1.56 mg/kg/day	3.12 mg/kg/day	6.25 mg/kg/day
Female				
Clinical Chemistry				
n	10	10	10	10
Urea Nitrogen (mg/dL)	17.2 ± 1.0	18.1 ± 0.5	21.3 ± 0.8**	22.1 ± 1.4**
Total Protein (g/dL)	6.3 ± 0.1	6.6 ± 0.1*	6.7 ± 0.1**	6.7 ± 0.1**
Albumin (g/dL)	4.4 ± 0.1	4.9 ± 0.1**	5.1 ± 0.1**	5.2 ± 0.1**
Globulin (g/dL)	1.9 ± 0.1	1.6 ± 0.1*	1.6 ± 0.1**	1.5 ± 0.0**
Albumin/Globulin Ratio	2.4 ± 0.1	3.0 ± 0.1**	3.2 ± 0.1**	3.5 ± 0.1**
Direct Bilirubin (mg/dL)	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00*
Alanine Aminotransferase (IU/L)	45 ± 3	47 ± 1	53 ± 1**	59 ± 3**
Alkaline Phosphatase (IU/L)	144 ± 7	159 ± 5	183 ± 8**	199 ± 13**
Sorbitol Dehydrogenase (IU/L)	7 ± 1	9 ± 1*	9 ± 1**	10 ± 1**
Bile Salts/Acids (µmol/L)	9.9 ± 2.1	15.1 ± 2.4	21.1 ± 1.5**	28.0 ± 4.5**
Thyroid Stimulating Hormone (ng/mL)	14.64 ± 1.66	15.52 ± 1.53	14.11 ± 1.13	14.33 ± 1.08
Triiodothyronine (ng/dL)	93.70 ± 6.03	84.10 ± 4.15	83.26 ± 2.67	89.90 ± 6.82
Free Thyroxine (ng/dL)	1.70 ± 0.20	1.47 ± 0.15	1.10 ± 0.10*	0.80 ± 0.10**
Total Thyroxine (µg/dL)	4.37 ± 0.41	3.57 ± 0.28	2.81 ± 0.17**	2.61 ± 0.24**
Testosterone (ng/mL)	0.30 ± 0.02	0.39 ± 0.03*	0.44 ± 0.04**	0.49 ± 0.04**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. No data are reported for the 5 or 10 (male) or 12.5 or 25 (female) mg/kg/day groups due to high mortality.
[b]n = 2.
[c]n = 9.
[d]n = 4.
[e]n = 7.

Total bilirubin concentrations (driven by both direct and indirect bilirubin) and AST and SDH activities were significantly increased in 1.25 and 2.5 mg/kg/day males compared to the vehicle control group (Table 22). ALT and ALP activities were significantly increased in 1.25 mg/kg males compared to the vehicle control group, and bile acid concentrations were significantly increased in all dose groups. In females, ALT and ALP activities and bile acid concentrations were significantly increased at 3.12 and 6.25 mg/kg/day compared to the vehicle control group; direct bilirubin was increased in the 6.25 mg/kg/day females compared to the vehicle control group. SDH activities were significantly increased in all female dose groups compared to the vehicle control group.

Free T4 concentrations were significantly decreased in all male dose groups compared to the vehicle control group, and total T4 concentrations were decreased in 0.625 and 1.25 mg/kg/day males (Table 22). TSH concentration was significantly decreased, and T3 concentration was significantly increased in 2.5 mg/kg/day males. Free T4 and total T4 concentrations were significantly decreased in 3.12 and 6.25 mg/kg/day females compared to the vehicle control

group. Testosterone concentrations were significantly decreased in 2.5 mg/kg males and increased in all female dose groups compared to the respective vehicle control groups.

Male and female rats administered PFNA exhibited a significant increase in gene expression of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* compared to respective controls, suggesting a significant increase in PPARα and CAR activity (Table 23). Male rats exhibited the highest fold change for *Cyp4a1* and in female rats, it was *Cyp2b1* when compared to respective controls. *Cyp2b1* and *Cyp2b2* had the lowest fold change in males compared to controls, indicating weaker CAR activation. Acyl-CoA oxidase activity was increased in all dosed males.

Table 23. Hepatic Parameters for Rats in the 28-day Gavage Study of Perfluorononanoic Acid[a]

	Vehicle Control	**0.625 mg/kg/day**	**1.25 mg/kg/day**	**2.5 mg/kg/day**	**5 mg/kg/day**
Male					
n	10	10	10	10	2
Enzyme Activity					
Acetyl-CoA (nmol/min/mg)	2.179 ± 0.049	10.694 ± 0.390**	15.400 ± 0.374**	13.060 ± 0.306**	10.600 ± 0.200**
Gene Expression					
Acox1	1.06 ± 0.13	4.33 ± 0.67**	4.96 ± 0.56**	6.26 ± 0.71**	6.62 ± 1.34**
Cyp4a1	1.11 ± 0.16	22.08 ± 4.87**	24.10 ± 4.88**	20.56 ± 2.73**	22.62 ± 6.93*
Cyp2b1	1.40 ± 0.32	9.01 ± 2.13**	9.45 ± 1.36**	5.69 ± 0.80*	4.18 ± 0.02
Cyp2b2	1.22 ± 0.27	3.85 ± 0.48**	4.98 ± 0.52**	3.94 ± 0.77**	4.66 ± 1.67*
	Vehicle Control	**1.56 mg/kg/day**	**3.12 mg/kg/day**	**6.25 mg/kg/day**	**12.5 mg/kg/day**
Female					
n	10	10	10	10	1
Gene Expression					
Acox1	1.02 ± 0.06	3.13 ± 0.57**	4.13 ± 0.48**	5.27 ± 0.99**	4.92[b]
Cyp4a1	1.05 ± 0.10	8.08 ± 3.36**	10.64 ± 3.83**	13.82 ± 3.05**	18.77
Cyp2b1	2.68 ± 1.37	29.83 ± 9.57**	38.20 ± 9.24**	36.40 ± 8.65**	33.85
Cyp2b2	2.06 ± 0.85	14.42 ± 1.50**	21.28 ± 2.17**	22.95 ± 3.82**	27.65

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's or Dunn's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values. No data available for the 10 (males) or 25 (females) mg/kg/day groups due to 100% mortality.
[b]Statistical tests were not performed due to there being only one survivor.

Male rats assessed for reproductive toxicity were administered 0, 0.625, 1.25, or 2.5 mg PFNA/kg/day for 28 days, which is much less than the time needed to make a full assessment of the effect on spermatogenesis. Epididymal weights were lower in the rats administered to 1.25 or 2.5 mg/kg/day compared to the vehicle controls (11% and 33% respectively; statistically significant) (Table 24). Cauda epididymal sperm counts in these rats were also lower (18% and 31%, respectively; statistically significant). When normalized to total sperm count/g cauda epididymis, counts in dosed rats were similar to vehicle controls. Histopathologic findings in the epididymis were noted in multiple rats administered 2.5 mg/kg/day or greater (hypospermia, exfoliated germ cells, apoptosis of the epididymal epithelium, granuloma sperm) (Table 26). Rats administered 1.25 or 2.5 mg/kg/day had lower testis weights than vehicle controls (7% and 20%, respectively; statistically significant). Testicular spermatid counts and normalized counts were variable (no apparent dose responses). Multiple histopathologic findings in the testis were noted in rats administered 2.5 mg/kg/day or greater (degeneration, spermatid retention, interstitial cell atrophy) (Table 26). Males administered 1.25, and 2.5 mg/kg/day displayed lower serum testosterone levels (28% and 81%, lower) (Table 22).

Table 24. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Perfluorononanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
n	10	10	10	10
Weights (g)				
Necropsy Body Wt.	344 ± 6	332 ± 6	286 ± 5	193 ± 9
L. Cauda Epididymis	0.195 ± 0.005	0.189 ± 0.005	0.173 ± 0.004**	0.130 ± 0.008**
L. Epididymis	0.555 ± 0.013	0.515 ± 0.010*	0.482 ± 0.005**	0.363 ± 0.022**
L. Testis	1.885 ± 0.041	1.820 ± 0.035	1.762 ± 0.031*	1.507 ± 0.052**
Spermatid Measurements				
Spermatid Heads (10^6/testis)	230.2 ± 10.7	192.5 ± 7.1*	220.8 ± 10.8	205.1 ± 9.5
Spermatid Heads (10^6/g testis)	121.9 ± 4.3	105.8 ± 3.6	125.3 ± 5.8	136.4 ± 5.3
Epididymal Spermatozoal Measurements				
Sperm Motility (%)	85.2 ± 0.5	85.9 ± 0.4	86.4 ± 0.7	85.8 ± 0.6
Sperm (10^6/cauda epididymis)	142.3 ± 9.4	136.2 ± 7.9	116.0 ± 6.3*	98.1 ± 9.0**
Sperm (10^6/g cauda epididymis)	731.2 ± 49.2	725.7 ± 47.7	674.4 ± 40.4	753.5 ± 47.2

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test (weights) or Shirley's or Dunn's test (spermatid and epididymal spermatozoal measurements).
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error.

Female rats assessed for reproductive toxicity were administered 0, 1.56, 3.12, or 6.25 mg PFNA/kg/day. Inspection of daily vaginal cytology data indicated that the rats were cycling similar to vehicle controls (**Error! Reference source not found.**Table 25 and Table A-4). Females in the 6.25 mg/kg/day dose group had fewer mean cycles (1.3 versus 1.7 in vehicle controls) and fewer were cycling (6 versus 9 in vehicle controls). Female rats in the 1.56, 3.12, and 6.25 mg/kg/day groups displayed 34%, 49%, and 66% higher testosterone levels (Table 22), the precursor of 17β-estradiol via aromatase, than vehicle controls.

Table 25. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Perfluorononanoic Acid[a]

	Vehicle Control	1.56 mg/kg/day	3.12 mg/kg/day	6.25 mg/kg/day
Number Weighed at Necropsy	10	10	10	10
Necropsy Body Wt. (g)	230 ± 5	231 ± 3	217 ± 3*	207 ± 4**
Proportion of Regular Cycling Females[b]	9/10	10/10	9/10	6/10
Estrous Cycle Length (days)	5.1 ± 0.10	5.7 ± 0.71	5.1 ± 0.11	5.0 ± 0.00
Mean Number of Cycles	1.7	1.9	2.0	1.3
Estrous Stages (% of cycle)				
Diestrus	67.5	60.6	61.3	75.0
Proestrus	5.0	9.4	8.8	5.0
Estrus	27.5	30.0	29.4	18.8
Metestrus	0.0	0.0	0.0	1.3
Uncertain	0.0	0.0	0.6	0.0

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test (body weight).
**$p \leq 0.01$.
[a]Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant Dunn's test (estrous cycle length). Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated that 3.12 mg/kg/day females had extended diestrus.
[b]Number of females with a regular cycle/number of females cycling.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following the Wyeth-14,643 results.

Liver: Compared to the respective vehicle control groups, the incidences of hepatocyte cytoplasmic alteration were significantly increased in all male dose groups and in 1.56, 3.12, 6.25, and 12.5 mg/kg/day female groups (Table 26) compared to the respective vehicle control groups. The incidences of hepatocyte hypertrophy were significantly increased in all dosed males and in 6.25 mg/kg/day or greater females compared to the respective vehicle control groups. The incidences of hepatocyte necrosis were significantly increased in 2.5 mg/kg/day or greater males and 12.5 mg/kg/day females compared to the respective vehicle control groups. The incidences of cytoplasmic vacuolization were significantly increased at 1.25 and 2.5 mg/kg/day in males compared to the vehicle control group. Severity of the hepatocellular lesions generally varied from minimal to marked among the dosed rats.

Nose: The incidence of olfactory epithelium degeneration was increased in 10 mg/kg/day males and significantly increased in 25 mg/kg/day females compared to the respective vehicle control groups (Table 26). The incidence of olfactory epithelium suppurative inflammation was increased in 25 mg/kg/day females compared to the vehicle control group; however, the increase was not statistically significant.

Table 26. Incidences of Selected Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Perfluorononanoic Acid

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
Male						
Liver[a]	(10)	(10)	(10)	(10)	(9)	(10)
Hepatocyte, Cytoplasmic Alteration[b]	0	10** (1.5)[c]	10** (3.7)	10** (4.0)	9** (3.1)	10** (2.1)
Hepatocyte, Hypertrophy	0	7** (2.3)	10** (4.0)	10** (4.0)	9** (3.6)	10** (3.0)
Hepatocyte, Necrosis	0	0	1 (1.0)	5* (1.2)	6** (2.0)	9** (1.8)
Hepatocyte, Vacuolization, Cytoplasmic	0	0	6** (1.5)	9** (3.7)	0	0
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory Epithelium Degeneration	0	0	0	0	1 (1.0)	3 (1.3)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	0	0	10** (3.4)	10** (4.0)	10** (3.1)
Spleen	(10)	(10)	(10)	(10)	(8)	(10)
Atrophy	0	0	0	0	7** (3.6)	9** (4.0)
Thymus	(10)	(10)	(10)	(10)	(10)	(8)
Atrophy	0	0	0	10** (3.3)	10** (4.0)	8** (4.0)
Lymph Node, Mandibular	(10)	(10)	(10)	(10)	(7)	(10)
Atrophy	0	0	0	2 (1.5)	5** (3.8)	5* (2.2)
Lymph Node, Mesenteric	(10)	(10)	(10)	(10)	(8)	(10)
Atrophy	0	0	0	3 (3.0)	6** (3.7)	9** (3.2)
Stomach, Forestomach	(10)	(10)	(10)	(10)	(8)	(10)
Epithelium, Hyperplasia	0	0	0	6** (2.3)	6** (1.8)	8** (1.8)
Inflammation, Chronic Active	0	0	0	5* (2.0)	0	0
Testes	(10)	(10)	(10)	(10)	(9)	(10)
Germinal Epithelium, Degeneration	0	0	0	6** (2.2)	9** (3.1)	10** (3.7)
Interstitial Cell, Atrophy	0	0	1 (1.0)	10** (1.7)	9** (3.1)	10** (3.5)
Seminiferous Tubule, Spermatid Retention	0	0	0	6** (2.8)	9** (3.0)	10** (3.0)
Epididymis	(10)	(10)	(10)	(10)	(9)	(10)
Hypospermia	0	0	0	2 (2.5)	9** (3.7)	10** (2.9)
Duct, Exfoliated Germ Cell	0	0	0	6** (2.7)	9** (3.7)	10** (3.6)
Epithelium, Apoptosis	0	0	0	0	8** (2.0)	10** (2.1)
Granuloma Sperm	0	0	0	0	0	4* (2.0)

	Vehicle Control	1.56 mg/kg/day	3.12 mg/kg/day	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
Female						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	5* (1.0)	10** (1.0)	10** (2.4)	9** (1.6)	0
Hepatocyte, Hypertrophy	0	0	2 (1.0)	10** (4.0)	10** (3.0)	10** (2.6)
Hepatocyte, Necrosis	0	0	0	0	4* (1.3)	3 (1.0)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory Epithelium Degeneration	0	0	0	0	0	5* (2.0)
Olfactory Epithelium, Inflammation, Suppurative	0	0	0	0	0	3 (1.7)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	0	0	0	10** (3.8)	10** (3.1)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy	0	0	0	0	8** (4.0)	10** (4.0)
Thymus	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy	0	0	0	0	9** (4.0)	10** (4.0)
Lymph Node, Mandibular	(10)	(10)	(10)	(10)	(8)	(9)
Atrophy	0	0	0	0	8** (3.4)	9** (3.1)
Lymph Node, Mesenteric	(10)	(10)	(10)	(10)	(9)	(10)
Atrophy	0	0	0	0	8** (4.0)	9** (2.9)
Stomach, Forestomach	(10)	(10)	(10)	(10)	(10)	(10)
Epithelium, Hyperplasia	0	0	0	0	7** (2.0)	6** (1.5)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Bone marrow: The incidences of bone marrow hypocellularity were significantly increased in 2.5 mg/kg/day or greater males and 12.5 and 25 mg/kg/day females compared to the respective vehicle control groups (Table 26). The severity of bone marrow hypocellularity varied from moderate to marked among dosed rats.

Spleen: The incidences of splenic atrophy were significantly increased in 5 and 10 mg/kg/day males and 12.5 and 25 mg/kg/day females compared to the respective vehicle control groups (Table 26). The severity was moderate to marked.

Thymus: The incidences of atrophy were significantly increased in males administered 2.5 mg/kg/day or greater and in 12.5 and 25 mg/kg/day females compared to the respective vehicle control groups (Table 26). The severity was generally marked.

Mandibular and Mesenteric Lymph Nodes: The incidences of atrophy were significantly increased in 5 and 10 mg/kg/day males and 12.5 and 25 mg/kg/day females compared to the respective vehicle control groups (Table 26). Severities ranged from moderate to marked.

Forestomach: The incidences of epithelial hyperplasia were significantly increased in 2.5 mg/kg/day or greater males and 12.5 and 25 mg/kg/day females compared to the respective vehicle control groups (Table 26). Average severities ranged from minimal to mild. The incidence of chronic inflammation was significantly increased compared to the vehicle control group with mild severity in 2.5 mg/kg/day males.

Testes: The incidences of germinal epithelium degeneration, interstitial cell atrophy, and spermatid retention in the seminiferous tubule were significantly increased in 2.5 mg/kg/day or greater males compared to those of the vehicle control group (Table 26). In general, the severities of the testicular lesions ranged from mild to marked.

Epididymis: The incidences of hypospermia and epithelial apoptosis in the 5 and 10 mg/kg/day dose groups and the incidences of duct, exfoliated germ cells in males administered 2.5 mg/kg/day or greater were significantly increased compared to those of the vehicle control group (Table 26). The incidence of granuloma sperm was significantly increased in the 10 mg/kg/day group compared to the vehicle control group. In general, the severities of the epididymal lesions ranged from mild to marked.

Perfluorodecanoic Acid (PFDA)

All rats survived to the end of the study (Table 27). No clinical observations related to chemical exposure were observed. The final mean body weights of the 1.25 and the 2.5 mg/kg/day males were 21% and 38% less, respectively, than that of the vehicle control group; the mean body weights of 1.25 and 2.5 mg/kg/day females were 12% and 36% less, respectively, than that of the vehicle control group (Table 27; Figure 4).

Plasma concentrations of PFDA increased with increasing dose in both males and females and were marginally higher in females (30% or less) compared to males across the corresponding dose groups (Table 28). Normalized plasma concentrations (µM/mmol/kg) in males and females increased with dose, with a 1.9-fold increase in males and a 1.4-fold increase in females from lowest to highest dose.

Liver concentrations of PFDA were measured in males only and increased with dose, but when normalized to dose (µM/mol/kg), these values decreased with increasing dose (Table 28). Liver/plasma ratios in males decreased with dose from 5.29 to 1.63.

Table 27. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Perfluorodecanoic Acid

	Vehicle Control		0.156 mg/kg/day			0.312 mg/kg/day			0.625 mg/kg/day			1.25 mg/kg/day			2.5 mg/kg/day		
Day	**Av. Wt. (g)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**
Male																	
1	265	10	265	100	10	263	99	10	263	99	10	264	100	10	266	100	10
8	286	10	287	100	10	284	99	10	285	100	10	285	100	10	283	99	10
15	304	10	310	102	10	301	99	10	306	101	10	291	96	10	264	87	10
22	323	10	329	102	10	317	98	10	313	97	10	273	85	10	236	73	10
29	333	10	344	103	10	331	99	10	322	97	10	263	79	10	207	62	10
Female																	
1	193	10	196	102	10	195	101	10	193	100	10	194	101	10	194	101	10
8	202	10	209	104	10	208	103	10	204	101	10	203	101	10	199	99	10
15	211	10	219	104	10	212	101	10	212	101	10	208	99	10	178	84	10
22	219	10	226	103	10	225	103	10	222	101	10	205	94	10	164	75	10
29	227	10	237	104	10	236	104	10	227	100	10	199	88	10	145	64	10

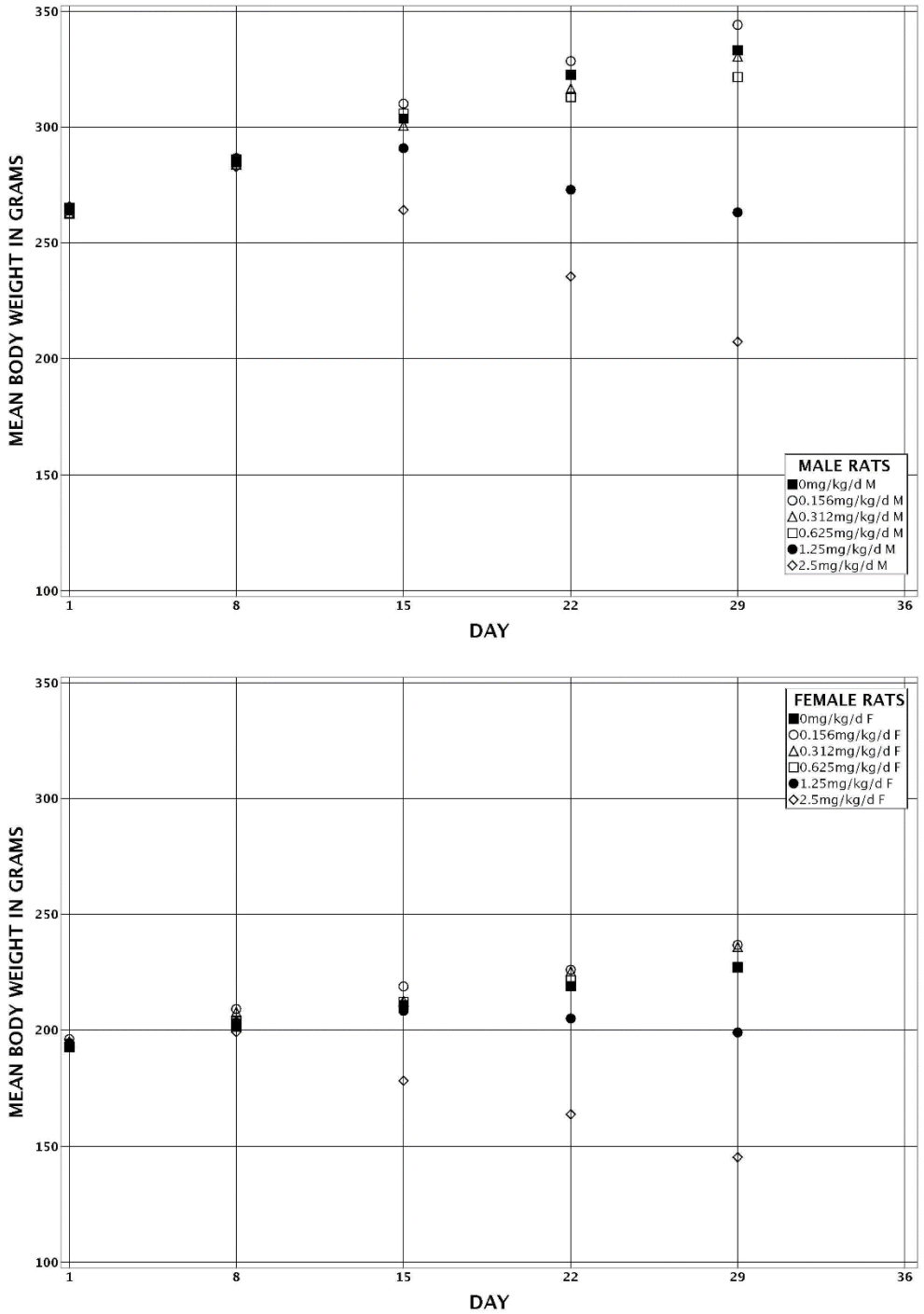


Figure 4. Growth Curves for Rats Administered Perfluorodecanoic Acid by Gavage for 28 Days

Table 28. Perfluorodecanoic Acid Concentrations in Tissues of Rats in the 28-day Gavage Study of Perfluorodecanoic Acid[a]

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Millimolar Dose (mmol/kg/day)	0	0.0003	0.00061	0.0012	0.0024	0.0049
Male						
n	10	10	10	10	10	10
Plasma						
Plasma Concentration (ng/mL)	21.9 ± 3.9	8,505.0 ± 577.8**	23,030.0 ± 1,770.5**	42,720.0 ± 2,959.9**	101,580.0 ± 4,009.2**	259,400.0 ± 20,196.4**
Plasma Concentration (µM)	0.0 ± 0.0	16.5 ± 1.1**	44.8 ± 3.4**	83.1 ± 5.8**	197.6 ± 7.8**	504.6 ± 39.3**
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	54,519.2 ± 3,703.9	73,814.1 ± 5,674.7	68,352.0 ± 4,735.9	81,264.0 ± 3,207.4	103,760.0 ± 8,078.5
Liver						
Liver Concentration (ng/mL)[b]	BD	44,680.0 ± 1,485.4	87,150.0 ± 1,674.6	163,900.0 ± 2,798.6	308,800.0 ± 8,176.9	403,600.0 ± 13,838.1
Liver Concentration (µM)	BD	86.9 ± 2.9	169.5 ± 3.3	318.8 ± 5.4	600.7 ± 15.9	785.1 ± 26.9
Normalized Liver Concentration (µM/mmol/kg/day)	NA	286,410.3 ± 9,521.8	279,326.9 ± 5,367.3	262,240.0 ± 4,477.8	247,040.0 ± 6,541.5	161,440.0 ± 5,535.2
Liver/Plasma Ratio	NA	5.49 ± 0.43	4.04 ± 0.40	4.00 ± 0.27	3.09 ± 0.15	1.63 ± 0.12
Female						
n	10	9	10	10	10	8
Plasma						
Plasma Concentration (ng/mL)	42.1 ± 16.8	11,207.8 ± 436.2**	25,700.0 ± 1,048.2**	50,290.0 ± 3,309.1**	117,150.0 ± 6,498.1**	246,875.0 ± 13,291.3**
Plasma Concentration (µM)	0.1 ± 0.0	21.8 ± 0.8**	50.0 ± 2.0**	97.8 ± 6.4**	227.9 ± 12.6**	480.2 ± 25.9**
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	71,844.7 ± 2,796.2	82,371.8 ± 3,359.5	80,464.0 ± 5,294.5	93,720.0 ± 5,198.5	98,750.0 ± 5,316.5

**Significantly different ($p \leq 0.01$) from the vehicle control group by Shirley's or Dunn's test.
BD = below detection; group did not have more than 20% of its values above the limit of quantification and no statistical analyses were conducted for the endpoint; NA = not applicable; value could not be calculated when dose value was 0.
[a]Tissue concentration data are presented as mean ± standard error. If more than 20% of the values in a group were above the limit of quantification (plasma LOQ = 25 ng/mL; liver LOQ = 500 ng/g), values that were below the limit of quantification were substituted with one-half the limit of quantification value. One-half the dose was administered twice daily.
[b]Density is assumed to be 1.0 g/mL.

There were dose-related, significant increases in the mean absolute and relative liver weights in treated male and female rats compared to those of the respective vehicle control groups (Table 29).

The mean relative kidney weights of males and females administered 0.625 mg/kg/day or greater were significantly greater than those of the respective vehicle control groups (Table 29). The mean absolute kidney weights of 2.5 mg/kg/day males and females were significantly less than those of the respective vehicle control groups; however, those of 0.312 and 0.625 mg/kg/day females were significantly greater.

The mean absolute spleen weight of 1.25 mg/kg/day males and the mean absolute and relative spleen weights of 2.5 mg/kg/day males were significantly less than those of the vehicle control group (Table 29). The mean relative spleen weight of 0.625 mg/kg/day females and the absolute and relative spleen weights of 1.25 and 2.5 mg/kg/day females were significantly less than those of the vehicle control group.

The mean absolute and relative thymus weights of 1.25 and 2.5 mg/kg/day males and 2.5 mg/kg/day females were significantly less than those of the respective vehicle control groups (Table 29).

The mean relative thyroid gland weights of the 1.25 and 2.5 mg/kg/day males were significantly greater than that of the vehicle control group (Table 29). The mean absolute and relative thyroid gland weights of 0.312, 0.625, and 1.25 mg/kg/day females and mean relative thyroid gland weight of 2.5 mg/kg/day females were significantly greater than those of the vehicle control group.

The mean absolute adrenal gland weights of all dosed males and 2.5 mg/kg/day females and the mean relative adrenal gland weight of 2.5 mg/kg/day males were significantly less than those of the respective vehicle control groups (Table 29).

The mean absolute testis weight of 2.5 mg/kg/day males was significantly less than that of the vehicle control group (Table 29).

Table 29. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Perfluorodecanoic Acid[a]

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
n	10	10	10	10	10	10
Male						
Necropsy Body Wt.	333 ± 6	344 ± 7	331 ± 6	322 ± 5	263 ± 4**	207 ± 8**
R. Adrenal Gland						
Absolute	0.0214 ± 0.0007	0.0182 ± 0.0007*	0.0200 ± 0.0007*	0.0185 ± 0.0009**	0.0169 ± 0.0006**	0.0176 ± 0.0009**
Relative	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.09 ± 0.01**
R. Kidney						
Absolute	0.96 ± 0.03	1.01 ± 0.02	0.95 ± 0.02	1.04 ± 0.02	0.94 ± 0.03	0.86 ± 0.02*
Relative	2.87 ± 0.04	2.93 ± 0.04	2.87 ± 0.04	3.22 ± 0.07**	3.56 ± 0.09**	4.17 ± 0.07**
Liver						
Absolute	11.87 ± 0.51	13.54 ± 0.40*	14.10 ± 0.38**	14.65 ± 0.35**	14.40 ± 0.20**	14.11 ± 0.70**
Relative	35.50 ± 0.97	39.32 ± 0.53**	42.61 ± 0.56**	45.56 ± 0.84**	54.77 ± 0.68**	67.90 ± 1.19**
Spleen						
Absolute	0.624 ± 0.023	0.691 ± 0.030	0.622 ± 0.018	0.596 ± 0.020	0.460 ± 0.013**	0.319 ± 0.023**
Relative	1.87 ± 0.05	2.00 ± 0.05	1.88 ± 0.04	1.86 ± 0.06	1.75 ± 0.05	1.52 ± 0.06**
R. Testis						
Absolute	1.777 ± 0.057[b]	1.797 ± 0.048	1.742 ± 0.038	1.740 ± 0.030	1.695 ± 0.034	1.553 ± 0.062**
Thymus						
Absolute	0.393 ± 0.013	0.397 ± 0.010	0.392 ± 0.022	0.391 ± 0.011	0.221 ± 0.015**	0.099 ± 0.014**
Relative	1.18 ± 0.03	1.16 ± 0.03	1.18 ± 0.05	1.22 ± 0.03	0.84 ± 0.05**	0.46 ± 0.05**
Thyroid						
Absolute	0.0239 ± 0.0012	0.0236 ± 0.0013	0.0242 ± 0.0029	0.0253 ± 0.0017	0.0268 ± 0.0012	0.0207 ± 0.0010
Relative	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.01	0.08 ± 0.01	0.10 ± 0.01**	0.10 ± 0.00**

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Female						
Necropsy Body Wt.	227 ± 4	237 ± 4	236 ± 3	227 ± 5.1	199 ± 4**	145 ± 4**
R. Adrenal Gland						
Absolute	0.0275 ± 0.0009	0.0286 ± 0.0017	0.0277 ± 0.0015	0.0289 ± 0.0005	0.0239 ± 0.0014	0.0176 ± 0.0010**
Relative	0.12 ± 0.00	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.00	0.12 ± 0.01	0.12 ± 0.01
R. Kidney						
Absolute	0.68 ± 0.01	0.72 ± 0.01	0.74 ± 0.01**	0.78 ± 0.01**	0.72 ± 0.02	0.58 ± 0.01**
Relative	3.00 ± 0.06	3.06 ± 0.04	3.15 ± 0.05	3.44 ± 0.06**	3.61 ± 0.08**	4.01 ± 0.08**
Liver						
Absolute	7.63 ± 0.28	8.94 ± 0.36**	9.46 ± 0.16**	10.06 ± 0.31**	10.09 ± 0.18**	9.85 ± 0.32**
Relative	33.52 ± 0.75	37.66 ± 0.89**	40.08 ± 0.56**	44.25 ± 0.82**	50.84 ± 0.67**	67.75 ± 0.90**
Spleen						
Absolute	0.591 ± 0.017	0.596 ± 0.021	0.582 ± 0.021	0.538 ± 0.017	0.377 ± 0.019**	0.264 ± 0.018**
Relative	2.60 ± 0.04	2.52 ± 0.06	2.46 ± 0.07	2.36 ± 0.05*	1.89 ± 0.06**	1.81 ± 0.10**
Thymus						
Absolute	0.290 ± 0.016	0.304 ± 0.020	0.342 ± 0.035	0.316 ± 0.016	0.233 ± 0.017	0.102 ± 0.015**
Relative	1.28 ± 0.07	1.29 ± 0.09	1.44 ± 0.14	1.39 ± 0.06	1.18 ± 0.10	0.69 ± 0.09**
Thyroid						
Absolute	0.0253 ± 0.0010	0.0288 ± 0.0019	0.0336 ± 0.0016**	0.0340 ± 0.0022**	0.0321 ± 0.0023*	0.0236 ± 0.0012
Relative	0.11 ± 0.00	0.12 ± 0.01	0.14 ± 0.01*	0.15 ± 0.01**	0.16 ± 0.01**	0.16 ± 0.01**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b]n = 9.

In male and female rats, reticulocyte counts were significantly decreased in the 1.25 and 2.5 mg/kg/day dose groups, decreasing by approximately 90% in the 2.5 mg/kg/day groups compared to the respective vehicle control groups (Table 30). These decreases generally caused significant decreases in the mean cell volumes and significant increases in the mean cell hemoglobin concentrations in the same dose groups. In addition, the erythrocyte count was increased in 2.5 mg/kg/day male rats compared to the vehicle control group; the erythrocyte counts, hematocrit, and hemoglobin concentrations were significantly increased in 1.25 and 2.5 mg/kg/day females compared to the vehicle control group. The platelet count was decreased in the 2.5 mg/kg/day females compared to the vehicle control group.

Urea nitrogen concentrations were increased in 1.25 and 2.5 mg/kg/day males and females compared to the vehicle control groups (Table 30). Glucose concentrations were decreased in 1.25 and 2.5 mg/kg/day males and females compared to the vehicle control groups, and creatinine concentrations were significantly decreased in 1.25 mg/kg/day males and 2.5 mg/kg/day males and females; these decreases were likely related to the decreased food intake and body weight. In males, the cholesterol concentrations were decreased in all dose groups, although not to the level of significance in the two highest dose groups; the triglyceride concentrations were also significantly decreased in 1.25 and 2.5 mg/kg/day males compared to the vehicle control group. The mean cholesterol concentration was significantly decreased in 2.5 mg/kg females compared to the vehicle control group.

Significant dose-dependent decreases were observed in total protein and globulin concentrations in male rats compared to the vehicle control group (Table 30). The albumin concentrations were also significantly decreased in 1.25 and 2.5 mg/kg/day males compared to the vehicle control group; the overall effect of these changes resulted in an increase in the albumin/globulin ratio for all male dose groups. The globulin concentration was significantly decreased and the albumin/globulin ratio significantly increased in 0.312 mg/kg/day or greater females compared to the vehicle control group.

ALT, AST, and ALP activities were generally significantly increased in 0.312 mg/kg and greater males compared to the vehicle control group (Table 30). Total bilirubin and bile acid concentrations were significantly elevated in 1.25 and 2.5 mg/kg/day males compared to the vehicle control group; the total bilirubin elevations were driven by significant increases in both direct and indirect bilirubin concentrations. ALP activity was significantly increased in 0.312 mg/kg or greater females compared to the vehicle control group. ALT and AST activities, as well as bile acid and total bilirubin concentrations, were increased in 1.25 and 2.5 mg/kg/day females. Similar to the males, the bilirubin elevations were driven by both the direct and indirect bilirubin concentrations. Creatinine kinase activity was significantly increased in 1.25 and 2.5 mg/kg/day females compared to the vehicle control group.

Free T4 concentrations were significantly decreased in 0.312 mg/kg/day and greater males and 1.25 and 2.5 mg/kg/day females compared to the respective vehicle control groups (Table 30).

Male and female rats administered PFDA exhibited a significant increase in gene expression of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* compared to respective controls indicating a significant increase in PPARα and CAR activity (Table 31). Except for *Cyp4a1* in females, genes of interest were significantly increased in males and females at all doses.

Table 30. Selected Hematology and Clinical Chemistry Data for Rats in the 28-day Gavage Study of Perfluorodecanoic Acid[a]

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Male						
Hematology						
n	10	10	10	10	10	10
Erythrocytes (10^6/μL)	8.62 ± 0.09	8.72 ± 0.08	8.56 ± 0.08	8.77 ± 0.09	8.91 ± 0.17	9.78 ± 0.26**
Reticulocytes (10^3/μL)	152.8 ± 9.5	164.2 ± 6.2	143.3 ± 6.2	137.5 ± 7.0	69.7 ± 9.4**	13.0 ± 3.1**
Mean Cell Volume (fL)	60.6 ± 0.4	60.7 ± 0.4	60.3 ± 0.3	59.7 ± 0.5	58.6 ± 0.4**	56.4 ± 0.4**
Mean Cell Hemoglobin Concentration (g/dL)	30.3 ± 0.1	30.6 ± 0.1	30.7 ± 0.1	30.8 ± 0.1**	31.1 ± 0.1**	31.3 ± 0.2**
Clinical Chemistry						
n	10	10	10	10	10	9
Urea Nitrogen (mg/dL)	18.2 ± 0.9	16.5 ± 0.4	15.9 ± 0.4	19.1 ± 0.6	22.8 ± 0.9**	22.8 ± 1.5*
Creatinine (mg/dL)	0.71 ± 0.02	0.71 ± 0.02	0.74 ± 0.02	0.65 ± 0.02	0.63 ± 0.02*	0.63 ± 0.02*
Glucose (mg/dL)	214 ± 21	203 ± 17	252 ± 24	180 ± 9	148 ± 10.0**	119 ± 6**
Total Protein (g/dL)	6.4 ± 0.1	6.2 ± 0.1*	6.1 ± 0.1*	5.8 ± 0.1**	5.3 ± 0.1**	4.6 ± 0.1**
Albumin (g/dL)	4.2 ± 0.0	4.3 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	3.9 ± 0.1**	3.3 ± 0.1**
Globulin (g/dL)	2.2 ± 0.1	1.9 ± 0.0**	1.8 ± 0.1**	1.6 ± 0.1**	1.4 ± 0.1**	1.3 ± 0.1**
Albumin/Globulin Ratio	1.9 ± 0.1	2.2 ± 0.1**	2.5 ± 0.1**	2.7 ± 0.2**	2.9 ± 0.2**	2.6 ± 0.1**
Total Bilirubin (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0**	0.6 ± 0.1**
Direct Bilirubin (mg/dL)	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.01**	0.23 ± 0.04**
Indirect Bilirubin (mg/dL)	0.108 ± 0.007	0.120 ± 0.008	0.116 ± 0.006	0.128 ± 0.012	0.148 ± 0.007**	0.383 ± 0.054**[c]
Cholesterol (mg/dL)	107 ± 5	78 ± 3*	66 ± 2**	78 ± 2**	94 ± 4	92 ± 8
Triglycerides (mg/dL)	112 ± 10	128 ± 12	109 ± 10	88 ± 7	72 ± 6**	53 ± 8**
Alanine Aminotransferase (IU/L)	58 ± 4	70 ± 3	84 ± 9*	84 ± 5**	74 ± 6	62 ± 4
Alkaline Phosphatase (IU/L)	196 ± 8	213 ± 10	240 ± 8**	276 ± 11**	372 ± 32**	275 ± 18**

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Aspartate Aminotransferase (IU/L)	65 ± 3	74 ± 3*	77 ± 5*	81 ± 3**	88 ± 5**	93 ± 3**
Bile Salts/Acids (µmol/L)	16.9 ± 3.7	8.0 ± 1.7	10.3 ± 2.0	23.2 ± 3.6	91.2 ± 11.6**	220.8 ± 30.9**[b]
Thyroid Stimulating Hormone (ng/mL)	19.79 ± 3.75	16.16 ± 1.88	17.73 ± 2.70	15.52 ± 2.50	11.63 ± 1.18[c]	8.97 ± 2.28[d]
Triiodothyronine (ng/dL)	95.86 ± 4.01	72.87 ± 2.66	66.14 ± 2.84	74.74 ± 5.45	147.95 ± 9.69	179.99 ± 13.73[e]
Free Thyroxine (ng/dL)	2.02 ± 0.21	1.90 ± 0.22	1.17 ± 0.11**	1.13 ± 0.06**	0.65 ± 0.05**	0.36 ± 0.05**[e]
Total Thyroxine (µg/dL)	4.36 ± 0.32	4.27 ± 0.23	3.24 ± 0.18*	3.82 ± 0.09	4.59 ± 0.26	4.64 ± 0.15
Testosterone (ng/mL)	2.59 ± 0.65	6.36 ± 1.69	3.42 ± 0.87	1.94 ± 0.41	0.93 ± 0.19*	0.65 ± 0.26*[e]
Female						
Hematology						
n	10	8	10	10	10	10
Hematocrit (%)	47.7 ± 0.6	46.5 ± 0.6	46.6 ± 0.4	47.6 ± 0.4	50.5 ± 0.7*	54.4 ± 1.3**
Manual Hematocrit (%)	43 ± 1	42 ± 1	42 ± 0	43 ± 1	46 ± 1**	50 ± 1**[c]
Hemoglobin (g/dL)	14.8 ± 0.2	14.5 ± 0.2	14.5 ± 0.1	14.7 ± 0.1	15.8 ± 0.2**	17.6 ± 0.5**
Erythrocytes (10^6/µL)	7.90 ± 0.09	7.80 ± 0.11	7.74 ± 0.05	7.91 ± 0.07	8.61 ± 0.16*	9.70 ± 0.23**
Reticulocytes (10^3/µL)	190.3 ± 14.6	180.8 ± 8.6	183.2 ± 12.1	179.1 ± 11.0	71.6 ± 7.6**	19.3 ± 5.3**
Mean Cell Volume (fL)	60.4 ± 0.4	59.6 ± 0.3	60.2 ± 0.4	60.2 ± 0.4	58.7 ± 0.5*	56.1 ± 0.5**
Mean Cell Hemoglobin Concentration (g/dL)	31.1 ± 0.1	31.3 ± 0.1	31.2 ± 0.2	30.8 ± 0.1	31.2 ± 0.2	32.4 ± 0.3**
Clinical Chemistry						
n	9	9	10	10	10	7
Urea Nitrogen (mg/dL)	16.7 ± 0.8	16.0 ± 0.7	16.3 ± 0.6	18.5 ± 1.3	23.1 ± 0.5**	25.0 ± 1.5**
Creatinine (mg/dL)	0.77 ± 0.02	0.74 ± 0.02	0.73 ± 0.02	0.75 ± 0.02	0.74 ± 0.02	0.69 ± 0.03*
Glucose (mg/dL)	210 ± 7	192 ± 9	187 ± 10	204 ± 10	145 ± 7**	103 ± 8**
Total Protein (g/dL)	6.3 ± 0.1	6.5 ± 0.2	6.5 ± 0.1	6.5 ± 0.1	6.2 ± 0.1	5.6 ± 0.3

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Albumin (g/dL)	4.4 ± 0.0	4.7 ± 0.1	4.9 ± 0.1**	5.0 ± 0.1**	4.7 ± 0.1	4.0 ± 0.1
Globulin (g/dL)	1.9 ± 0.1	1.7 ± 0.1	1.5 ± 0.1**	1.5 ± 0.1**	1.5 ± 0.1**	1.6 ± 0.2**
Albumin/Globulin Ratio	2.4 ± 0.1	2.8 ± 0.1**	3.2 ± 0.1**	3.5 ± 0.1**	3.2 ± 0.1**	2.7 ± 0.4**
Total Bilirubin (mg/dL)	0.1 ± 0.0[f]	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0*	0.6 ± 0.1**
Direct Bilirubin (mg/dL)	0.02 ± 0.00[f]	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00**	0.19 ± 0.06**
Indirect Bilirubin (mg/dL)	0.101 ± 0.003	0.094 ± 0.008	0.090 ± 0.005	0.087 ± 0.006	0.111 ± 0.004	0.379 ± 0.026**
Cholesterol (mg/dL)	99 ± 4	100 ± 7	91 ± 4	99 ± 4	91 ± 5	65 ± 8**
Alanine Aminotransferase (IU/L)	48 ± 3	47 ± 1	49 ± 3	54 ± 2	69 ± 4**	57 ± 5*
Alkaline Phosphatase (IU/L)	136 ± 6	156 ± 8	183 ± 12**	184 ± 11**	281 ± 23**	262 ± 23**
Aspartate Aminotransferase (IU/L)	63 ± 3	60 ± 2	58 ± 1	63 ± 2	82 ± 3**	113 ± 9**
Creatine Kinase (IU/L)	87 ± 4	82 ± 5	87 ± 3	91 ± 3	107 ± 5**	158 ± 15**
Bile Salts/Acids (µmol/L)	13.0 ± 3.5	12.2 ± 2.9	20.2 ± 1.9	17.4 ± 3.2	39.7 ± 7.3**	98.6 ± 31.4**
Thyroid Stimulating Hormone (ng/mL)	12.10 ± 0.96	15.44 ± 1.23	15.36 ± 1.21	12.50 ± 0.99	16.28 ± 2.34	15.42 ± 1.72[g]
Triiodothyronine (ng/dL)	100.68 ± 8.81	107.27 ± 6.41	97.14 ± 4.51	106.02 ± 5.72	124.45 ± 10.26*	210.61 ± 37.65**[d]
Free Thyroxine (ng/dL)	1.78 ± 0.22	2.14 ± 0.19	2.35 ± 0.16	1.95 ± 0.22	1.093 ± 0.08*	0.47 ± 0.02*[d]
Total Thyroxine (µg/dL)	3.87 ± 0.35	4.28 ± 0.31	4.20 ± 0.25	3.92 ± 0.37	3.53 ± 0.21	4.39 ± 0.14
Testosterone (ng/mL)	0.31 ± 0.02	0.36 ± 0.02	0.41 ± 0.03*	0.54 ± 0.06**	0.76 ± 0.09**	1.41 ± 0.19**[h]

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error.
[b]n = 8.
[c]n = 9.
[d]n = 3.
[e]n = 6.
[f]n = 10.
[g]n = 2.
[h]n = 4.

Table 31. Hepatic Parameters for Rats in the 28-day Gavage Study of Perfluorodecanoic Acid[a]

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Male						
n	9	10	10	10	10	10
Enzyme Activity						
Acetyl-CoA (nmol/min/mg)	1.892 ± 0.051[b]	2.299 ± 0.055**	4.580 ± 0.088**	9.364 ± 0.367**	11.856 ± 0.321**	7.645 ± 0.335**
Gene Expression						
Acox1	1.03 ± 0.10	1.70 ± 0.17**	4.39 ± 0.39**	6.39 ± 0.62**	8.16 ± 0.47**	9.34 ± 0.73**
Cyp4a1	1.04 ± 0.10	10.85 ± 1.11**	33.46 ± 3.52**	41.12 ± 4.19**	62.69 ± 4.39**	38.19 ± 3.64**
Cyp2b1	1.06 ± 0.21	3.33 ± 0.57**	6.17 ± 0.85**	8.24 ± 1.36**	10.87 ± 1.50**	4.07 ± 0.64**
Cyp2b2	1.00 ± 0.13	4.24 ± 0.52**	5.94 ± 0.61**	6.26 ± 0.75**	10.91 ± 0.86**	6.33 ± 0.96**
Female						
n	10	10	10	10	10	10
Gene Expression						
Acox1	1.06 ± 0.12	1.58 ± 0.12*	2.30 ± 0.16**	3.50 ± 0.35**	5.46 ± 0.45**	5.73 ± 0.67**
Cyp4a1	1.09 ± 0.14	1.42 ± 0.17	1.47 ± 0.18	3.71 ± 0.91**	18.76 ± 1.91**	10.17 ± 1.53**
Cyp2b1	1.44 ± 0.37	14.39 ± 4.01**	45.16 ± 9.69**	49.71 ± 7.67**	128.63 ± 19.57**	33.14 ± 5.68**
Cyp2b2	1.47 ± 0.42	12.28 ± 2.95**	26.48 ± 3.15**	32.89 ± 3.76**	52.84 ± 5.30**	16.79 ± 3.48**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's or Dunn's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values.
[b]n = 10.

Male rats assessed for reproductive toxicity were administered 0, 0.625, 1.25, or 2.5 mg PFDA/kg/day for 28 days, which is much less than the time needed to make a full assessment of the effect on spermatogenesis. Cauda epididymal sperm counts in rats administered 1.25 or 2.5 mg/kg/day were lower than vehicle controls (17% and 30% respectively; statistically significant at 2.5 mg/kg) and occurred in the presence of lower epididymal weights (10% and 23% respectively; statistically significant) (Table 32). Exfoliated germ cell in the duct of the epididymis was observed in four rats administered 2.5 mg/kg/day. Males administered 0.625, 1.25, or 2.5 mg/kg/day had lower serum testosterone levels (25%, 64%, and 75%; statistically significant at 2.5 mg/kg/day) (Table 30). Testis weight was lower in the 2.5 mg/kg/day group (11%; statistically significant). Spermatid and spermatid/g testis counts in this group were similar to the vehicle control group. Multiple histopathologic findings in the testis were noted in rats exposed to 2.5 mg/kg/day (degeneration, spermatid retention, interstitial cell atrophy; interstitial atrophy was also noted in eight rats in the 1.25 mg/kg/day group) (Table 34).

Table 32. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Perfluorodecanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
n	10	10	10	10
Weights (g)				
Necropsy Body Wt.	333 ± 6	322 ± 5	263 ± 4**	207 ± 8**
L. Cauda Epididymis	0.184 ± 0.005	0.178 ± 0.004	0.164 ± 0.006*	0.138 ± 0.009**
L. Epididymis	0.528 ± 0.015	0.508 ± 0.009	0.474 ± 0.013*	0.407 ± 0.024**
L. Testis	1.786 ± 0.060	1.777 ± 0.031	1.730 ± 0.031	1.594 ± 0.047**
Spermatid Measurements				
Spermatid Heads (10^6/testis)	230.3 ± 12.5	208.2 ± 8.8	181.6 ± 10.7*	217.0 ± 7.5
Spermatid Heads (10^6/g testis)	129.8 ± 7.8	117.0 ± 3.8	104.8 ± 5.6*	137.3 ± 6.3
Epididymal Spermatozoal Measurements				
Sperm Motility (%)	85.7 ± 0.7	85.5 ± 1.0	84.1 ± 0.7	76.2 ± 7.8
Sperm (10^6/cauda epididymis)	136.3 ± 10.2	120.8 ± 5.5	112.9 ± 7.3	95.7 ± 11.5**
Sperm (10^6/g cauda epididymis)	735.9 ± 41.8	678.6 ± 30.6	687.1 ± 36.4	672.9 ± 78.2

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test (weights) or Shirley's or Dunn's test (spermatid and epididymal spermatozoal measurements).
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error.

Female rats administered 0, 0.625, 1.25, or 2.5 mg PFDA/kg/day were assessed for reproductive toxicity. Inspection of the daily vaginal cytology data indicated that none of the 2.5 mg/kg/day rats were cycling, (93.8% of the time in diestrus) (

Table 33). Markov analyses demonstrated that rats administered 1.25 or 2.5 mg/kg/day had a higher probability than the control group of transitioning to extended diestrus from normal cycling (Table A-5; Figure A-4). Females administered 0.312 mg/kg/day or greater had significantly higher serum testosterone levels (41%, 54%, 76%, and 141%, respectively) relative to the vehicle control group (Table 30).

Table 33. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Perfluorodecanoic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Number Weighed at Necropsy	10	10	10	10
Necropsy Body Wt. (g)	227 ± 4	227 ± 5	199 ± 4**	145 ± 4**
Proportion of Regular Cycling Females[b]	8/10	9/10	7/10	0/10
Estrous Cycle Length (days)	5.3 ± 0.57	4.8 ± 0.14	5.6 ± 0.28	0[c]
Estrous Stages (% of cycle)				
Diestrus	57.5	63.1	73.1	93.8
Proestrus	3.1	6.3	6.9	0.6
Estrus	34.4	26.9	20.0	5.6

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Metestrus	3.1	3.8	0.0	0.0
Uncertain	1.9	0.0	0.0	0.0

**Significantly different ($p \leq 0.01$) from the vehicle control group by Dunnett's test (body weight).
[a]Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunn's test (estrous cycle length). Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated that 1.25 and 2.5 mg/kg/day females had extended diestrus.
[b]Number of females with a regular cycle/number of females cycling.
[c]No cycles observed.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following the Wyeth-14,643 results.

Liver: The incidences of hepatocyte cytoplasmic alteration were significantly increased in 0.625 mg/kg/day or greater males and females compared to the respective vehicle control groups (Table 34). The incidences of hepatocyte hypertrophy in 1.25 and 2.5 mg/kg/day males and females, cytoplasmic vacuolization in 1.25 and 2.5 mg/kg/day males and 2.5 mg/kg/day females, and hepatocyte, necrosis in 2.5 mg/kg/day females were significantly greater than those of the respective vehicle control groups. The severity of the hepatocellular lesions generally increased with increasing dose. Hepatocyte cytoplasmic alteration was mostly of marked severity in the 1.25 and 2.5 mg/kg/day males and minimal to mild severity in the females. Hepatocyte hypertrophy was generally of mild to marked severity in males and minimal to moderate in females. In general, hepatocyte cytoplasmic vacuolization was mostly of mild to moderate severity in males, and moderate to marked in females.

Table 34. Incidences of Selected Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Perfluorodecanoic Acid

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Male						
Liver[a]	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration[b]	0	0	0	10** (1.1)[c]	10** (4.0)	10** (3.8)
Hepatocyte, Hypertrophy	0	0	0	2 (1.5)	10** (3.1)**	10** (4.0)
Hepatocyte, Vacuolization Cytoplasmic	0	0	0	0	9** (1.9)	10** (2.9)
Stomach, Forestomach	(10)	(10)	(10)	(10)	(10)	(10)
Inflammation	0	0	0	0	0	2 (2.0)
Ulcer	0	0	0	0	0	1 (1.0)
Epithelium, Hyperplasia	0	0	0	0	0	2 (2.5)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	0	0	0	0	10** (2.5)
Thymus	(10)	(10)	(10)	(10)	(10)	(10)
Lymphocyte, Apoptosis	0	0	0	0	8** (1.3)	0
Atrophy	0	0	0	0	0	9** (2.8)
Testes	(10)	(10)	(10)	(10)	(10)	(10)
Interstitial Cell, Atrophy	0	0	0	0	8** (1.6)	10** (1.5)
Seminiferous Tubule, Spermatid Retention	0	0	0	0	0	4* (2.0)
Germinal Epithelium, Degeneration	1 (1.0)	0	0	0	0	4 (1.8)
Epididymis	(10)	(0)	(0)	(10)	(10)	(10)
Duct, Exfoliated Germ Cell	1 (1.0)	0	0	0	0	4 (2.0)

	Vehicle Control	0.156 mg/kg/day	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
Female						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	0	0	8** (1.0)	10** (1.3)	10** (1.6)
Hepatocyte, Hypertrophy	0	0	0	0	8** (1.1)	10** (2.9)
Hepatocyte, Vacuolization Cytoplasmic	0	0	0	0	1 (1.0)	10** (3.2)
Hepatocyte, Necrosis	0	0	0	0	1 (1.0)	4* (1.0)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	0	0	0	0	10** (2.5)
Thymus	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy	0	0	0	0	0	8** (2.8)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Bone Marrow: The incidences of bone marrow hypocellularity were significantly increased in 2.5 mg/kg/day males and females compared to the respective vehicle control groups (Table 34).

Thymus: The incidences of lymphocyte apoptosis in the thymus of 1.25 mg/kg/day males and atrophy of the thymus in 2.5 mg/kg/day males and females were significantly increased compared to the respective vehicle control groups (Table 34).

Testis and Epididymis: The incidences of interstitial cell atrophy of the testes were significantly increased in 1.25 and 2.5 mg/kg/day males compared to the vehicle control group (Table 34). The incidence of seminiferous tubule spermatid retention was significantly increased in 2.5 mg/kg/day males. The incidences of minimal germinal epithelium degeneration in the testes and epididymis, duct, exfoliated germ cells were increased in 2.5 mg/kg/day males; although these increases were not statistically significant, they may be an indication of toxicity in the testes. Germinal epithelium degeneration in the testes and in the epididymis, duct, exfoliated germ cells occurred in the same rats. The severities in three of the four rats with germinal epithelium degeneration or epididymis, duct, exfoliated germ cells were minimal or mild, and moderate or severe in the fourth rat, respectively.

Forestomach: Incidences of epithelial hyperplasia, ulcer, and inflammation in the forestomach were increased in the 2.5 mg/kg/day males compared to the vehicle control group; however, the incidences were not statistically significant (Table 34).

Wyeth-14,643

Wyeth-14,643 (WY) was used as a positive control for PPARα agonists. In the WY study, all rats survived to scheduled euthanasia (Table 35). There were no significant treatment-related clinical observations in male or female rats. The mean body weights of dose groups of males and females were within 10% of the respective vehicle control groups throughout the study (Table 35; Figure 5).

Table 35. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Wyeth-14,643

Day	Vehicle Control		6.25 mg/kg/day			12.5 mg/kg/day			25 mg/kg/day		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
Male											
1	271	10	273	101	10	272	100	10	272	100	10
8	291	10	297	102	10	292	101	10	293	101	10
15	310	10	310	100	10	303	98	10	300	97	10
22	321	10	320	100	10	313	97	10	309	96	10
29	336	10	324	96	10	316	94	10	308	92	10
Female											
1	197	10	198	100	10	198	100	10	198	100	10
8	204	10	208	102	10	208	102	10	209	102	10
15	215	10	218	102	10	216	101	10	216	101	10
22	220	10	224	102	10	225	103	10	224	102	10
29	224	10	230	103	10	229	102	10	229	103	10

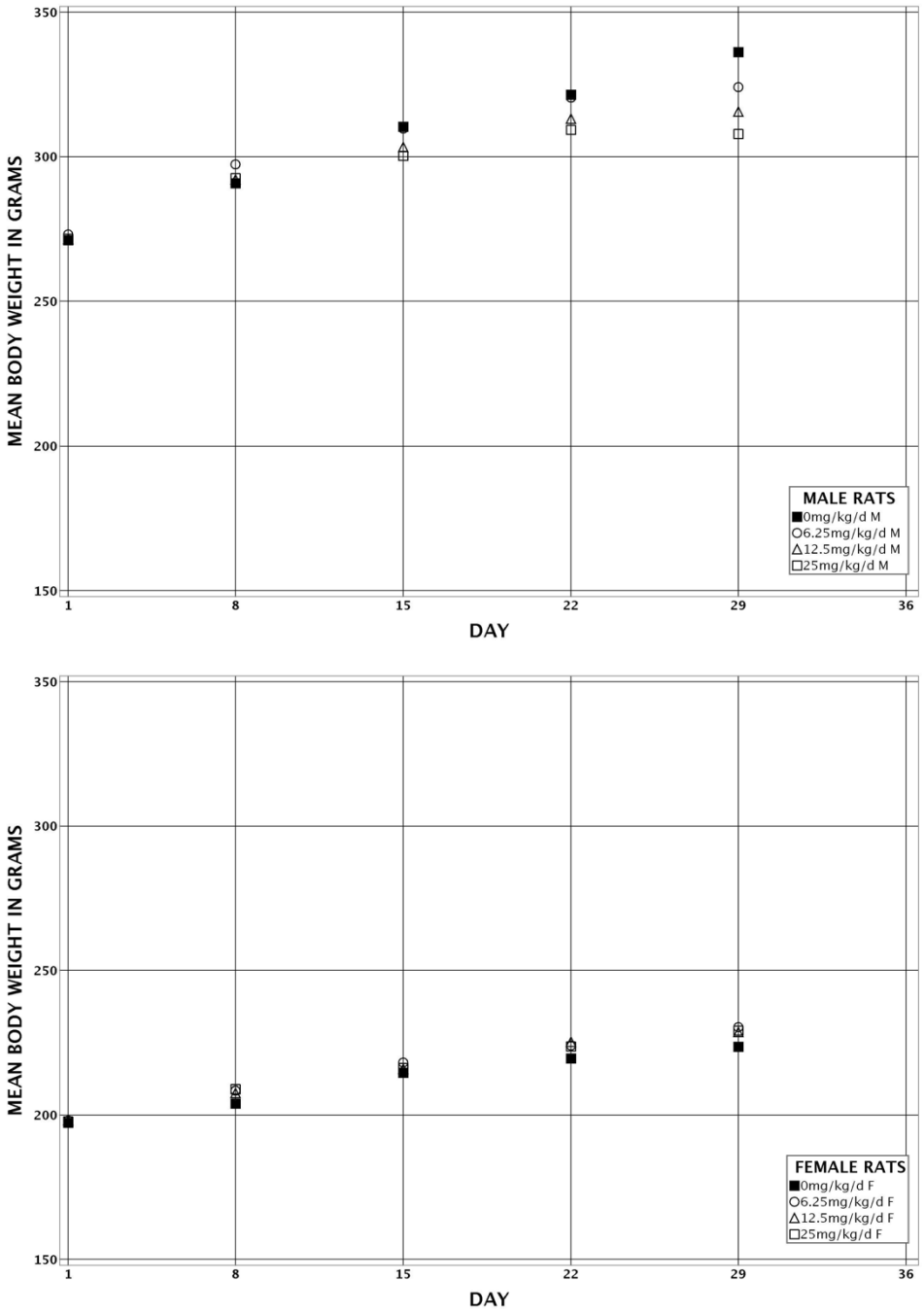


Figure 5. Growth Curves for Rats Administered Wyeth-14,643 by Gavage for 28 Days

In all WY exposed male and female groups, compared to vehicle controls, there were dose-related significant increases in the absolute and relative liver and right kidney weights (Table 36). Changes in liver weights appear to correlate with histopathologic changes in the liver. Absolute heart weight in 25 mg/kg/day males and absolute spleen weight in 6.25 and 25 mg/kg/day males were significantly lower compared to those of the vehicle control group; biologic significance of these changes is not clear.

Table 36. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Male				
Necropsy Body Wt.	337 ± 4	324 ± 5	316 ± 5**	308 ± 5**
R. Kidney				
Absolute	1.00 ± 0.02	1.10 ± 0.04*	1.13 ± 0.03**	1.13 ± 0.02**
Relative	2.98 ± 0.07	3.38 ± 0.08**	3.59 ± 0.08**	3.68 ± 0.05**
Liver				
Absolute	11.57 ± 0.21	15.35 ± 0.31**	15.97 ± 0.49**	16.56 ± 0.44**
Relative	34.33 ± 0.49	47.35 ± 0.46**	50.55 ± 1.22**	53.90 ± 1.67**
Female				
Necropsy Body Wt.	224 ± 4	230 ± 4	229 ± 3	229 ± 5
R. Kidney				
Absolute	0.67 ± 0.01	0.74 ± 0.02**	0.75 ± 0.02**	0.76 ± 0.01**
Relative	3.02 ± 0.05	3.22 ± 0.08*	3.30 ± 0.09**	3.31 ± 0.04**
Liver				
Absolute	7.32 ± 0.19	10.25 ± 0.27**	11.07 ± 0.27**	11.98 ± 0.40**
Relative	32.78 ± 0.71	44.52 ± 0.90**	48.45 ± 1.05**	52.21 ± 1.14**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

There were several statistically significant changes, when compared to the respective vehicle control groups, in the hematology parameters of male and female rats. These changes, however, were inconsistent across dose groups and not considered toxicologically relevant.

Blood urea nitrogen concentrations were significantly increased in all male dose groups compared to the vehicle control group (Table 37). Cholesterol concentrations were significantly decreased in all male dose groups compared to the vehicle control group. In all male dose groups, the total protein and globulin concentrations were significantly decreased, and the albumin concentrations were significantly increased compared to the vehicle control group; the combination of these changes resulted in significantly increased albumin/globulin ratios in male dose groups. In female rats, globulin concentrations were significantly decreased in all dose groups compared to the vehicle control group and total protein and albumin concentrations were significantly increased in most dose groups compared to the vehicle control group; the combination of these changes resulted in significantly increased albumin/globulin ratios in all dose groups compared to the vehicle control group. ALT, ALP, and SDH activities were significantly increased in all male dose groups compared to the vehicle control group; AST was significantly increased in 25 mg/kg/day males compared to the vehicle control group. In addition, direct bilirubin concentrations were significantly increased in 25 mg/kg/day males and bile acid concentrations were significantly increased in all male dose groups compared to the

vehicle control group. In females, ALP and SDH activities were significantly increased in all dose groups and ALT and AST activities were significantly increased in the 25 mg/kg/day group.

Table 37. Selected Clinical Chemistry Data for Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Male				
Urea Nitrogen (mg/dL)	18.6 ± 0.7	20.2 ± 0.5*	21.3 ± 0.5**	23.1 ± 0.6**
Total Protein (g/dL)	6.59 ± 0.05	6.24 ± 0.13*	6.37 ± 0.05*	6.25 ± 0.07**
Albumin (g/dL)	4.30 ± 0.03	4.74 ± 0.06**	4.78 ± 0.04**	4.86 ± 0.09**
Globulin (g/dL)	2.29 ± 0.03	1.50 ± 0.07**	1.59 ± 0.04**	1.39 ± 0.04**
Albumin/Globulin Ratio	1.88 ± 0.02	3.22 ± 0.13**	3.02 ± 0.08**	3.53 ± 0.14**
Cholesterol (mg/dL)	125.0 ± 2.7	106.6 ± 5.0**	116.2 ± 4.0*	101.8 ± 3.5**
Alanine Aminotransferase (IU/L)	52.20 ± 1.32	62.80 ± 4.29*	59.50 ± 2.36*	62.70 ± 2.10**
Alkaline Phosphatase (IU/L)	202.8 ± 7.5	286.2 ± 12.2**	325.1 ± 14.7**	386.5 ± 31.2**
Aspartate Aminotransferase (IU/L)	60.00 ± 1.26	64.70 ± 3.45	62.50 ± 1.90	70.00 ± 3.35*
Sorbitol Dehydrogenase (IU/L)	5.7 ± 0.4	9.9 ± 1.4**	7.9 ± 0.5**	8.4 ± 0.4**
Direct Bilirubin (mg/dL)	0.024 ± 0.003	0.021 ± 0.002	0.025 ± 0.002	0.030 ± 0.002*
Bile Acids (µmol/L)	11.8 ± 1.6	40.9 ± 6.5**	73.1 ± 11.2**	83.2 ± 9.5**
Total Thyroxine (µg/dL)	3.58 ± 0.13	2.90 ± 0.15**	2.94 ± 0.20*	2.78 ± 0.17**
Free Thyroxine (ng/dL)	2.35 ± 0.09	1.43 ± 0.13**	1.41 ± 0.14**	1.31 ± 0.13**
Thyroid Stimulating Hormone (ng/mL)	18.56 ± 1.16	21.46 ± 3.59	15.64 ± 2.13	13.07 ± 1.01*
Testosterone (ng/mL)	4.03 ± 0.48	3.14 ± 0.91	4.37 ± 1.29	1.56 ± 0.39*
Female				
Total Protein (g/dL)	6.37 ± 0.08	6.54 ± 0.05	6.85 ± 0.12**	6.85 ± 0.08**
Albumin (g/dL)	4.46 ± 0.05	5.03 ± 0.05**	5.20 ± 0.08**	5.20 ± 0.05**
Globulin (g/dL)	1.91 ± 0.05	1.51 ± 0.04**	1.65 ± 0.05*	1.65 ± 0.05*
Albumin/Globulin Ratio	2.34 ± 0.05	3.35 ± 0.10**	3.17 ± 0.08**	3.17 ± 0.09**
Alanine Aminotransferase (IU/L)	41.80 ± 1.24	49.50 ± 3.85	46.80 ± 1.85	54.80 ± 2.58**
Alkaline Phosphatase (IU/L)	142.6 ± 6.9	181.7 ± 10.2**	179.2 ± 5.4**	217.3 ± 11.4**
Aspartate Aminotransferase (IU/L)	55.30 ± 1.08	64.70 ± 8.15	56.50 ± 1.70	61.90 ± 1.55*
Sorbitol Dehydrogenase (IU/L)	4.2 ± 0.5	9.9 ± 2.4**	7.7 ± 0.7**	8.3 ± 0.6**
Total Triiodothyronine (ng/dL)	65.66 ± 2.47	71.86 ± 4.312	83.09 ± 4.79**	83.57 ± 3.72**
Thyroid Stimulating Hormone (ng/mL)	10.07 ± 0.51	16.19 ± 2.03**	14.96 ± 1.71**	17.21 ± 1.25**
Testosterone (ng/mL)	0.33 ± 0.03	0.52 ± 0.07*	0.49 ± 0.06	0.57 ± 0.08*

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error.

Free T4 and total T4 concentrations were significantly decreased in all male dose groups compared to the vehicle control group, and TSH was significantly decreased in the 25 mg/kg/day group compared to the vehicle control group (Table 37). Additionally, testosterone concentrations were significantly decreased in 25 mg/kg/day males compared to the vehicle control group. In females, T3 and TSH concentrations were significantly increased in most dose groups compared to the vehicle control group, and testosterone concentrations were significantly increased at 6.25 and 25 mg/kg/day compared to the vehicle control group.

There were significant increases in expression of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* compared to controls indicating significantly increased PPARα and CAR activities in treated male and female rats. Males displayed a greater fold increase in *Cyp4a1* expression compared to controls than did females compared to their controls, whereas CAR increases compared to controls were more prominent in females than in males (Table 38). Acyl-CoA oxidase activity was increased in male rats at all dose levels.

Table 38. Hepatic Parameters for Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Male				
Enzyme Activity				
Acyl-CoA Oxidase (nmol/min/mg)	2.028 ± 0.044	11.731 ± 0.692**	14.560 ± 1.027**	17.370 ± 0.592**
Gene Expression				
Acox1	1.05 ± 0.10	3.54 ± 0.60**	4.77 ± 0.55**	6.86 ± 0.44**
Cyp4a1	1.06 ± 0.10	29.92 ± 4.63**	33.96 ± 2.45**	45.82 ± 3.31**
Cyp2b1	1.21 ± 0.21	3.77 ± 0.94**	3.69 ± 0.67**	4.45 ± 0.95**
Cyp2b2	1.27 ± 0.28	2.32 ± 0.23**	1.93 ± 0.20*	2.43 ± 0.19**
Female				
Gene Expression				
Acox1	1.01 ± 0.05	3.11 ± 0.26**	4.08 ± 0.27**	5.65 ± 0.49**
Cyp4a1	1.02 ± 0.07	2.90 ± 0.52**	3.85 ± 1.12**	7.26 ± 1.22**
Cyp2b1	1.18 ± 0.24	24.05 ± 4.59**	28.82 ± 3.36**	38.79 ± 4.74**
Cyp2b2	1.18 ± 0.21	19.59 ± 2.67**	29.41 ± 1.48**	30.41 ± 3.22**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values.

Male rats administered 6.25, 12.5, or 25 mg/kg/day for 28 days displayed lower testicular spermatid counts (9%, 14%, and 13%, respectively) relative to vehicle controls (Table 39). When normalized to total testicular weight, counts were slightly lower in the dose groups, with the 12.5 and 25 mg/kg/day groups exhibiting lower (11% and 10%) counts relative to the vehicle control group. These differences did not attain statistical significance, but the trend was significant. WY administration did not affect motility or testis weight. Cauda epididymal sperm counts (both total and normalized to total weight) were generally lower (22% to 25%) in the dosed rats; however,

none of these changes attained statistical significance. Epididymis weights in treated males were 10% to 12% lower than vehicle controls with the observed decrease in 25 mg/kg/day males attaining statistical significance. The trend test was also significant. One rat in the 25 mg/kg/day group displayed histopathologic findings in the testis (germinal epithelium degeneration) and epididymis (duct, exfoliated germ cell) (Table 41). Testosterone measured at time of necropsy was significantly lower (61%) in the 25 mg/kg/day group (positive trend test) (Table 37).

Table 39. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Weights (g)				
Necropsy Body Wt.	337 ± 4	324 ± 5	316 ± 5	308 ± 5**
L. Cauda Epididymis	0.230 ± 0.035	0.197 ± 0.007	0.188 ± 0.004	0.179 ± 0.006
L. Epididymis	0.584 ± 0.037	0.519 ± 0.008	0.524 ± 0.012	0.512 ± 0.011*
L. Testis	1.840 ± 0.029	1.786 ± 0.040	1.780 ± 0.043	1.774 ± 0.036
Spermatid Measurements				
Spermatid Heads (10^6/testis)	294.3 ± 13.6	269.2 ± 13.7	253.9 ± 13.7	255.8 ± 11.5
Spermatid Heads (10^6/g testis)	159.8 ± 6.3	150.7 ± 6.9	141.9 ± 4.8	144.4 ± 6.4
Epididymal Spermatozoal Measurements				
Sperm Motility (%)	88.5 ± 0.5	87.9 ± 0.3	87.9 ± 0.3	88.2 ± 0.5
Sperm (10^6/g cauda epididymis)	148.4 ± 17.4	111.0 ± 8.2	115.3 ± 7.8	116.3 ± 8.4
Sperm (10^6/cauda epididymis)	671 ± 46	562 ± 31	616 ± 44	643 ± 33

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Williams' or Dunnett's test (cauda epididymis and testis weights) or Shirley's or Dunn's test (spermatid and epididymal spermatozoal measurements).

Dosed female rats displayed similar amounts of time in each respective estrous stage compared to control (Table 40, Figure A-5). However, Markov analyses demonstrated that females administered WY displayed alterations in the estrous cycle (extended diestrus was significantly higher in all dose groups) (Table 40 and Table A-6). Under the conditions of this study, these data indicate that WY via oral gavage exhibits the potential to be a reproductive toxicant in female Sprague Dawley rats on the basis of altered estrus cyclicity (extended diestrus).

Table 40. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	9	10
Necropsy Body Wt. (g)	224 ± 4	230 ± 4	229 ± 3	229 ± 5
Number of Estrus Cycles	2.0 ± 0.15	2.0 ± 0.00	1.9 ± 0.11	1.8 ± 0.13
Estrous Cycle Length (days)	5.0 ± 0.16	5.0 ± 0.09	4.8 ± 0.26	5.1 ± 0.16
Estrous Stages (% of cycle)				
Diestrus	55.6	61.9	58.1	61.9
Proestrus	4.4	3.8	12.5	6.3
Estrus	38.1	32.5	25.6	28.8
Metestrus	1.9	1.9	3.1	3.1
Uncertain diagnosis	0.0	0.0	0.6	0.0

[a]Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated that dosed females spent significantly more time in extended diestrus (p = 0.009 or less) than did the vehicle control group.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following these results.

Liver: The incidences of hepatocyte hypertrophy were significantly increased in all dose groups of males and in 25 mg/kg/day females (Table 41). Hepatocyte hypertrophy was generally of minimal to mild severity in males and minimal in females. The average severity increased with increasing dose in males. The incidences of hepatocyte cytoplasmic alteration were significantly increased in all dose groups of males and females, and the lesion was generally of minimal to marked severity in males and minimal to mild in females. The average severity increased with increasing dose.

Table 41. Incidences of Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Wyeth-14,643

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
Male				
Liver[a]	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration[b]	0	9** (1.7)[c]	10** (2.2)	10** (4.0)
Hepatocyte, Hypertrophy	0	5* (1.4)	10** (1.5)	10** (2.0)
Testes	(10)	(10)	(10)	(10)
Germinal Epithelium, Degeneration	0	0	0	1 (2.0)
Epididymis	(10)	(0)	(10)	(10)
Duct, Exfoliated Germ Cell	0	0	0	1 (1.0)
Female				
Liver	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	7** (1.0)	10** (1.2)	10** (1.9)
Hepatocyte, Hypertrophy	0	0	2 (1.0)	8** (1.1)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Histopathologic Descriptions for PFHxA, PFOA, PFNA, PFDA, and WY

The treatment-related lesions in the liver, nose, bone marrow, spleen, thymus, mandibular and mesenteric lymph nodes, testes and epididymis, thyroid gland, and forestomach were generally morphologically similar across the studies in which they occurred; however, the severities of the lesions varied among studies.

Liver: Figure 6 and Figure 7 show the normal histological morphology of the liver. Hepatocyte cytoplasmic alteration was generally of minimal to marked severity characterized by an accumulation of eosinophilic granules within the cytoplasm of centrilobular hepatocytes (Figure 8 and Figure 9). A grade of minimal (Grade 1) was used when eosinophilic granules were present within occasional centrilobular hepatocytes (up to 10% of the liver involved); mild (Grade 2) when 10% to 33% of the liver was involved with most or all centrilobular zones affected; moderate (Grade 3) when 33% to 67% of the liver was involved with widespread centrilobular and midzonal hepatocytes affected; and marked (Grade 4) when greater than 67% of the liver was involved.

Hepatocyte hypertrophy was generally of minimal to marked severity characterized by an increase in the size of primarily centrilobular hepatocytes due to an accumulation of cytoplasmic granules or an increase in homogenous eosinophilic cytoplasm in the absence of distinct eosinophilic cytoplasmic granules (Figure 10 and Figure 11). Hepatocyte hypertrophy was graded as minimal (Grade 1) when hepatocytes were enlarged in occasional lobules (up to 10% of the liver involved); mild (Grade 2) when 10% to 33% of the liver was involved; moderate (Grade 3) when 33% to 67% of the liver was involved; and marked (Grade 4) when greater than 67% of the liver was involved.

Hepatocyte cytoplasmic vacuolization was generally of mild to marked severity in males and moderate to marked in females; it was largely centrilobular in distribution and characterized by accumulation of microvacuoles within the cytoplasm (Figure 12). Hepatocyte cytoplasmic vacuolization was graded as minimal (Grade 1) when cytoplasmic vacuolization was present within occasional centrilobular hepatocytes (with up to 10% of the liver involved); mild (Grade 2) when 10% to 33% of the liver was involved with most or all centrilobular zones affected; moderate (Grade 3) when 33% to 67% of the liver was involved with widespread centrilobular and midzonal hepatocytes affected; and marked (Grade 4) when greater than 67% of the liver was involved.

In all cases, hepatocyte necrosis was of minimal severity and consisted of a few widely scattered, variably sized, randomly distributed foci of necrotic hepatocytes within the hepatic parenchyma, mixed with variable numbers of mononuclear inflammatory cells.

Nose: Compared to normal respiratory epithelium (Figure 13), respiratory epithelium hyperplasia was characterized by increased numbers of basal and nonciliated cuboidal to columnar epithelial cells of the mucosal respiratory epithelium primarily lining the nasal septum and terminates of Level I (Figure 14). The cell layers were frequently disorganized and often associated with chronic active inflammation. A grade of minimal (Grade 1) was assigned when respiratory epithelium hyperplasia was present as one to several foci on one or both sides of the nasal septum or a few small areas on the tips of the nasal turbinates and mild (Grade 2) when one entire side or both sides of the nasal septum were involved or, less commonly, the hyperplasia was primarily on the surface of multiple turbinates with little or no septum involvement.

Moderate (Grade 3) hyperplasia was diagnosed when the hyperplasia involved the majority of the nasal septum epithelium and up to 50% of the surface of the turbinates.

Chronic active inflammation of the respiratory epithelium generally occurred concurrently with respiratory epithelium hyperplasia and consisted of a mixed population of primarily mononuclear inflammatory cells (mostly lymphocytes) and lesser numbers of neutrophils within the lamina propria beneath the mucosal respiratory epithelium of the nasal turbinates (Figure 14). Neutrophils were also noted migrating from the lamina propria into the overlying respiratory epithelium. A grade of minimal (Grade 1) was used when a few foci of inflammatory cells were noted in propria beneath the septum or a few areas in the tips of turbinates; mild (Grade 2) when inflammatory cells were noted along the majority of the nasal septum propria and migrating into the overlying respiratory epithelium; and moderate (Grade 3) when inflammatory cells were noted in the majority of the nasal septum and in multiple turbinates with migration into the overlying respiratory epithelium.

Compared to normal olfactory epithelium (Figure 15), olfactory epithelium, degeneration was characterized by segmental decreased nuclear density and decreased height of the olfactory epithelium due to loss of the olfactory epithelial cells (Figure 16). In the affected segments, the remaining epithelial cells had lost the surface cilia/microvillus border and contained cytoplasmic vacuoles, and there were scattered pyknotic cell nuclei within the epithelium. Olfactory epithelium, degeneration was diagnosed as minimal (Grade 1) when epithelium on up to 25% of the nasal turbinates was affected; mild (Grade 2) when epithelium on 25% to 50% of the nasal turbinates was affected; moderate (Grade 3) when epithelium on 50% to 75% of the nasal turbinates was affected; and marked (Grade 4) when epithelium on greater than 75% of the nasal turbinates was affected.

Olfactory epithelium, hyperplasia was of minimal to mild severity and was characterized by multifocal to linear areas of proliferation and piling up of the olfactory epithelial cells resulting in disorganization and increased thickness of the epithelium (Figure 16). In some affected segments, there were focal proliferations of basal epithelial cells extending into the underlying propria effacing the normally distinct border between olfactory epithelium and the underlying lamina propria. A grade of minimal (Grade 1) was used when less than 15% of the olfactory epithelium in Level III was involved and mild (Grade 2) when greater than or equal to 16% to 50% of the Level III olfactory epithelium was hyperplastic.

Olfactory epithelium, suppurative inflammation was of minimal to moderate severity and was characterized by aggregates of neutrophils and cellular debris within the nasal passages and within the epithelium and lamina propria of the ethmoid turbinates (Figure 16). A grade of minimal (Grade 1) was used when up to 25% of the nasal cavity lumen contained free neutrophils; mild (Grade 2) when 25% up to 50% of the nasal cavity lumen contained free neutrophils; moderate (Grade 3) when 50% up to 75% of the nasal cavity lumen contained free neutrophils; and marked (Grade 4) when 75% or greater of the nasal cavity contained free neutrophils.

Bone Marrow: Hypocellularity was minimal to marked in severity and was characterized by a decrease in both erythroid and myeloid cell lines and a corresponding increase in fat cells in the marrow. Minimal (Grade 1) hypocellularity was diagnosed when the bone marrow contained approximately 10% to 15% more adipocytes and a corresponding fewer number of hematopoietic

cells than concurrent vehicle controls. Mild (Grade 2) hypocellularity was diagnosed when the bone marrow contained greater than 15% to 30% more adipocytes and a corresponding fewer number of hematopoietic cells than concurrent vehicle controls.

Spleen and Mandibular and Mesenteric Lymph Nodes: Compared to the normal spleen (Figure 17) and lymph node (Figure 19), atrophy was characterized by regional reduction in the number of lymphocytes within the splenic white pulp (Figure 18) and generalized decrease in the number of lymphocytes within the lymph nodes (Figure 20). The severity of atrophy was diagnosed as minimal (Grade 1) when there was up to a 25% reduction in mandibular lymph node lymphocyte number; mild (Grade 2) when there was a 26% to 50% reduction in mandibular lymph node lymphocyte number; moderate (Grade 3) when there was a 51% to 75% reduction in mandibular lymph node lymphocyte number; and marked (Grade 4) when there was over a 75% reduction in mandibular lymph node lymphocyte number.

Increased extramedullary hematopoiesis in the spleen was generally of minimal to mild severity and diagnosed when hematopoietic cells, largely of the erythroid lineage, were present within the splenic red pulp in excess of numbers normally present in the red pulp. The hematopoietic cells occurred as variably sized discrete clusters distributed diffusely throughout the red pulp. Extramedullary hematopoietic cell proliferation was graded as minimal (Grade 1) when approximately 25% of the splenic red pulp contained excess hematopoietic cells; mild (Grade 2) when 26% to 50% of the splenic red pulp contained excess hematopoietic cells; moderate (Grade 3) when 51% to 75% of the splenic red pulp contained excess hematopoietic cells; and marked (Grade 4) when greater than 75% of the splenic red pulp contained excess hematopoietic cells.

Thymus: Lymphocyte apoptosis was of mostly minimal severity characterized by an increased number of shrunken cells with dark nuclei and was diagnosed when ≤15% of the cortical lymphocytes were apoptotic.

Atrophy was of minimal to marked severity among dosed rats but was mostly marked in males and females. Compared to the normal thymus (Figure 21), atrophy was characterized by a decrease in the thickness of the thymic cortex considered to be due to a decrease in the number of cortical lymphocytes (Figure 22). Atrophy was diagnosed as minimal (Grade 1) when there was up to a 25% reduction in the cortex; mild (Grade 2) when there was a 26% to 50% reduction of the cortex; moderate (Grade 3) when there was a 51% to 75% reduction of the cortex; and marked (Grade 4) when there was over a 75% reduction in the cortex and loss of the corticomedullary junction. Increased tingible body macrophages that contained apoptotic bodies were scattered among the thymic lymphocytes.

Testes and Epididymis: Normal histology of a seminiferous tubule of the testis is shown in Figure 23. Interstitial cell atrophy of the testes was of minimal to marked severity characterized by decreased numbers of interstitial cells; affected cells also seemingly had decreased amounts of cytoplasm (Figure 24). A minimal severity grade (Grade 1) was assigned when less than 25% of the interstitial cells had decreased amounts of cytoplasm; mild (Grade 2) when there was a decrease in cytoplasm of 26% to 50%; moderate (Grade 3) when there was a 51% to 75% decrease in cytoplasm accompanied by an approximate decrease in cell numbers by up to 30%; and marked (Grade 4) when almost no cytoplasm could be visualized around interstitial cell nuclei and numbers of interstitial cells had decreased by approximately 31% to 60%.

Seminiferous tubule spermatid retention was of minimal to marked severity characterized by either retained spermatids at the surface of the germinal epithelium and/or retained spermatid heads at the basement membrane of the germinal epithelium of Stage IX through Stage XIV tubules (Figure 25). A severity grade of minimal (Grade 1) was assigned if greater than three retained spermatids (combined surface or heads at the basement membrane) were noted in over seven tubules; mild (Grade 2) when greater than 50% of the late stage tubules had greater than three retained spermatids; and moderate (Grade 3) when retention at the surface or basement membrane could be noted at low power and involved the majority of the late stage tubules (although not all spermatids being produced by the testes were retained).

Germinal epithelium degeneration in the testes was of minimal to marked severity characterized by loss of elongating spermatids in late stage tubules; cell-to-cell disassociation; vacuolization of Sertoli cells; pyknotic pachytene, diplotene, and meiotic spermatocytes; focal areas of cell drop out; multinucleated giant cells; loss of spermatogonia; vacuolated nuclei of round spermatids; loss of round spermatids in early stages; and residual bodies in the tubular lumens (Figure 24, Figure 25, Figure 26, and Figure 27). Germinal epithelial degeneration was graded as minimal (Grade 1) when the primary finding was increased residual bodies in lumens of tubules; mild (Grade 2) was characterized by increased residual bodies in tubular lumens, decreased elongating spermatids in late stages, and necrotic pachytene spermatocytes; moderate (Grade 3) was used when many or all of the above findings listed for degeneration were noted and involved up to 67% of the tubules; and marked (Grade 4) was used when greater than 67% of tubules were affected.

Normal histology of the epididymis is shown in Figure 28. Epididymis, duct, exfoliated germ cells was of mild to marked severity characterized by sloughed germinal epithelial cells and cytoplasmic debris, including residual bodies, in some cases, in the lumen of the duct of the epididymis (Figure 29). A minimal severity grade (Grade 1) was used when the average of the five largest profiles in the cauda of the epididymis contained 5 to 12 exfoliated germ cells or bits of cellular debris, mild (Grade 2) was used when the average was 13 to 25, moderate (Grade 3) was used when the average was greater than 25 but less than marked (Grade 4), which was used when the lumen of the duct was almost completely filled with sloughed cells and cytoplasmic debris.

Epididymis hypospermia was of mild to marked severity characterized by decreased sperm within the duct of the epididymis (Figure 29 and Figure 30). An estimate of the decrease was made by a semiquantitative and qualitative assessment of the duct, particularly the cauda portion, for density of the sperm within the duct and the degree of distension of the duct lumen as the lumen tends to become decreased in diameter with less sperm. Degree of decrease in lumen diameter with hypospermia was not recorded separately. A minimal severity grade (Grade 1) was used when the duct only contained a slight decrease in sperm density and a slight decrease in duct diameter compared to concurrent vehicle controls.

Epididymis, epithelium apoptosis occurred primarily in the head of the epididymis with extension into the body where this change was most prominent (Figure 30). Apoptosis consisted of shrunken, hypereosinophilic cells with fragmented pyknotic nuclei within the epithelium of the ducts. Apoptosis was graded as minimal (Grade 1) if only scattered cells in the epithelium of the head were affected; mild (Grade 2) when up to 30% of the cells were affected; and moderate

(Grade 3) when greater than 30% of the cells were affected with extension into the body of the epididymis.

Epididymis sperm granuloma was of mild severity characterized as a focal aggregated mass of spermatozoa in the interstitial tissue around the epididymal duct (Figure 31 and Figure 32). The mass of spermatozoa was mixed with neutrophils and necrotic cell debris and, in some areas, surrounded by fibrous tissue and inflammatory cells, primarily macrophages and neutrophils and with lesser numbers of lymphocytes.

Thyroid Gland: Follicle cell hypertrophy was of minimal severity characterized by enlarged follicle cells approximately twice as tall as normal and frequently with a vacuolated pale amphophilic cytoplasm. Minimal (Grade 1) hypertrophy was used when the number of enlarged follicular cells was less than 15% of the total number of cells involved, and the affected cells were generally no taller than twice the height of follicular cells in concurrent vehicle controls.

Forestomach: Epithelial hyperplasia of the forestomach mucosa was characterized by a focal area of increased cell numbers resulting in an increased thickness and folding of the mucosa. The epithelial cells maintained normal orientation, and atypia was not noted.

Inflammation chronic was diagnosed as mild (Grade 2) when infiltrates of lymphocytes and plasma cells were randomly scattered in the submucosal lamina propria.

Genetic Toxicology

The four perfluoroalkyl carboxylates were tested in assays that measure the potential for induction of mutations in bacteria (*Salmonella typhimurium* strains TA100 and TA98 and *Escherichia coli* strain WP2 *uvr*A pKM101) and chromosomal damage in erythrocyte precursor cells in the bone marrow of male and female Sprague Dawley rats. Although the damage is induced in the erythroblast population in the bone marrow, it is measured as micronuclei in immature red blood cells (reticulocytes, RET; polychromatic erythrocytes, PCEs) in peripheral blood.

PFHxA was negative in bacterial mutagenicity assays with and without 10% rat liver S9; it was tested at doses up to 750 µg/plate in TA100 and TA98 and up to 2,000 µg/plate in the *E. coli* strain in the absence of S9 (Table C-1). With S9, all strains were tested up to the assay limit of 10,000 µg/plate.

No increases in micronucleated reticulocytes were seen in peripheral blood of male or female rats administered PFHxA by two daily gavage treatments (31.3 to 500 mg/kg twice daily) for 28 days; however, the percentage of circulating immature erythrocytes (% PCEs) was markedly increased over the dose range in both sexes, suggesting a stimulation of erythropoiesis in the bone marrow of PFHxA-treated rats (Table B-5).

PFOA was equivocal in bacterial mutagenicity assays using strain TA 98 without 10% rat liver S9 and negative in bacterial mutagenicity assays using TA 100 and *E. coli* with and without 10% rat liver S9, and in TA 98 with S9;[a] it was tested at doses up to 1,000 µg/plate in all three strains

[a]ERRATUM: An error was identified in the NTP Toxicity Report on Perfluoroalkyl Carboxylates (TOX 97). The text was changed from “negative in bacterial mutagenicity tests” to “equivocal in TA98 bacterial mutagenicity tests” without S9 for PFOA. [20 May 2020]

in the absence of S9 (Table C-2). PFOA was tested at doses up to 5,000 µg/plate with S9 in TA100 and TA98 and 10,000 µg/plate in the *E. coli* strain.

No increases in micronucleated reticulocytes were seen in peripheral blood of female rats administered PFOA (6.25 to 100 mg/kg/day) by gavage once daily for 28 days (Table B-6). A statistically significant increase in micronucleated reticulocytes was observed over the administered dose range of 0.625 to 10 mg/kg/day in male rats. However, this increase was within the laboratory's historical control range (95% confidence limits); therefore, the biologic significance of this small increase is questionable. No changes were noted in the percentage of immature erythrocytes in peripheral blood of male or female rats, suggesting that PFOA did not induce bone marrow toxicity.

PFNA was negative in bacterial mutagenicity assays with and without 10% rat liver S9; it was tested at doses up to 500 µg/plate in TA100 and TA98 and up to 5,000 µg/plate in the *E. coli* strain in the absence of S9 (Table C-3). With S9, PFNA was tested up to 1,000 µg/plate in TA100 and TA98 and up to the assay limit of 10,000 µg/plate in the *E. coli* strain.

No increases in micronucleated reticulocytes were seen in peripheral blood of male rats administered PFNA once daily by gavage for 28 days; only two treatment groups could be evaluated for this endpoint due to severe toxicity to the bone marrow in the high-dose male rats, evidenced by marked reduction in circulating reticulocytes (Table B-7). In female rats, no significant increases in micronucleated reticulocytes were seen over the dose range of 1.56 to 6.25 mg/kg/day, and no indication of bone marrow toxicity was seen due to the percentage of immature erythrocytes in circulation, which was unaffected by treatment.

PFDA was negative in bacterial mutagenicity assays with and without 10% rat liver S9; it was tested at doses up to 1,000 µg/plate in TA100 and TA98 and up to 5,000 µg/plate in the *E. coli* strain in the absence of S9 (Table C-4). With S9, all strains were tested up to the assay limit of 10,000 µg/plate.

No increases in micronucleated reticulocytes were seen in peripheral blood of female rats administered PFDA once daily by gavage for 28 days over a dose range of 0.156 to 1.25 mg/kg/day (Table B-8). However, in males, a significant increase in micronucleated reticulocytes was seen at the highest dose administered (2.5 mg/kg/day), although two of the five rats in this treatment group had a severe reduction in the number of reticulocytes, precluding accurate evaluation of the % PCEs in these two rats. Thus, in male rats, the increase in micronuclei was only seen at a dose level that induced severe toxicity to the bone marrow, and the % PCEs value was at the top end of the laboratory's historical control range (95% confidence limits) for male Sprague Dawley rats.

No increases in the frequencies of micronucleated erythrocytes (either immature or mature [NCEs]) were observed in peripheral blood of rats administered WY (6.25 to 25 mg/kg/day) by gavage for 28 days (Table B-9). However, WY caused significant, dose-dependent decreases in % PCEs in the peripheral blood of female rats, suggesting that the bone marrow was a target for cytotoxicity.

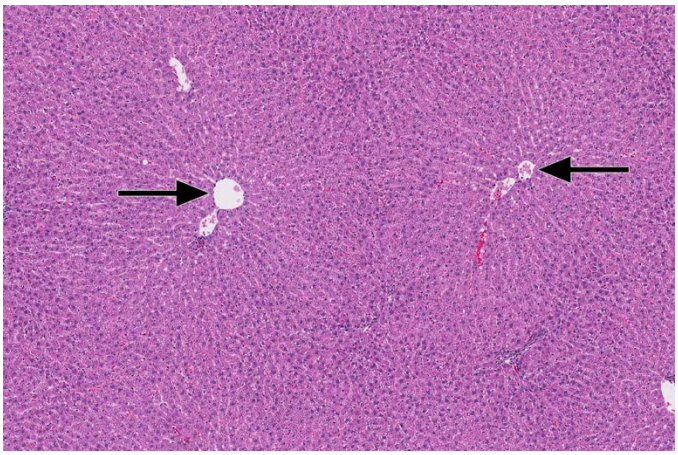


Figure 6. Normal Liver of a Male Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorononanoic Acid (H&E)

Central veins (arrows).

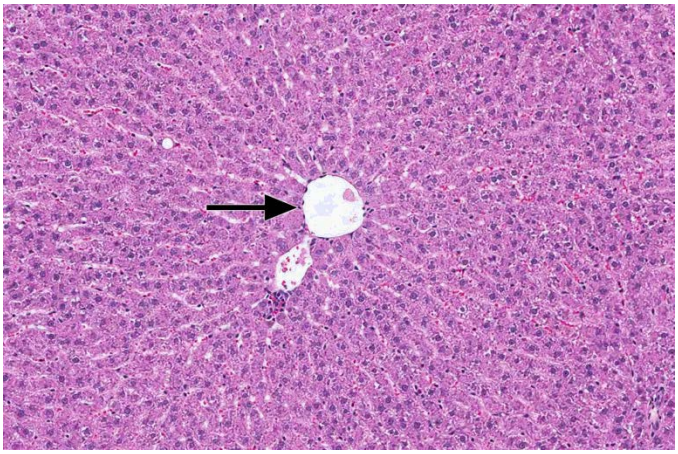


Figure 7. Higher Magnification of Figure 6 (H&E)

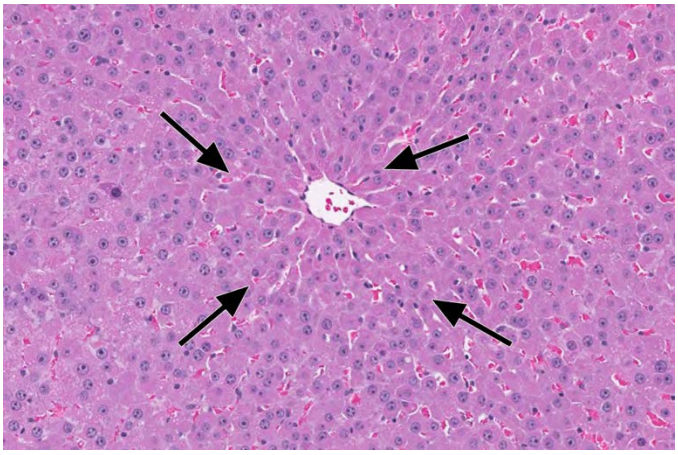


Figure 8. Centrilobular Areas of Cytoplasmic Alteration (Arrows) in a Male Rat Administered Perfluorononanoic Acid 2.5 mg/kg/day by Gavage for 28 Days (H&E)

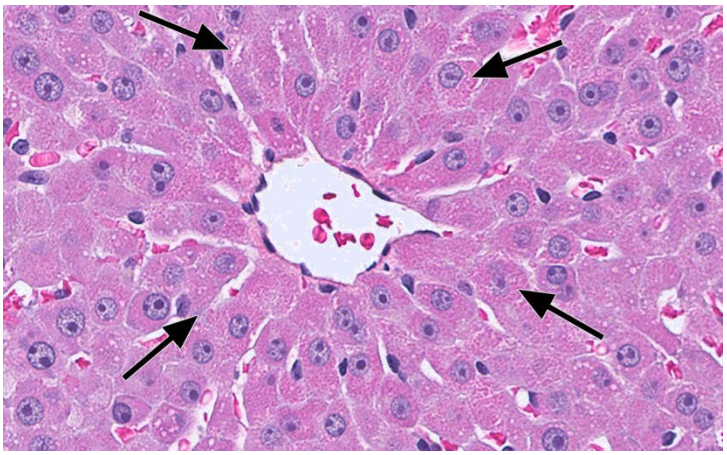


Figure 9. Higher Magnification of Figure 8 (H&E)

Hepatocyte Cytoplasmic alteration is characterized by enlarged centrilobular hepatocytes that contain granular eosinophilic cytoplasm (arrows).

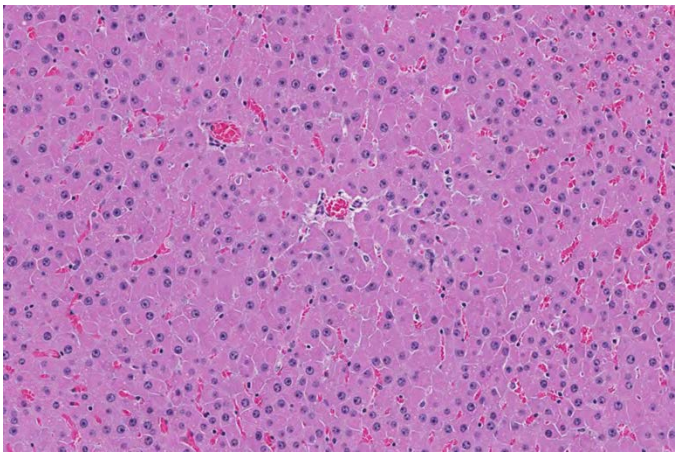


Figure 10. Hepatocyte Hypertrophy in a Male Rat Administered Perfluorobutane Sulfonic Acid 2.5 mg/kg/day by Gavage for 28 Days (H&E)

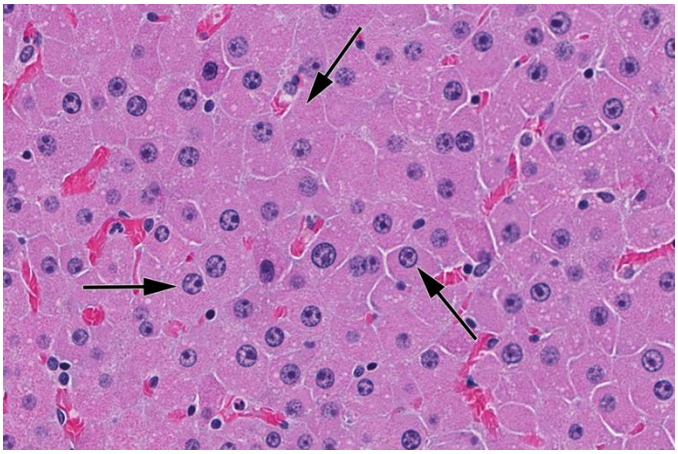


Figure 11. Higher Magnification of Figure 10 (H&E)

Centrilobular hepatocytes are enlarged (arrows) but lack cytoplasmic granules.

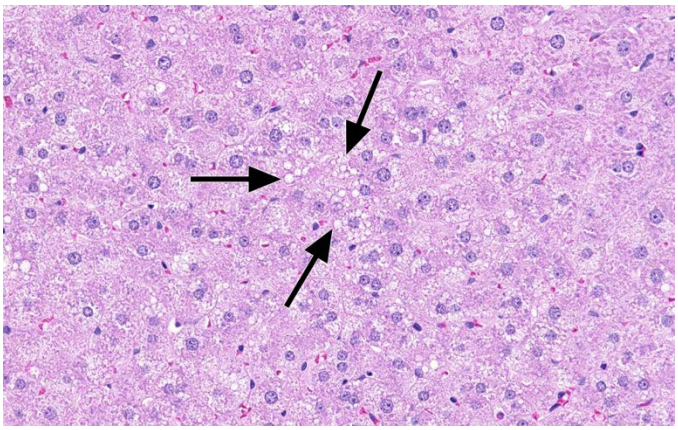


Figure 12. Hepatocyte Cytoplasmic Vacuolation in a Male Rat Administered Perfluorononanoic Acid 2.5 mg/kg/day by Gavage for 28 Days (H&E)

Note clear vacuoles (arrows) within the hepatocytes.

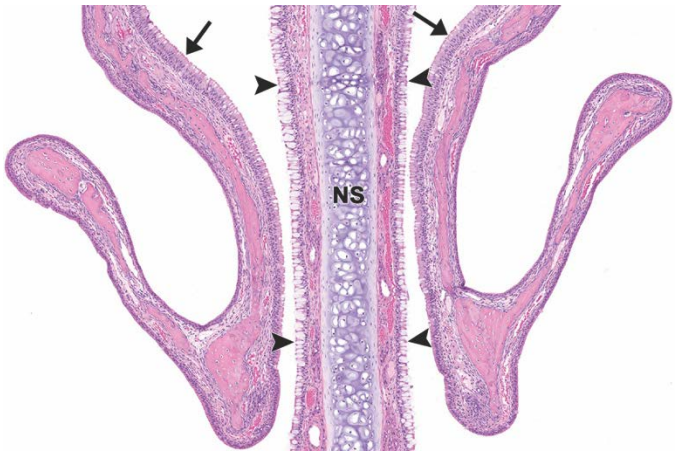


Figure 13. Normal Respiratory Epithelium in the Nose of a Female Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorooctanoic Acid (H&E)

The epithelium lining the turbinates is composed of a single layer of tall columnar, ciliated epithelial cells (arrows). The epithelium lining the nasal septum is composed of a single layer of tall columnar epithelial cells (arrowheads), which contain clear goblet cells within the cytoplasm. NS = nasal septum.

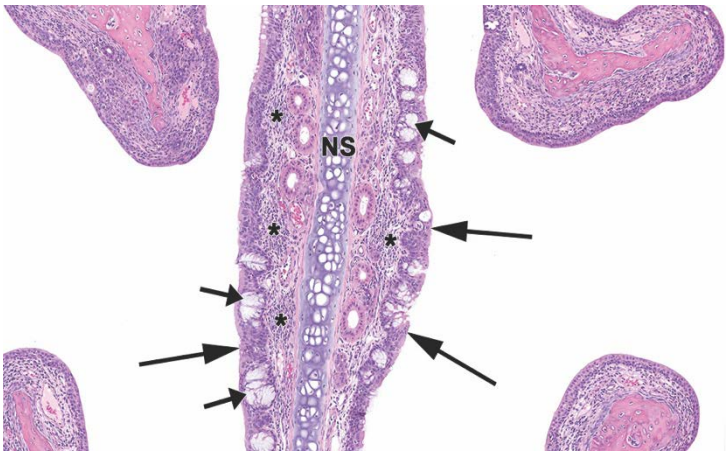


Figure 14. Respiratory Epithelium Hyperplasia and Chronic Active Inflammation in the Nose of a Female Rat Administered Perfluorooctanoic Acid 100 mg/kg/day by Gavage for 28 Days (H&E)

Respiratory epithelium hyperplasia is characterized by disorganization of the epithelium and increased numbers of epithelial cells including basal epithelial cells (long arrows). There is also focal proliferation of goblet cells (short arrows). Chronic active inflammation consists of a mixture of mononuclear inflammatory cells and neutrophils in the submucosa (asterisks). NS = nasal septum.

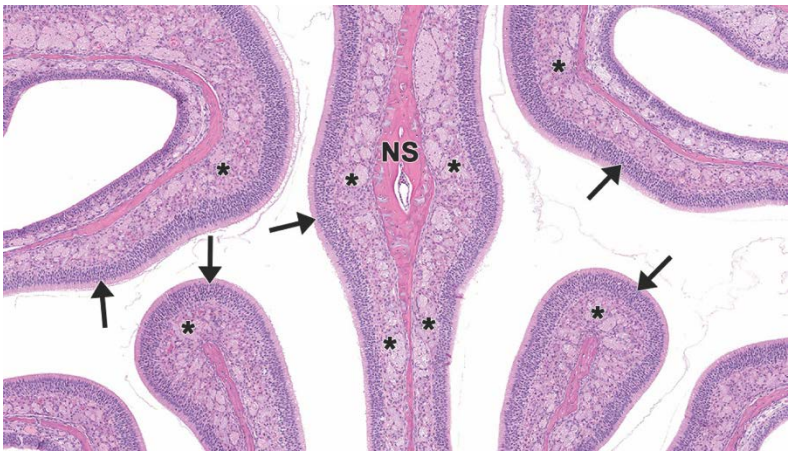


Figure 15. Normal Olfactory Epithelium in the Nose of a Female Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorooctanoic Acid (H&E)

The epithelium (arrows) lining the turbinates and nasal septum is pseudostratified and has a prominent brush border. Note prominent olfactory nerve bundles (asterisks) within the submucosa. NS = nasal septum.

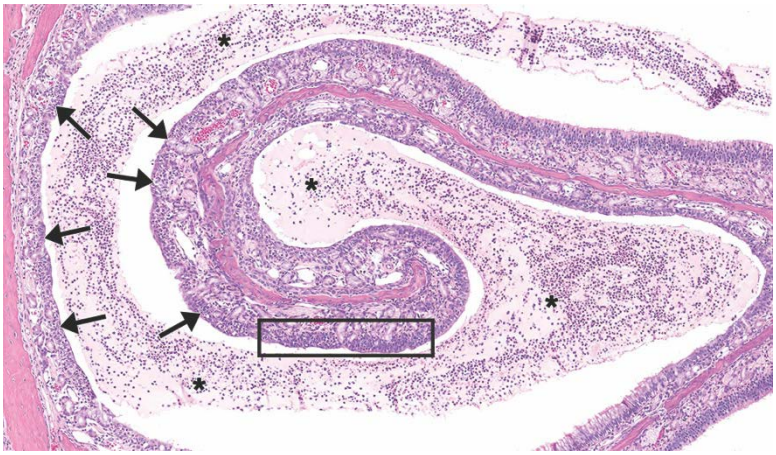


Figure 16. Olfactory Epithelium, Degeneration, Hyperplasia, and Suppurative Inflammation in a Female Rat Administered Perfluorooctanoic Acid 100 mg/kg/day by Gavage for 28 Days (H&E)

Epithelial degeneration (arrows) is characterized by disorganization of the epithelium, decreased density of epithelial cells, degenerate cells within the epithelium, and loss of the surface microvilli. Epithelial hyperplasia (square) is characterized by disorganized proliferation of the olfactory epithelial cells. Suppurative inflammation consists of accumulations of neutrophils and a pale fibrinous exudate within the nasal passages (asterisks).

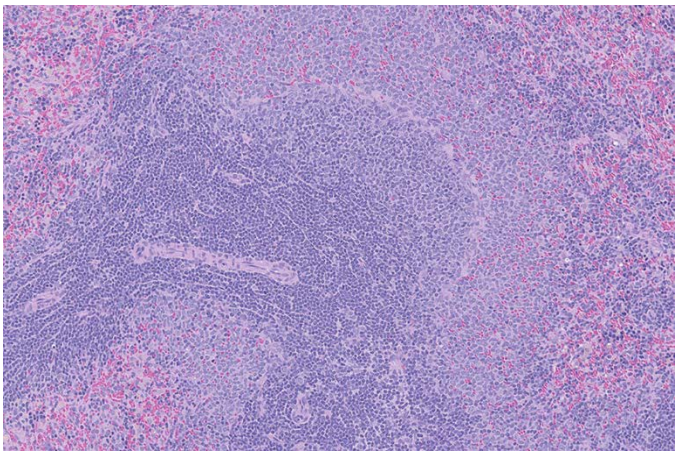


Figure 17. Normal Spleen of a Male Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorononanoic Acid (H&E)

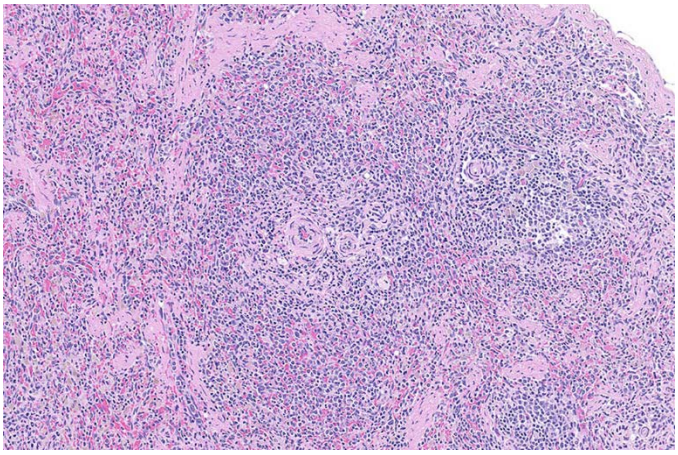


Figure 18. Atrophy in the Spleen of a Male Rat Administered Perfluorononanoic Acid 5 mg/kg/day (H&E)

Atrophy is characterized by a generalized decrease in the number of lymphocytes throughout the red and white pulp.

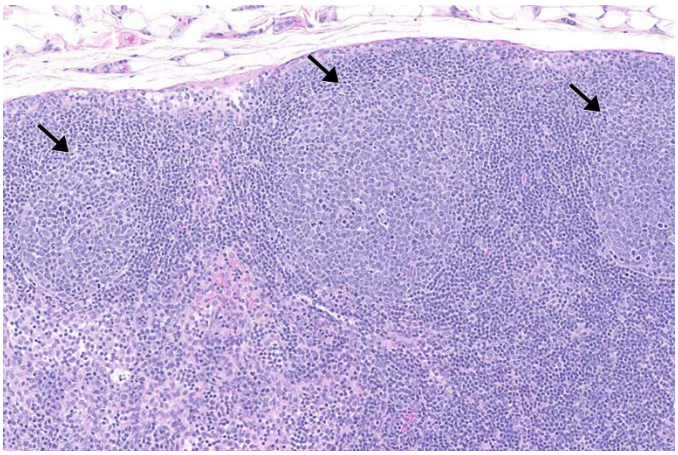


Figure 19. Normal Mesenteric Lymph Node of a Male Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorononanoic Acid (H&E)

Lymphoid follicles (arrows).

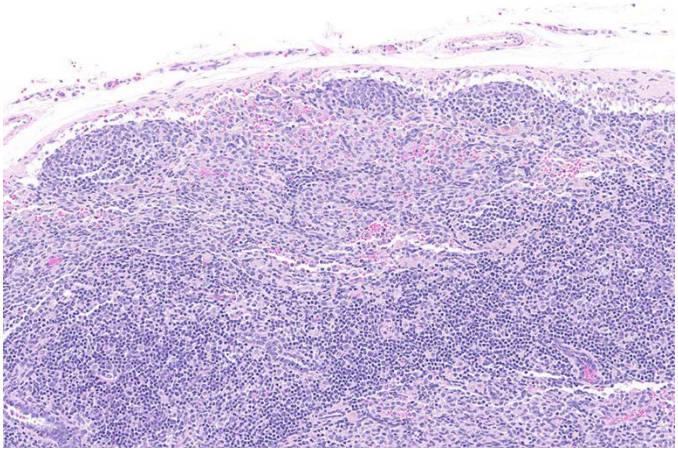


Figure 20. Atrophy in the Mesenteric Lymph Node of a Male Rat Administered Perfluorononanoic Acid 5 mg/kg/day (H&E)

Atrophy is characterized by a generalized decrease in the number of lymphocytes within the lymphoid follicles

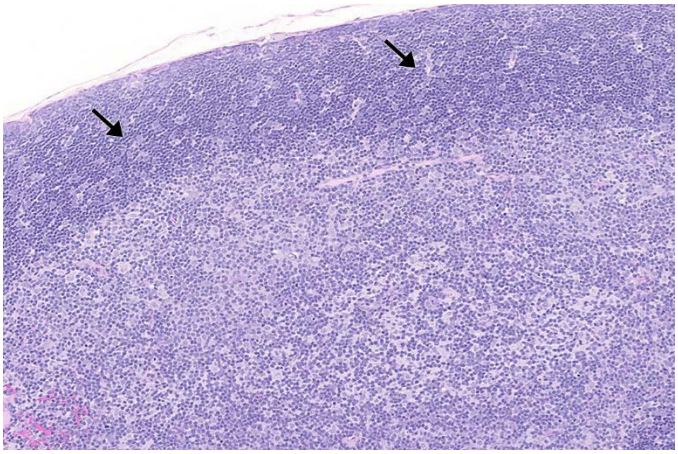


Figure 21. Normal Thymus of a Male Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorononanoic Acid (H&E)

Cortex (arrows).

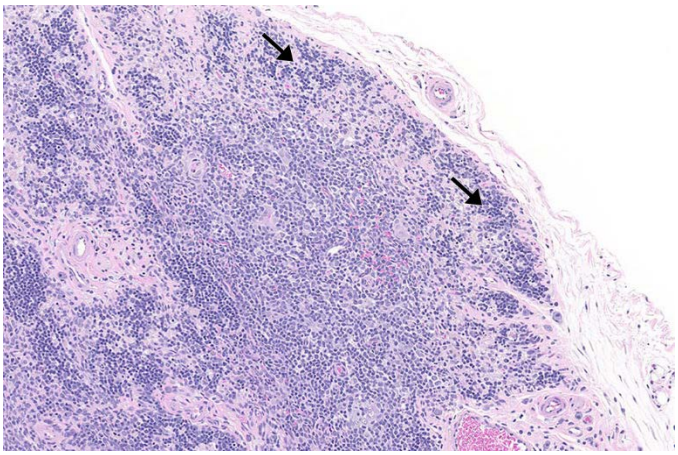


Figure 22. Atrophy in the Thymus of a Male Rat Administered Perfluorodecanoic Acid 2.5 mg/kg/day by Gavage for 28 Days (H&E)

Atrophy is characterized by a generalized decrease in the number of cortical lymphocytes (arrows).

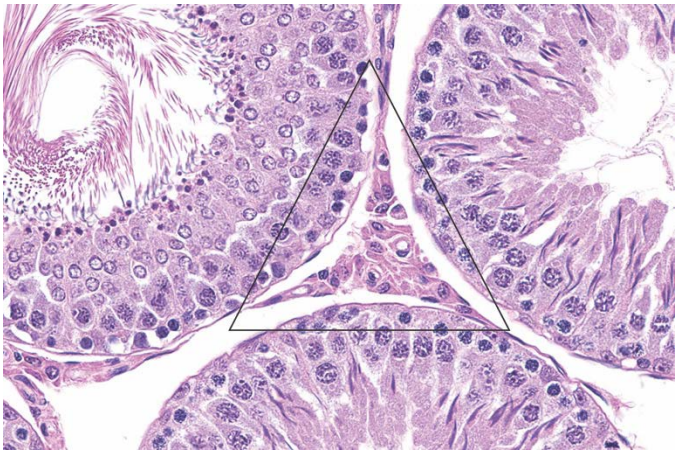


Figure 23. Normal Testis of a Male Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorononanoic Acid (H&E)

Note normal germinal epithelium with typical residual bodies observed in this stage VIII seminiferous tubule (upper left). Also note plump cytoplasm of the interstitial (Leydig) cells (triangle).

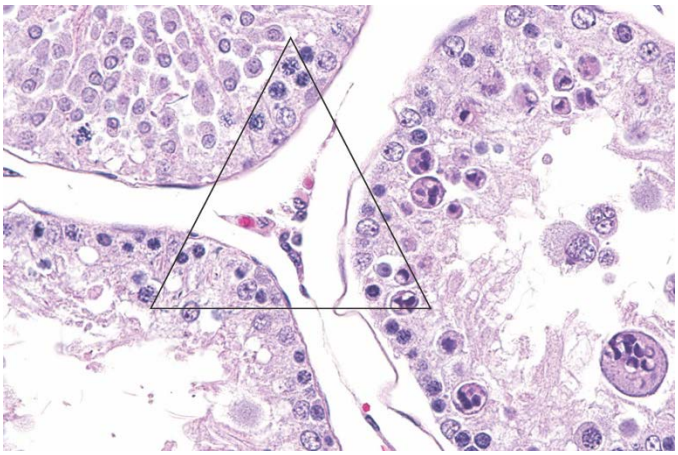


Figure 24. Interstitial Cell Atrophy and Degeneration in the Testis of a Male Rat Administered Perfluorononanoic Acid 10 mg/kg/day (H&E)

Compared to Figure 23, atrophic interstitial cells are shrunken and have scant cytoplasm. Also note degeneration characterized by decreased tubular diameters, depletion of germ cells in all tubules, and the multinucleated giant cell. Magnification and triangle size are the same as in Figure 23.

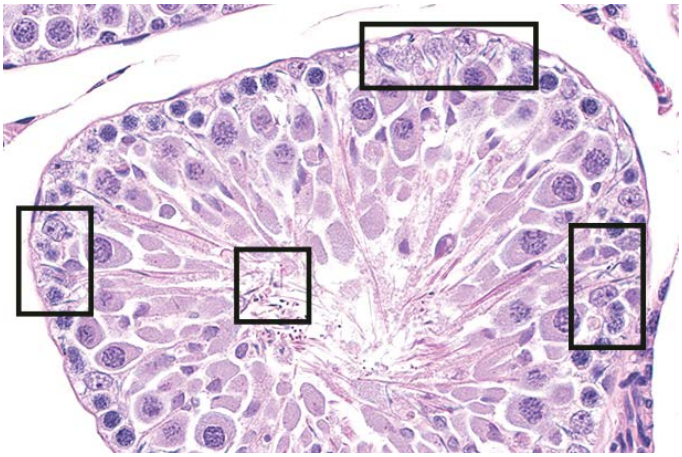


Figure 25. Testis of a Male Rat Administered Perfluorononanoic Acid 10 mg/kg/day for 28 Days (H&E)

Note retained spermatid heads at the basement membrane (rectangles) and surface (square) of the germinal epithelium of a Stage 10 seminiferous tubule.

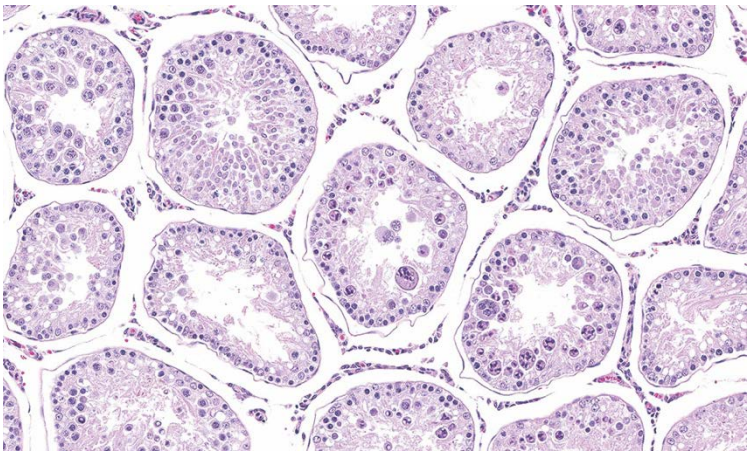


Figure 26. Marked Degeneration in the Testis of a Male Rat Administered Perfluorononanoic Acid 2.5 mg/kg/day for 28 Days (H&E)

Note that all seminiferous tubules in the section are affected. The primary changes are disorganization of the germinal epithelium, significant exfoliation, and loss of germinal epithelial cells. Also, note Sertoli cell vacuolation and the presence of numerous atypical residual bodies, apoptotic cells, and multinucleate cells.

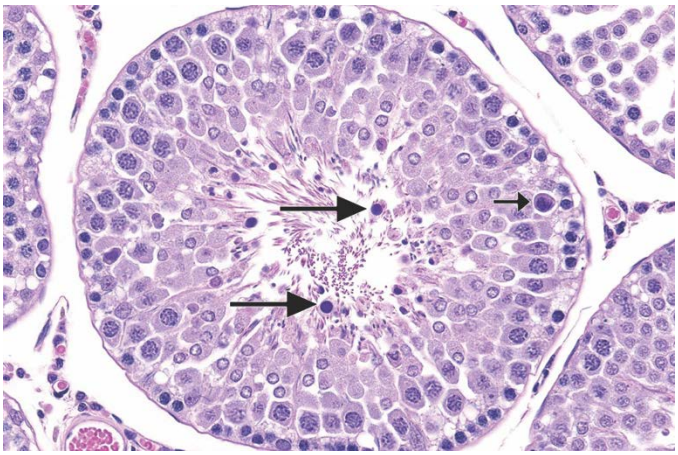


Figure 27. Degeneration in the Testis of a Male Rat Administered Perfluorononanoic Acid 2.5 mg/kg/day (H&E)

Note the atypical residual bodies (long arrow) and the apoptotic pachytene spermatocyte (short arrow) in a Stage VIII seminiferous tubule. The residual bodies are larger than the typical residual bodies in the Stage VIII tubule in the control testis shown in Figure 23.

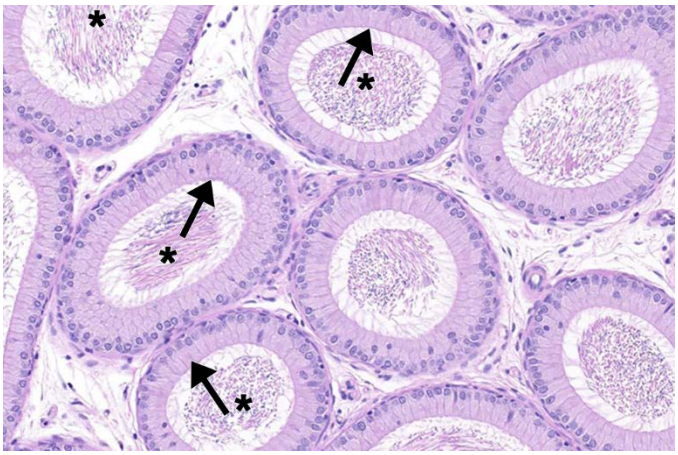


Figure 28. Normal Epididymis of a Male Vehicle Control Rat in the 28-day Gavage Toxicity Study of Perfluorononanoic Acid (H&E)

Epididymal duct is lined by a single row of columnar epithelial cells (arrows) and contain tangled masses of mature spermatozoa (asterisks).

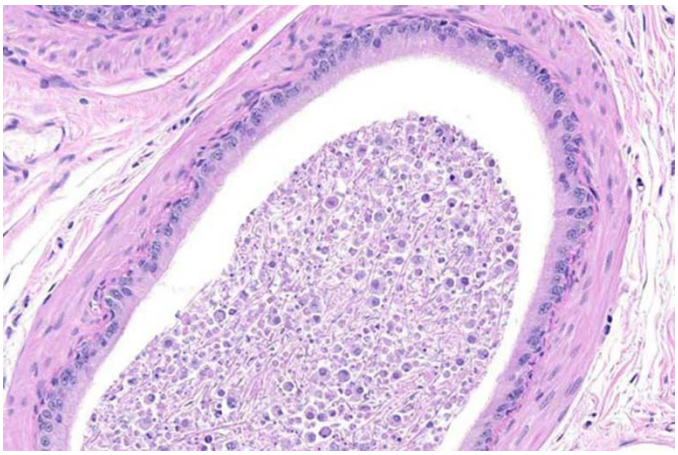


Figure 29. Exfoliated Germ Cells and Hypospermia in the Epididymal Duct of a Male Rat Administered Perfluorononanoic Acid 10 mg/kg/day for 28 Days (H&E)

Note that the epididymal duct from cauda epididymis is filled mostly with exfoliated germinal epithelial and apoptotic cells mixed with low numbers of mature spermatozoa (hypospermia).

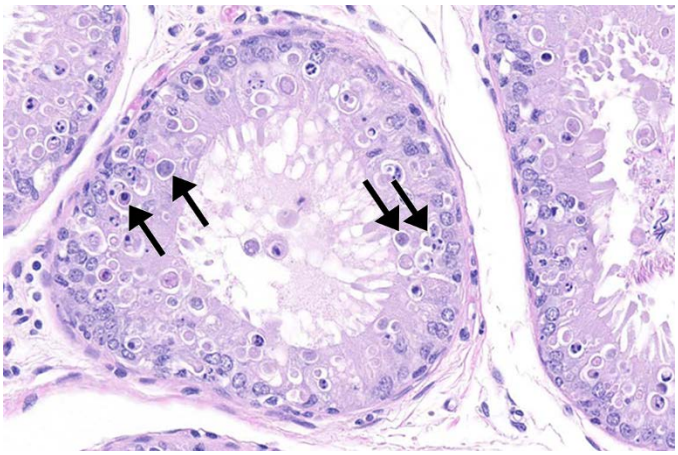


Figure 30. Apoptosis in the Head of the Epididymis of a Male Rat Administered Perfluorononanoic Acid 10 mg/kg/day for 28 Days (H&E)

Note numerous apoptotic cells (arrows) in the epithelium of the seminiferous tubules.

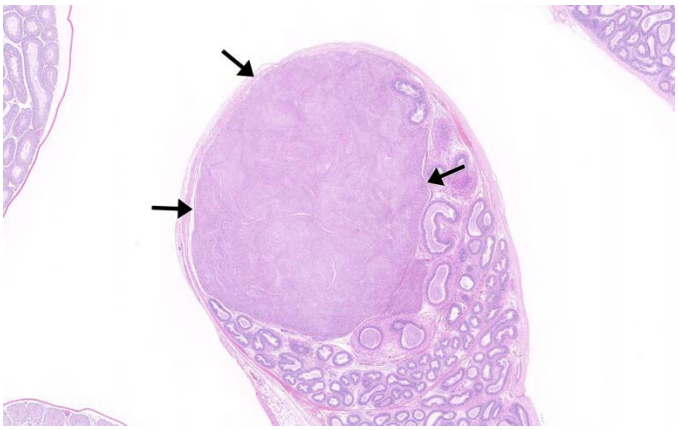


Figure 31. Sperm Granuloma in a Male Rat Administered Perfluorononanoic Acid 10 mg/kg/day (H&E)

The granuloma occurs as a discrete mass within the interstitium of the epididymis (arrows).

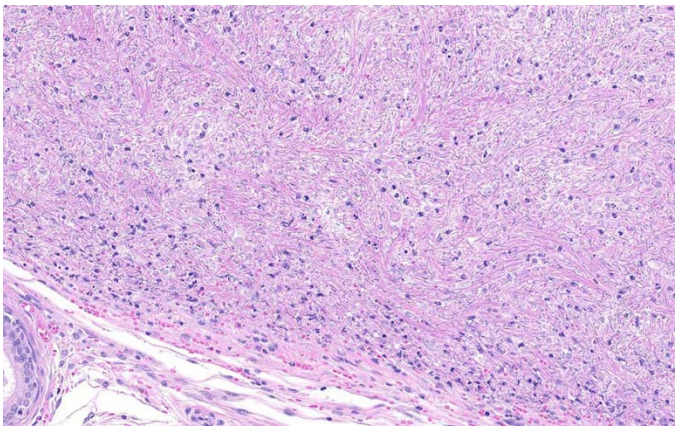


Figure 32. Higher Magnification of Figure 31 (H&E)

Sperm granuloma consists of a mass of tangled spermatozoa mixed with necrotic cell debris, inflammatory cells (macrophages, neutrophils, and lymphocytes) bordered by fibrous tissue.

Discussion

Widespread exposure to per- and polyfluorinated alkyl substances (PFAS), combined with a lack of data for some of the less commonly detected class members, has underscored the need for class comparisons. Although a large amount of research has focused on the higher-production entities, perfluorooctane sulfonic acid (PFOS) and perfluorooctane octanoic acid (PFOA), few studies to date have compared toxicities across the class of straight-chained PFAS. Toxicity Study Report 96[124] and this report, Toxicity Study Report 97, detail results from 28-day studies evaluating the toxicity of seven straight-chained sulfonate and carboxylate PFAS of varying chain length. Importantly, these studies included biosampling of PFAS concentrations in the plasma and liver (males only) to facilitate potency comparisons and future dose-response modeling. Wyeth-14,643 (WY) was included with the comparison studies as an indicator of potential peroxisome proliferator-activated receptor alpha (PPARα)-mediated toxicity. This section provides an overview of major endpoints of toxicity, a brief comparison across the evaluated PFAS, and a discussion of potential mechanisms of toxicity.

To aid in determining relative potency of PFAS, these studies evaluated the plasma and liver (males only) concentrations of perfluorohexanoic acid (PFHxA), PFOA, perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) to provide a direct comparison of internal exposure. In general, the levels of each chemical followed a similar pattern to that described in the literature on the kinetics of that PFAS. Shorter-chain PFAS typically have shorter half-lives than do longer-chain PFAS[25; 28], reflected herein by the higher plasma concentrations as chain length increased; for example, as illustrated with the dose-normalized plasma concentrations across the females (2 to 6 µM/mmol/kg in PFHxA compared to 70,000 to 100,000 mM/mmol/kg in PFDA). Longer-chain PFAS (PFNA, PFDA) are reported to have preferential biliary elimination over urinary elimination[29; 33], and the higher liver levels and liver/plasma ratios in those PFAS compared to PFHxA and PFOA—which had liver/plasma ratios approximately 0.5 and 1, respectively—reflect this difference. Sex differences in the urinary elimination of PFAS in rodents have also been reported, specifically for the shorter-chain PFAS, with females exhibiting more rapid elimination than males[27; 125]. The plasma levels observed in male and female rats administered PFOA underscore this contrast (15,000 to 80,000 µM/mmol/kg in males compared to 80 to 200 µM/mmol/kg in females). In rats administered PFHxA, relatively low plasma levels precluded determination of sex-related differences in exposure. In general, the levels of PFAS measured in these studies were orders of magnitude higher than those measured in humans[8].

The toxicity comparisons of a chemical could be evaluated from an exposure standpoint in several ways (e.g., comparing external dose versus internal dose as C_{max}, area under the curve [AUC], or final blood measurements). In addition, each toxicity or effect may be better explained using a different exposure paradigm. An extensive evaluation of these possible exposure comparisons and response models is beyond the scope of this report, but a comparison of findings based on external dose (mmol/kg/day) and plasma levels on day 29 (µM) allows for a qualitative summary of the findings and highlights the challenges in potency estimations. Highlighted are select liver endpoints and thyroid hormone measurements graphed against plasma levels or administered dose (Figure 33, Figure 34, Figure 35, Figure 36, Figure 37, Figure 38, Figure 39, Figure 40, Figure 41, Figure 42, Figure 43, Figure 44) because these findings were generally present across all the chemicals. Briefly, toxicity comparisons based on dose administered generally show PFDA to be the most potently toxic of the four PFAS

carboxylic acids and PFHxA to be the least potent, with PFNA and PFOA closer to PFDA in potency than PFHxA. These findings are described in more detail below.

A major target organ of toxicity for PFHxA, PFOA, PFNA, and PFDA was the liver (Figure 33, Figure 34), which is consistent with the greater PFAS class. Inclusion of WY allows for a direct comparison of a known PPARα with a PFAS, which have been shown to activate PPARα, constitutive androstane receptor (CAR), pregnane X receptor (PXR), and other receptors[126; 127]. Gene expression of *Cyp4a1*, *Acox1*, *Cyp2b1*, and *Cyp2b2* was measured to probe specific nuclear receptor pathways: *Cyp2b1*/*Cyp2b2* activation indicates CAR-mediated activity, and *Acox1*/*Cyp4a1* activation suggests PPARα activity. All five chemicals, at the highest surviving dose, induced the expression of all four genes in males and females. Males generally had a higher magnitude of induction for *Acox1*/*Cyp4a1* than females, and females exhibited a higher magnitude of induction for *Cyp2b1*/*Cyp2b2* than males. Sexually dimorphic responses to PPARα and CAR activation in rats have been reported previously[128; 129], thus different sensitivities to activation are not unexpected. When comparing the five chemicals using the administered dose as the exposure metric, PFHxA is the least potent and PFDA is generally the most potent for *Cyp* induction, with WY in between (Figure 33A–D, and Figure 34A–D). Using plasma levels (molarity) as the exposure metric, there is a high degree of overlap in males, whereas PFHxA is the most potent in females. These patterns of potency were also reflective of the changes in liver weight and the occurrences of hepatocellular hypertrophy (Figure 33E–H, Figure 34E–H). Overall, the similarities in gene induction patterns suggest a high degree of overlap between PFAS and WY with respect to nuclear receptor activation. Several toxicity endpoints described below may be mediated by PPARα and/or CAR-related activity.

In the present studies, all four perfluoroalkyl carboxylates increased liver weights in males and females; however, histologic lesions in the liver varied somewhat across these chemicals and between sexes (Figure 35, Figure 36, Figure 37 and Figure 38). Hepatocyte cytoplasmic alteration and hepatocyte hypertrophy were observed in males and females administered PFHxA, PFOA, PFNA, or PFDA. In the short-chain PFAS, hepatocyte necrosis was only observed in one male administered 1,000 mg/kg/day PFHxA and one male administered 5 mg/kg/day PFOA. Necrosis was more frequently observed in PFNA- and PFDA-treated males and females and occurred in a dose-dependent manner. Hepatocyte hypertrophy and cytoplasmic alteration were also observed in the PPARα agonist WY, but hepatocyte necrosis was not. Hepatocyte hypertrophy observed with PFAS is likely due to the peroxisome proliferation. This is also supported by the elevated *Acox1* and *Cyp4a1* enzymes known to be inducible by PPARα agonists. Hepatocyte hypertrophy can also be partially mediated through CAR. It is worth noting that the current studies were not designed to evaluate the other possible mechanisms; however, attributing the effects of PFAS solely to PPARα activation would misrepresent the complexity of the biological activity of this chemical class[130-134]. In short, it is possible that other mechanisms beyond PPARα- and CAR-mediated mechanisms are involved in the induction of hepatocyte hypertrophy.

Beyond histopathology, PFAS administration increased the levels of serum biomarkers associated with hepatobiliary injury. In general, bile acid concentrations in PFDA and PFNA studies had greater than a threefold increase in the higher-dose groups compared to concurrent vehicle controls. Increases of this magnitude are indicative of impaired bile flow or (intrahepatic) cholestasis, the causes of which include physical disruption of bile flow through the biliary system or perturbation of bile acid formation and excretion at the cellular level[135]. In addition,

total bilirubin concentration, another marker of cholestasis, was substantially elevated in most high-dose PFNA and PFDA groups. Increases in bile acid concentration were also observed with PFOA and PFHxA (males only) but to a lesser extent, and a small increase in direct (not total) bilirubin was observed only in the high-dose PFOA males. There were mild increases in alkaline phosphatase (ALP) activity in both sexes in all four carboxylate studies. ALP is considered a poor marker of cholestasis in rats[136; 137]; thus, although the increases in ALP may be due to cholestasis, limited studies have shown an association between the administration of PPARα agonists and mild increases in serum ALP activity when the only histologic effect observed was centrilobular hypertrophy[138]. In general, there were mild (≤onefold) increases in serum ALT, AST, and/or SDH activities in both sexes of the higher-dose groups of all the carboxylates. These enzymes are biomarkers of hepatocellular injury, and their increases correlate with the observed hepatocellular necrosis, as well as the finding of cholestasis as hepatocellular injury can occur secondary to cholestasis[136]. In some cases, indications of cholestasis and histopathologic necrosis were not found, suggesting reversible hepatocellular injury resulting in leakage of these enzymes from the cytoplasm of the hepatocytes. Additionally, and similar to ALP activity, it has been shown that mild (≤1.5-fold) increases in hepatic and serum transaminases are associated with the administration of hepatic microsomal enzyme inducer compounds, including PPARα agonists[136; 138; 139]. In addition to increases in liver enzyme activities, albumin concentrations were decreased in some dose groups across the four chemicals. These decreases may be related to the decreases in food intake and body weight, or they may be due to a functional perturbation of albumin metabolism by the liver. Hepatobiliary injury biomarker changes similar to those observed in the current study have been reported in the literature[44; 140]. Many of these markers of hepatic injury were present in WY-treated animals, including increased direct bilirubin and bile acids in males and increased ALT, ALP, SDH, and AST in males and females.

There were decreases in cholesterol concentrations in the PFHxA-, PFOA-, PFNA- and PFDA-dosed males, and the PFOA- and PFDA-dosed females. A decrease in triglyceride concentrations was also observed in PFOA, PFNA, and PFDA males. Changes in lipid markers are consistent with the known effects of PPARα activation on lipid metabolism, which includes increases in peroxisomal fatty acid β-oxidation and effects on lipid transport. Studies have shown that administration of PPARα agonists to rats, including carboxylated PFAS, results in lowered circulating triglyceride and cholesterol concentrations[44; 141; 142]. CAR is also an important regulator of cholesterol homeostasis, and its activation may also be related to the observed lipid alterations[143]. In addition, thyroid hormone triiodothyronine (T3) directly influences activities of critical enzymes in lipolytic pathways, and hypothyroidism in rats results in decreased blood triglyceride concentrations[144]. Globulin concentrations were decreased in males and females in all four perfluoroalkyl carboxylate studies. In each case, the decrease in the globulin concentration contributed to an increase in the albumin/globulin (A/G) ratio. The globulin fraction of serum total protein consists of the α -, β-, and γ-globulins. In general, α- and β-globulins are produced by hepatocytes and the γ-globulins (immunoglobulins) by B-lymphocytes. It is not known whether these chemicals cause perturbation of the hepatic or lymphocytic production/metabolism of these proteins. WY-dosed animals did not exhibit the same alterations in cholesterol and triglyceride levels as PFAS-dosed animals; however, they did exhibit decreased globulin concentration and increased A/G ratio.

Changes in thyroid hormone concentrations were observed across the four perfluoroalkyl carboxylates (Figure 39, Figure 40, Figure 41, Figure 42, Figure 43 and Figure 44). Total

thyroxine (T4) and free T4 were generally decreased in all animals administered carboxylated PFAS. Total T3 was not altered in a consistent manner across the chemicals or sexes, nor was thyroid stimulating hormone (TSH) increased in response to the lower T4 concentrations aside from females administered 10 mg/kg/day PFOA. Except for the high-dosed PFOA males and females, which exhibited thyroid gland follicular hypertrophy, there were no histopathologic changes in the thyroid gland. The few known studies evaluating effects of oral administration of these carboxylated PFAS (PFDA) on thyroid hormone status reported similar findings[145], and several studies of sulfonated PFAS reported decreases in thyroid hormones without compensatory increases in TSH[132; 146-148]. Production and release of thyroid hormones is regulated by thyroid-releasing hormone (hypothalamus) and TSH (pituitary) in what is referred to as the hypothalamic-pituitary-thyroid axis where decreases in circulating levels of free T4 and free T3 result in a compensatory increase in TSH. Archetypal xenobiotics that disrupt this axis in rats, causing decreases in thyroid hormones (either as a direct or indirect thyroid effect) with compensatory increases in TSH, and thyroid gland follicular cell hyperplasia, include sulfonamides, FD&C Red No. 3, and phenobarbital[149].

The reason for a lack of TSH response in the face of substantially low thyroid hormone concentrations in these perfluoroalkyl carboxylate studies is not clear and not consistent with a disruption in the hypothalamic-pituitary-thyroid axis. It has been shown that PFAS can bind to proteins, including albumin and transthyretin (previously prealbumin), which are transport proteins for thyroid hormones. Several PFOS studies (rat and monkey) have shown low free T4 levels as measured by analog radioimmunoassays (RIA) (the method used in the present studies), but no change in free T4 levels when measured by equilibrium dialysis followed by RIA (ED-RIA)[132; 148; 150; 151]. ED-RIA is considered the reference method for the determination of free T4 and is the standard by which other methods are compared. These findings are consistent with PFOS competing with T4 for binding to serum proteins, creating a negative bias in the (competitive binding) analog RIA method. A study evaluating the competitive binding of various PFAS to transthyretin confirmed the ability of these perfluoroalkyl carboxylates to bind to transthyretin[152]. Nevertheless, decreases in total T4 were found in the aforementioned studies and in the current studies. It is plausible that the decreases in total T4 may be related to activation of PPARα and CAR, resulting in an increase in thyroxine-UDP glucuronosyltransferase and accelerated degradation of thyroxine by the liver. While the total T4 was decreased, the lack of a TSH response in most cases and the findings by several researchers of an unaffected free T4 by ED-RIA do not support a state of classical hypothyroidism, and the effect is more similar to the profile of nonthyroidal illness syndrome (a.k.a. euthyroid sick syndrome)[153]. Other researchers have also concluded, on the basis of the thyroid-related results of their studies, that the administration of PFAS (PFDA, PFOS) does not cause a classical hypothyroid state[145; 148; 150].

Hematology parameters were also altered for these chemicals. In PFDA males and females and PFNA males, dose-dependent decreases in reticulocytes occurred, with their numbers being severely decreased in the higher-dose groups (≤10% of controls). In addition, increases in some erythron parameters characterized by increased erythrocyte counts, increased hematocrit, or increased hemoglobin concentration (in total indicative of hemoconcentration) were observed. The mean cell volume was decreased, which was driven by the severe decreases in the reticulocytes as reticulocytes are larger in size. Moderate to severe bone marrow hypocellularity in the higher-dose groups was also observed. The decreases in reticulocyte counts and the bone marrow hypocellularity are consistent with suppression of erythropoiesis. Because rat

erythrocytes have a circulating life span of approximately 60 days and the study was 28 days, it may be that the hypocellularity in the bone marrow was not fully reflected in the circulating total red blood cell mass (i.e., erythrocyte count). The bone marrow suppression was due in part to chronic stress, especially at higher doses, as evidenced by the decreases in body weight; however, a direct effect cannot be ruled out. The hemoconcentration (and increases in blood urea nitrogen) was due to a decrease in water intake. Bone marrow hypocellularity and decreases in the erythron were observed in the PFOA male and female rats and were likely secondary to chronic stress. In addition, low thyroid hormones can cause decreases in the erythron[154].

In the PFHxA-treated male and female rats there was a dose-dependent hypochromic, macrocytic regenerative decrease in the red blood cell mass (i.e., regenerative anemia). This particular combination of complete blood count (CBC) findings is indicative of either red blood cell loss (e.g., GI hemorrhage) or red blood cell destruction (hemolysis). Clinical observations and histopathologic evidence of hemorrhage were not observed. In general, hemolysis occurs within the vasculature (intravascular) or as erythrophagocytosis in the spleen or liver (extravascular). Indications of hemolysis were not seen on peripheral blood smears (e.g., Heinz bodies, blister cells), nor were indications of hemolysis observed on histopathology (e.g., increased pigment, erythrophagocytosis in the spleen). Thus, the basic underlying mechanism for the regenerative anemia is not known at this time. Similar hematology findings in PFHxA exposed rats have been reported in the literature[44; 75]. In PFNA and PFDA males and females, there were increases in thymic atrophy in the higher-dose groups. This change is most likely associated with stress as thymic atrophy is one of the commonly recognized stress responses in toxicity studies[155]. In addition, atrophy in the lymphoid organs and decreases in the lymphocyte counts (males) observed in PFNA-treated animals is also very likely associated with stress.

Interstitial cell atrophy in the testis was present in PFNA- and PFDA-treated males. Interstitial cell atrophy is known to occur with reduced testosterone levels, and there was a significant reduction of testosterone in PFNA- and PFDA-treated males. Increased apoptosis of epididymal epithelium in PFNA-treated males also supports the possibility of reduced testosterone levels[156]. However, other changes associated with reduced testosterone such as atrophy of accessory sex glands and the presence of increased numbers of apoptotic pachytene spermatocytes in Stage VII and VIII tubules were not observed[157; 158]. An epidemiology study found a negative association between PFAS and testosterone levels in humans[159]. In addition to interstitial cell atrophy, there were degenerate changes, spermatid retention in the seminiferous tubules. These changes can result from either a reduced testosterone level or from Sertoli cell toxicity[157]. The presence of exfoliated germ cells and hypospermia in the epididymis also supports the changes in the testis. Furthermore, testicular and epididymal weights were also significantly affected. In PFNA-treated males, there was an increase in the incidence of sperm granulomas in the epididymis. Damage to epididymal epithelium can result in reduced resorption of seminiferous tubule fluid and sperm granuloma formation[157]. Sperm parameters at the respective highest doses assessed were concordantly affected (to varying degrees). Epididymal sperm counts at the highest dose assessed were similarly lowered by PFHxA, PFNA, or PFDA exposure. Spermatid counts were not affected or were minimally affected, and sperm motility was not affected by exposure to any of the chemicals. This minimal testicular response in spermatid counts is consistent with the observed histopathologic findings of retained spermatids offsetting the epithelial degeneration response. The sperm that subsequently enter the cauda epididymis are motile. If the dosing duration would have been longer, the spermatid counts would have been expected to be

concomitantly decreased. The observed effects on spermatid number are consistent with the testosterone levels needed for normal spermatogenesis. Given the consistency of response in male endpoints affected, it appears that the mode of action for the testicular responses is consistent among the agents assessed. Irrespective of the mechanism(s) involved, it is possible that PFNA and PFDA target the testes. However, both PFNA and PFDA treatment also induced substantial decreases in the body weights. Therefore, the indirect effect of reduced body weight compared to controls as the cause of the testicular changes cannot be ruled out[160]. Testicular toxicity was not observed in the animals treated with WY, which was used as a positive control for PPARα induction. Therefore, testicular toxicity observed in PFNA- and PFDA-treated animals is not likely related to PPARα activation. Unlike the response observed in males, females exhibited different chemical-related responses, ranging from no apparent effects on cyclicity (PFHxA) to sustained diestrus (PFDA).

Other sites of toxicity identified across the carboxylated PFAS include the nose, and forestomach. Olfactory epithelium degeneration, hyperplasia, and inflammation were observed in PFHxA, PFOA (females), and to a lesser extent in PFNA-exposed animals. The pattern of nasal pathology in this study was not suggestive of gavage-related reflux described in rats[161]. Because the olfactory epithelium can metabolize xenobiotics[162], the changes could be a direct effect of administration of PFAS. However, it is not possible to determine the underlying mechanism with the available data. Hyperplasia of olfactory epithelium appeared to be a response to degeneration and loss of the olfactory epithelium. In addition to olfactory epithelial changes, the respiratory epithelium of the nasal cavity was also affected in animals treated with PFOA. Forestomach epithelium hyperplasia was observed in PFNA males and females and in PFDA males at the higher dose levels. This is most likely a result of mucosal irritation due to the administered chemical.

All four PFAS were negative in bacterial mutagenicity tests, similar to what has been reported for these chemicals in the literature, with the exception of PFOA, which gave nonreproducible responses over three independent trials in a single strain, leading to a call of equivocal.[b] No indication of chromosomal damage, as measured in an erythrocyte micronucleus assay, was seen in male or female rats exposed to PFHxA, PFNA, PFDA, or WY or female rats exposed to PFOA. Although a positive response was indicated for male rats exposed to PFOA, the response was within the laboratory's historical control range, and therefore the biologic significance of the increase is questionable. The bone marrow clearly was a target for these PFAS, as indicated by the changes in % PCEs observed in the micronucleus assay and hematological changes observed in clinical chemistry studies. The four PFAS showed differential effects on % PCEs. Whereas PFHxA increased the % PCE in male and female rats, PFOA did not affect % PCE in rats, and % PCE was severely reduced in male rats exposed to PFNA or PFDA, but not females. The stimulus of % PCE in rats exposed to PFHxA at the two highest doses and the reduction in % PCE with increasing chain length was consistent with hematological observations. Also, although PFOA did not appear to affect % PCE, hematological effects were observed in the clinical chemistry studies.

[b] ERRATUM: An error was identified in the NTP Toxicity Report on Perfluoroalkyl Carboxylates (TOX 97). The text was changed from "negative in bacterial mutagenicity tests" to "equivocal in TA98 bacterial mutagenicity tests" without S9 for PFOA. [20 May 2020]

The studies reported here and in Toxicity Study Report 96[124] provide data for a comparison of several members of the PFAS class. The liver and thyroid hormones were common targets for all seven PFAS evaluated in these studies. In addition, there were histopathologic findings in the forestomach and nose that were common between perfluoroalkyl sulfonates and carboxylates. Increases in PPARα- and CAR-related genes occurred in most doses for all seven PFAS. In some cases, this corresponded with increased liver weights at the lowest dose tested. With the inclusion of WY as a known PPARα agonist, toxicities that are present in animals administered PFAS, but not WY, may be attributed to mechanisms other than PPARα. This includes hematologic and reproductive toxicity, as well as several nasal and forestomach lesions. Complete quantitative comparisons of these chemicals will require a more in-depth evaluation of the exposures between chemicals that could include AUC or C_{max} evaluations. The 28-day studies discussed in this report help to provide exposure and mechanistic context to facilitate extrapolation of findings.

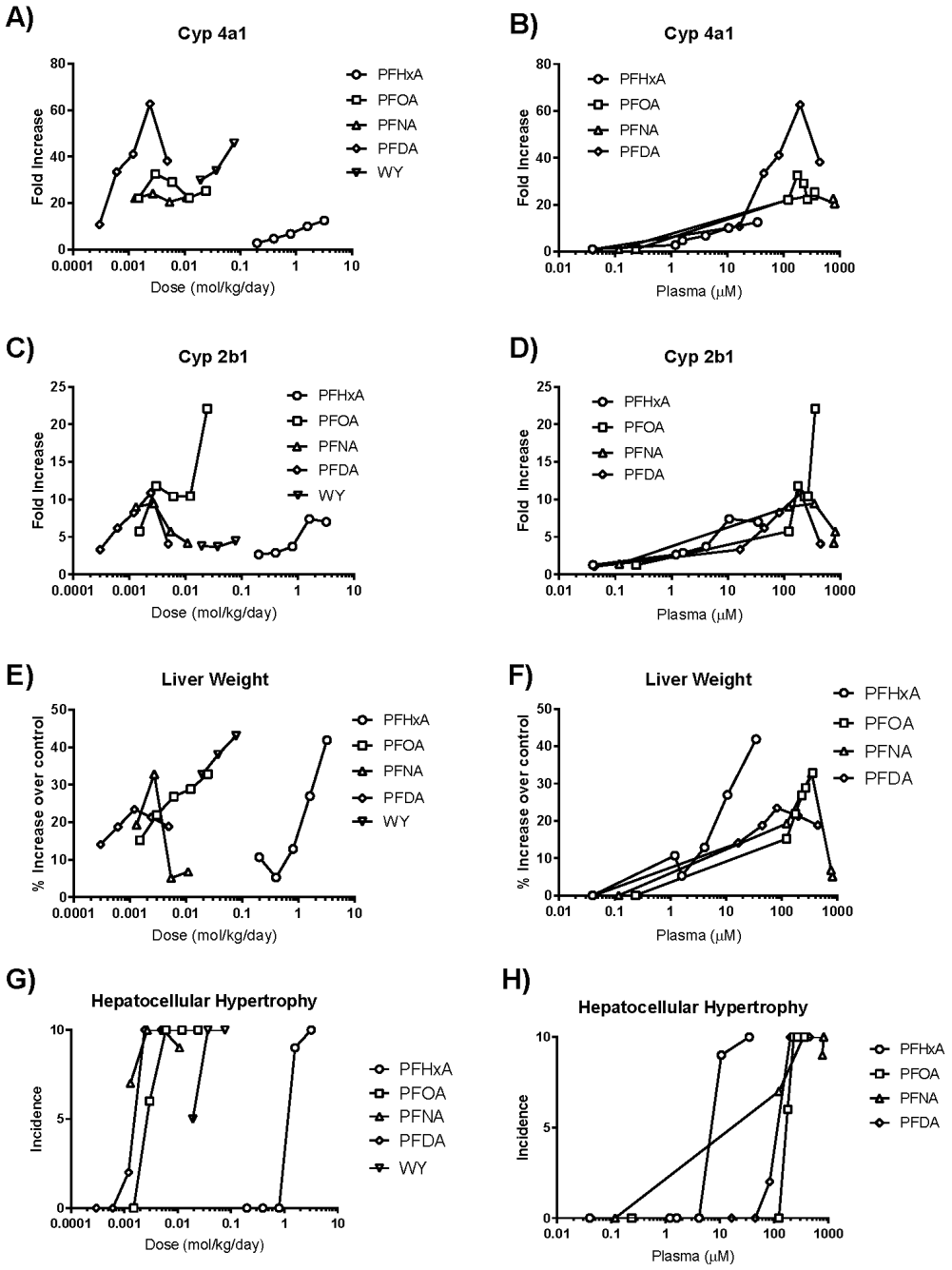


Figure 33. Average Magnitude of Change for *Cyp4a1* Expression (A, B), *Cyp2b1* Expression (C, D), Liver Weight (E, F), and Hepatocellular Hypertrophy (G, H) in Male Sprague Dawley Rats Administered Perfluorohexanoic Acid, Perfluorooctanoic Acid, Perfluorononanoic Acid, or Perfluorodecanoic Acid by Gavage for 28 Days

PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; WY = Wyeth-14,643.
Comparisons of a dose administered (mmol/kg/day) basis (A, C, E, G) versus a plasma level (μM) basis (B, D, F, H).
Wyeth-14,643 (PPARα agonist) is used for comparison on a dose administered (mmol/kg/day) basis.

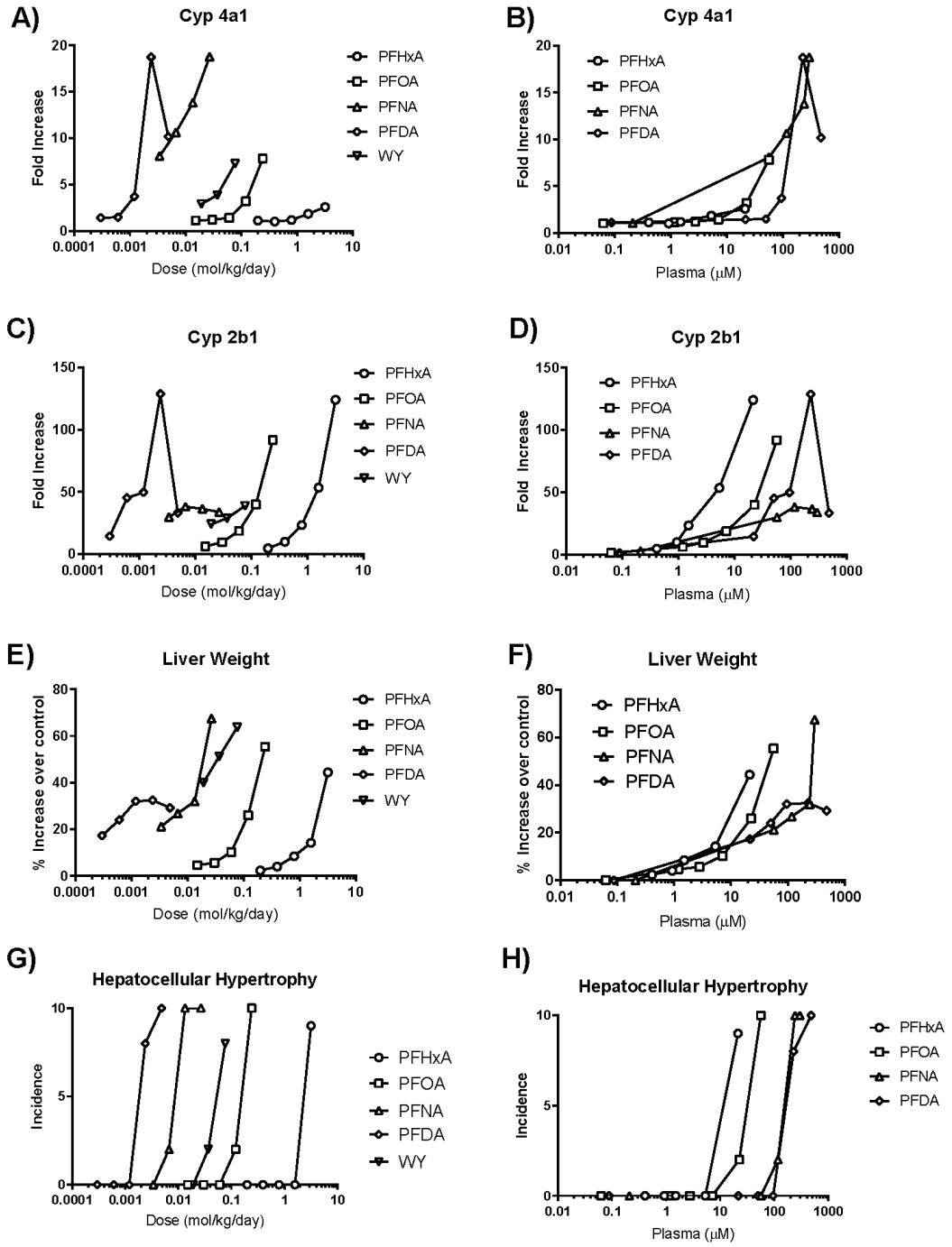


Figure 34. Average Magnitude of Change for *Cyp4a1* Expression (A, B), *Cyp2b1* Expression (C, D), Liver Weight (E, F), and Hepatocellular Hypertrophy (G, H) in Female Sprague Dawley Rats Administered Perfluorohexanoic Acid, Perfluorooctanoic Acid, Perfluorononanoic Acid, or Perfluorodecanoic Acid by Gavage for 28 Days

PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; WY = Wyeth-14,643.
Comparisons of a dose administered (mmol/kg/day) basis (A, C, E, G) versus a plasma level (μM) basis (B, D, F, H). Wyeth-14,643 (PPARα agonist) is used for comparison on a dose administered (mmol/kg/day) basis.

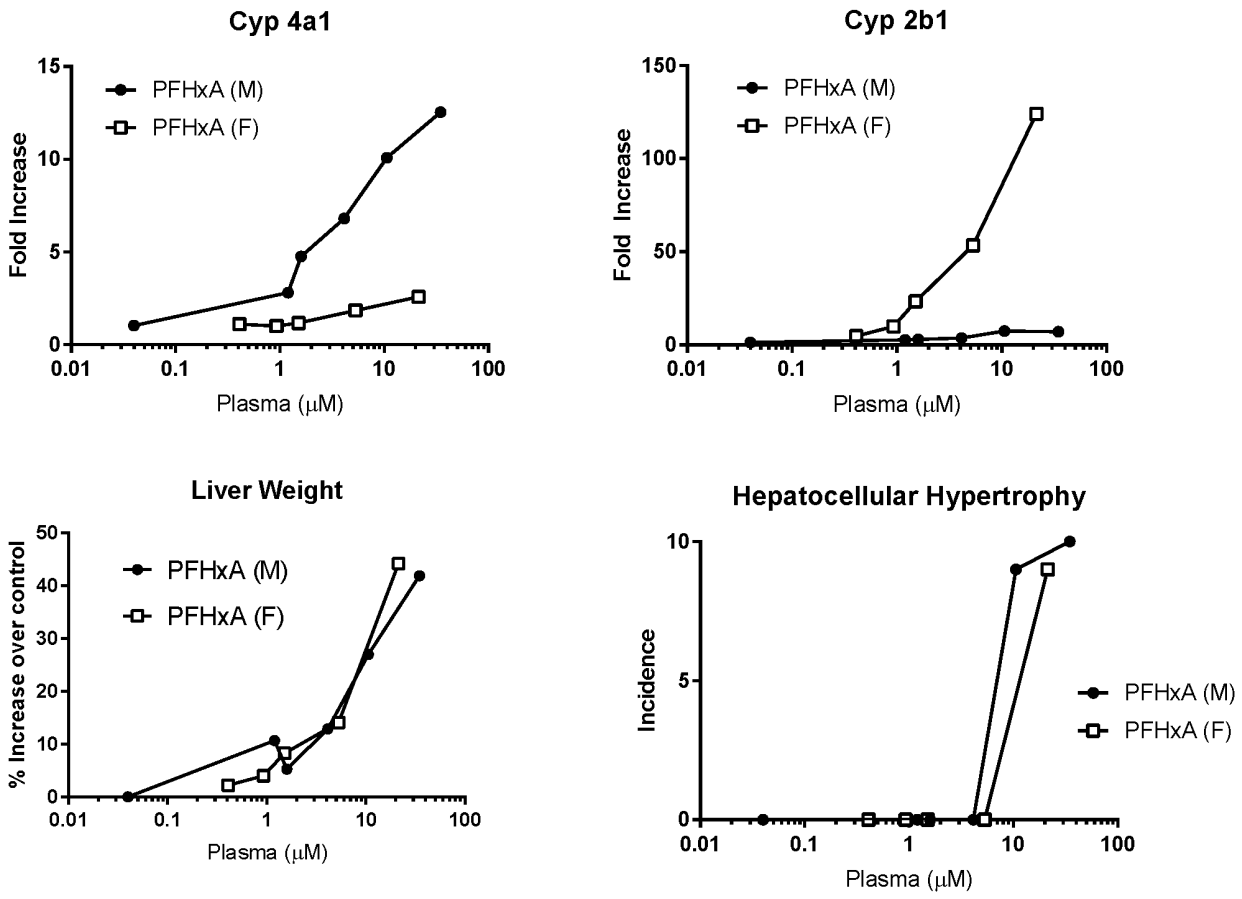


Figure 35. Comparison of Magnitude of Change for Select Liver Endpoints in Sprague Dawley Rats Administered Perfluorohexanoic Acid by Gavage for 28 Days

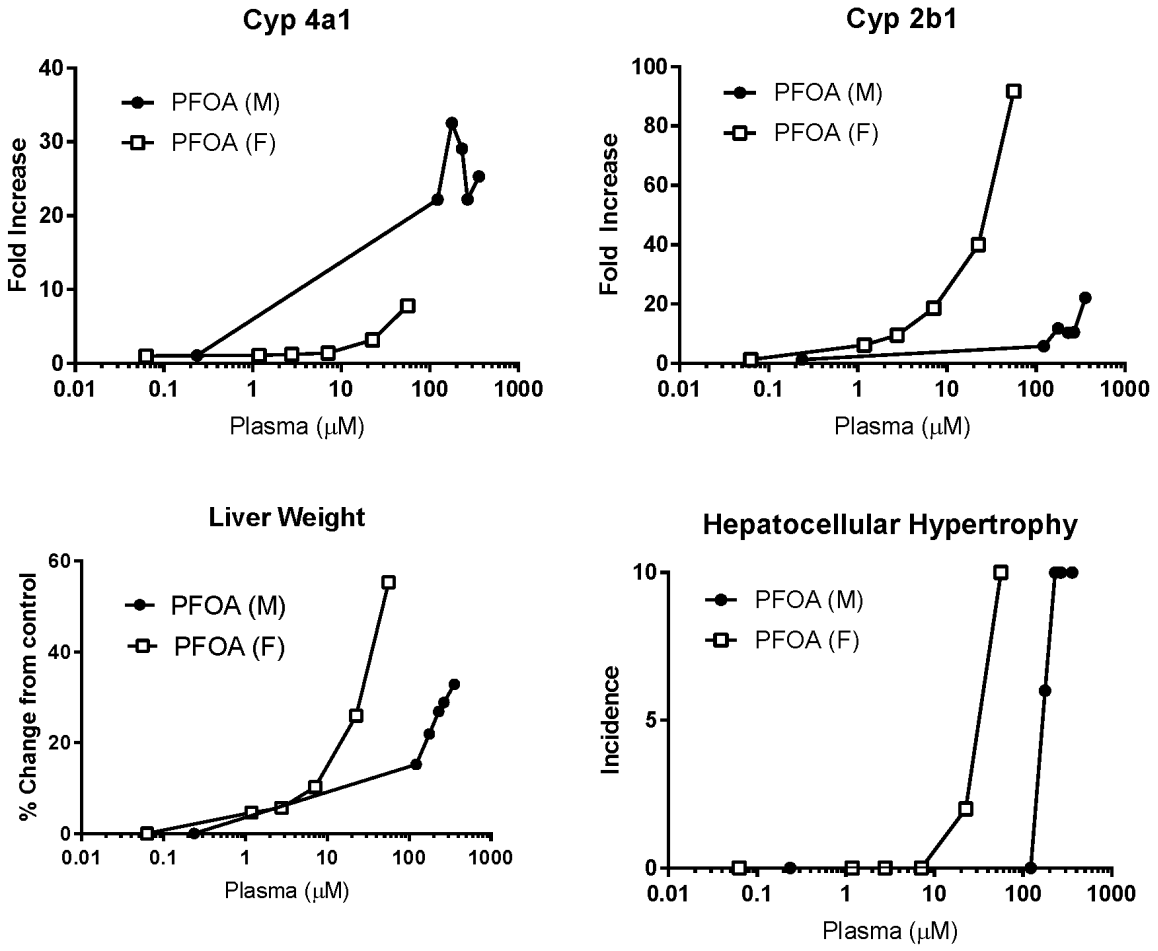


Figure 36. Comparison of Magnitude of Change for Select Liver Endpoints in Sprague Dawley Rats Administered Perfluorooctanoic Acid by Gavage for 28 Days

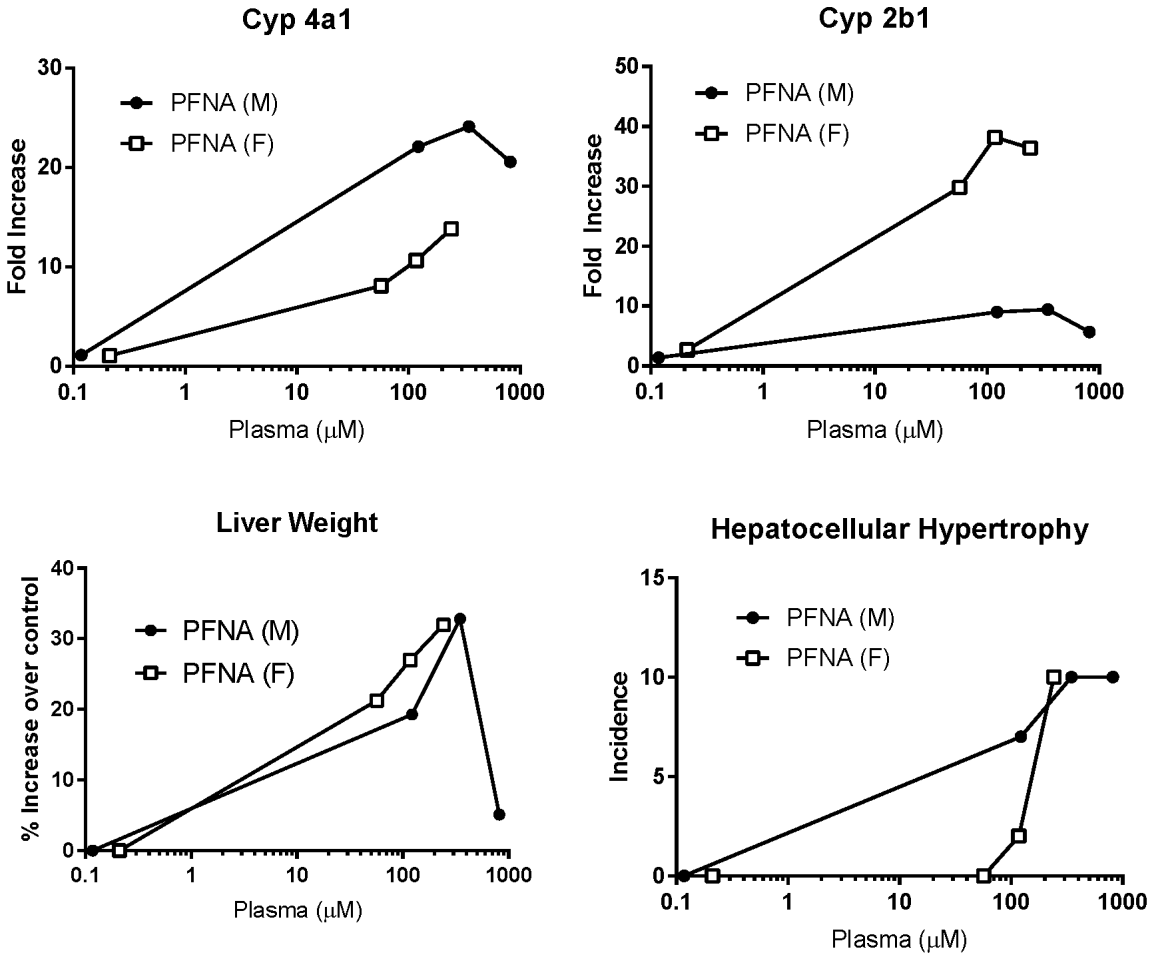


Figure 37. Comparison of Magnitude of Change for Select Liver Endpoints in Sprague Dawley Rats Administered Perfluorononanoic Acid by Gavage for 28 Days

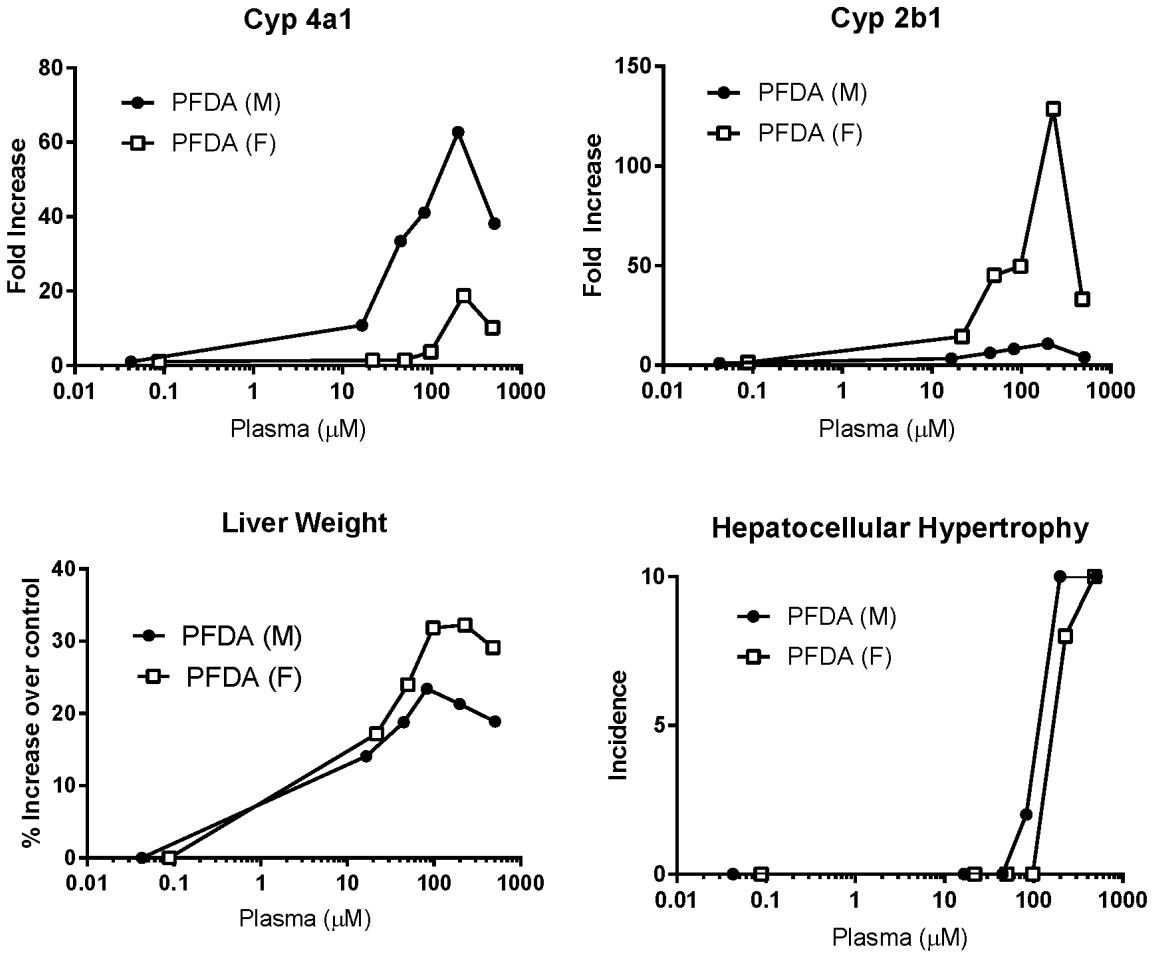


Figure 38. Comparison of Magnitude of Change for Select Liver Endpoints in Sprague Dawley Rats Administered Perfluorodecanoic Acid by Gavage for 28 Days

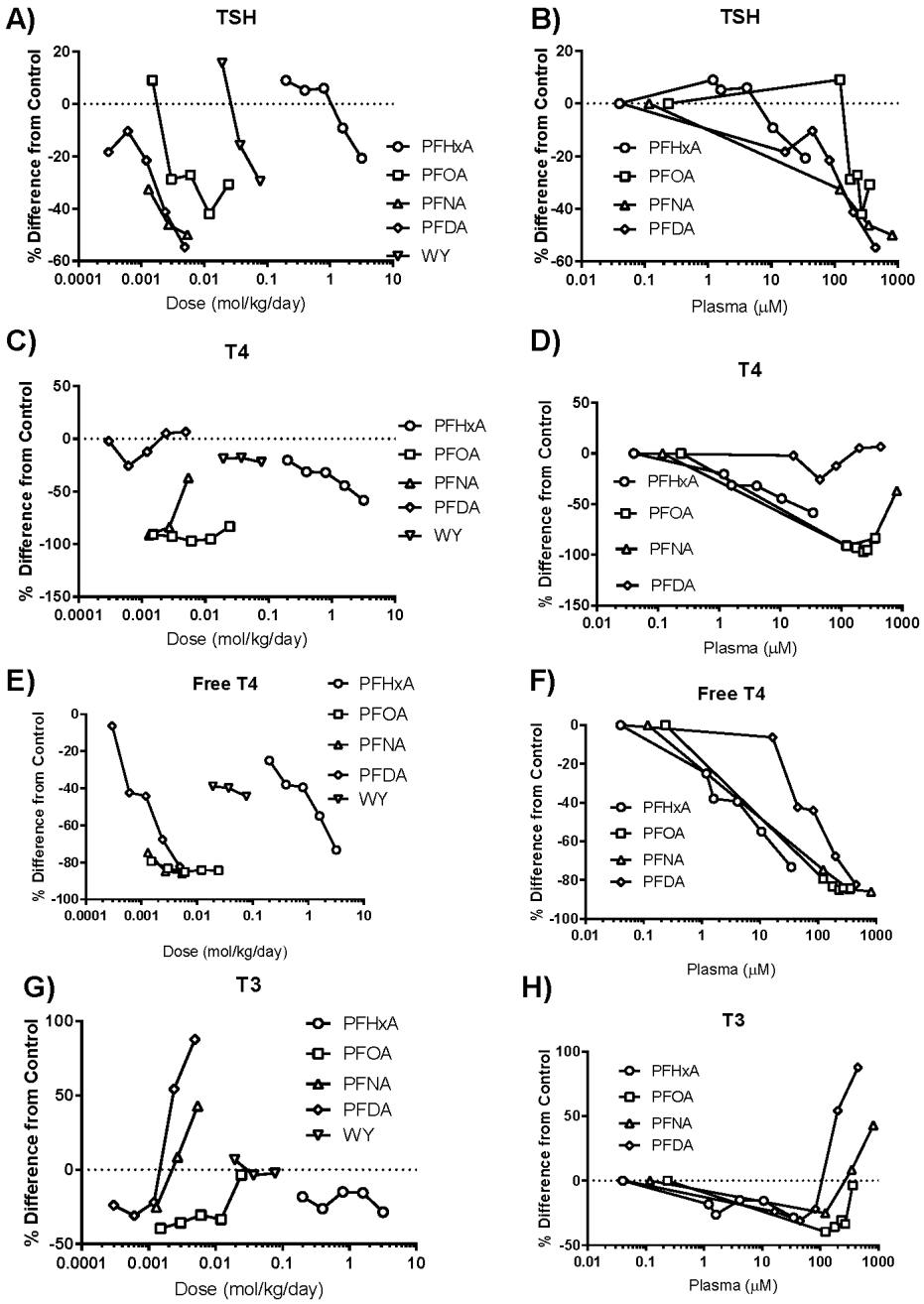


Figure 39. Average Magnitude of Change for Serum Levels of Thyroid Stimulating Hormone (A, B), Triiodothyronine (C, D), Thyroxine (E, F), and Free Thyroxine (G, H) in Male Sprague Dawley Rats Administered Perfluorohexanoic Acid, Perfluorooctanoic Acid, Perfluorononanoic Acid, or Perfluorodecanoic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine;
PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; WY = Wyeth-14,643.
Comparisons of a dose administered (mmol/kg/day) basis (A, C, E, G) versus a plasma level (μM) basis (B, D, F, H). Wyeth-14,643 (PPARα agonist) is used for comparison on a dose administered (mmol/kg/day) basis.

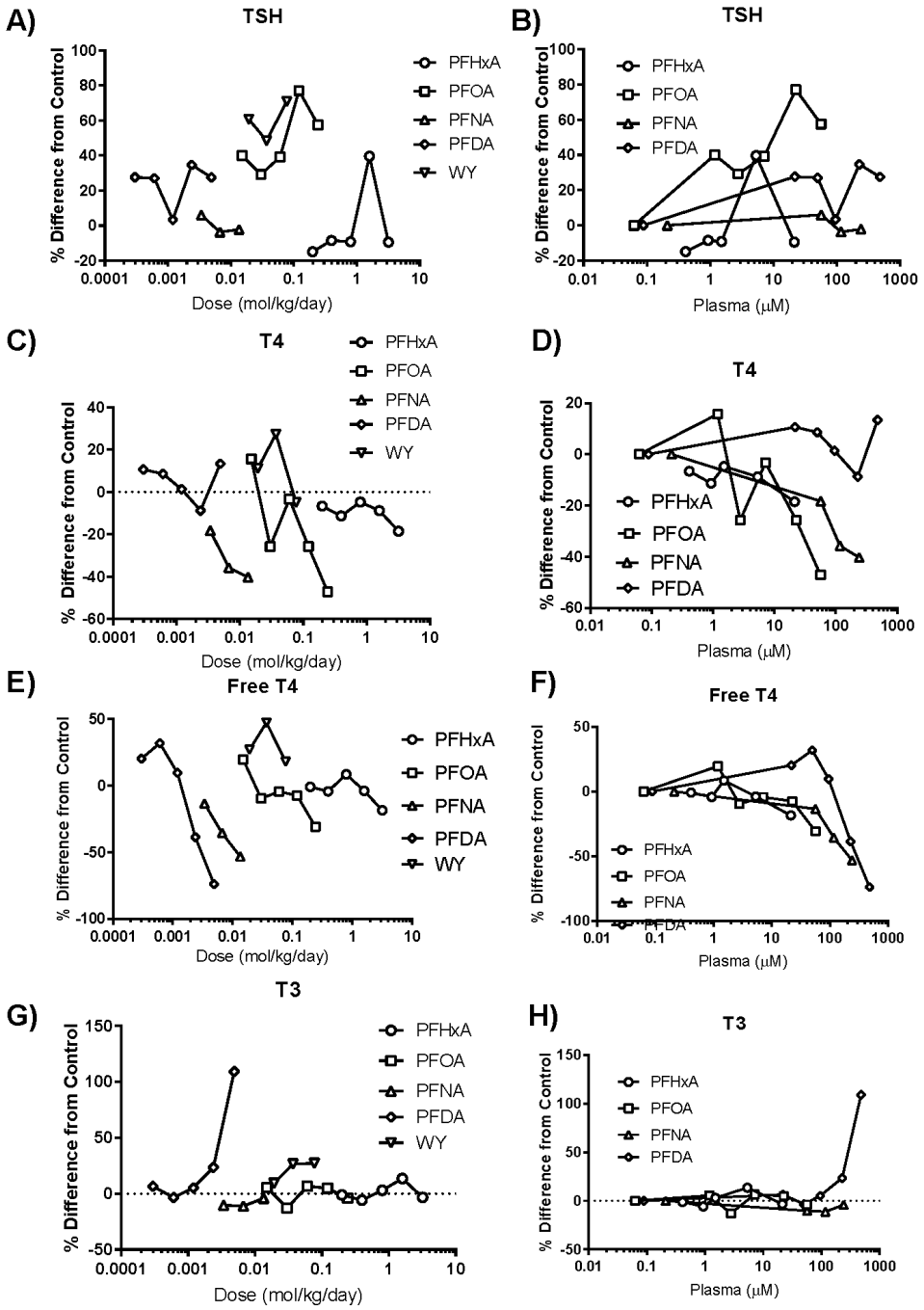


Figure 40. Average Magnitude of Change for Serum Levels of Thyroid Stimulating Hormone (A, B), Triiodothyronine (C, D), Thyroxine (E, F), and Free Thyroxine (G, H) in Female Sprague Dawley Rats Administered Perfluorohexanoic Acid, Perfluorooctanoic Acid, Perfluorononanoic Acid, or Perfluorodecanoic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine;
PFHxA = perfluorohexanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; WY = Wyeth-14,643.
Comparisons are made on a dose administered (mmol/kg/day) basis (A, C, E, G) and plasma level (μM) basis (B, D, F, H).
Wyeth-14,643 (PPARα agonist) is used for comparison on a dose administered (mmol/kg/day) basis.

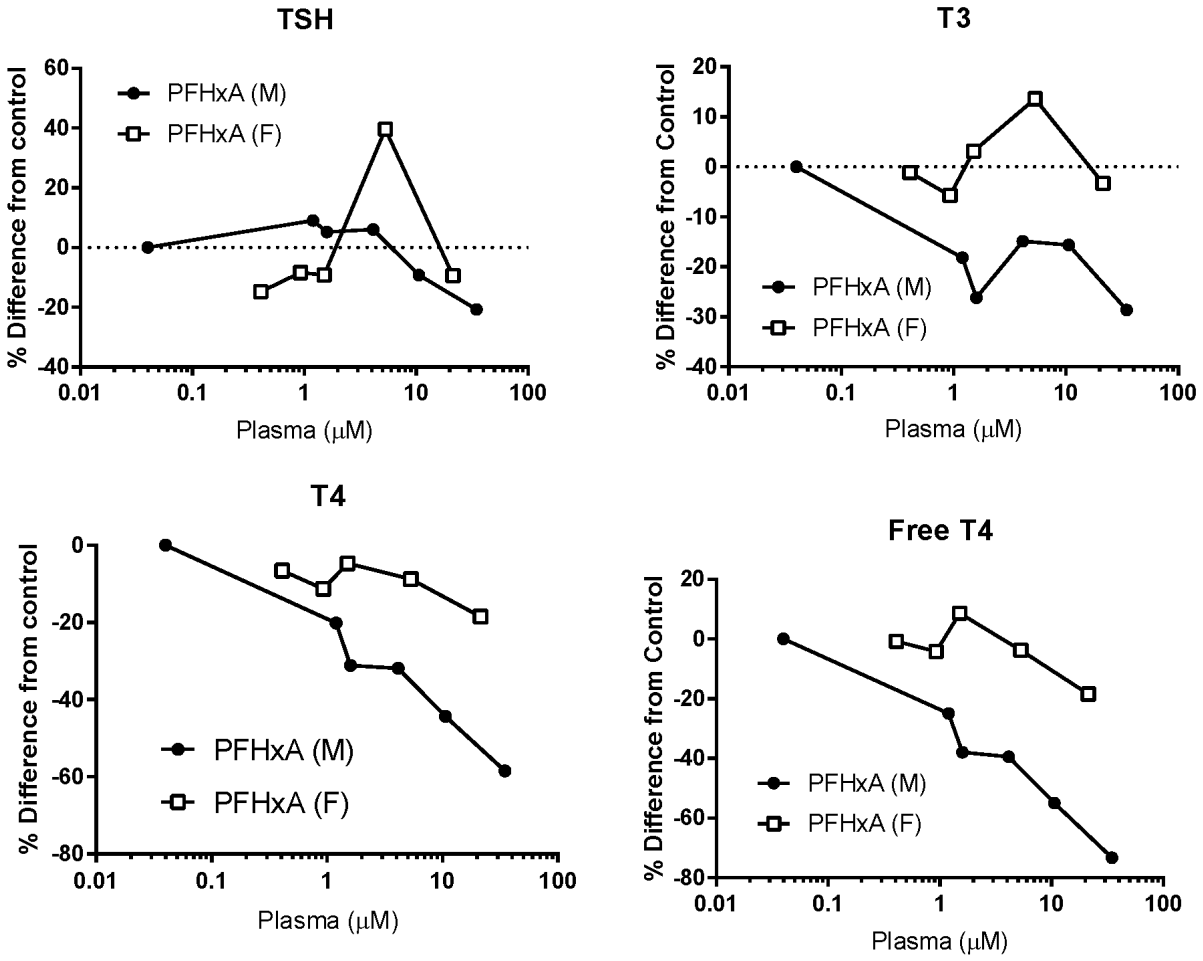


Figure 41. Comparison of Magnitude of Change for Thyroid Hormone Levels in Sprague Dawley Rats Administered Perfluorohexanoic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine.

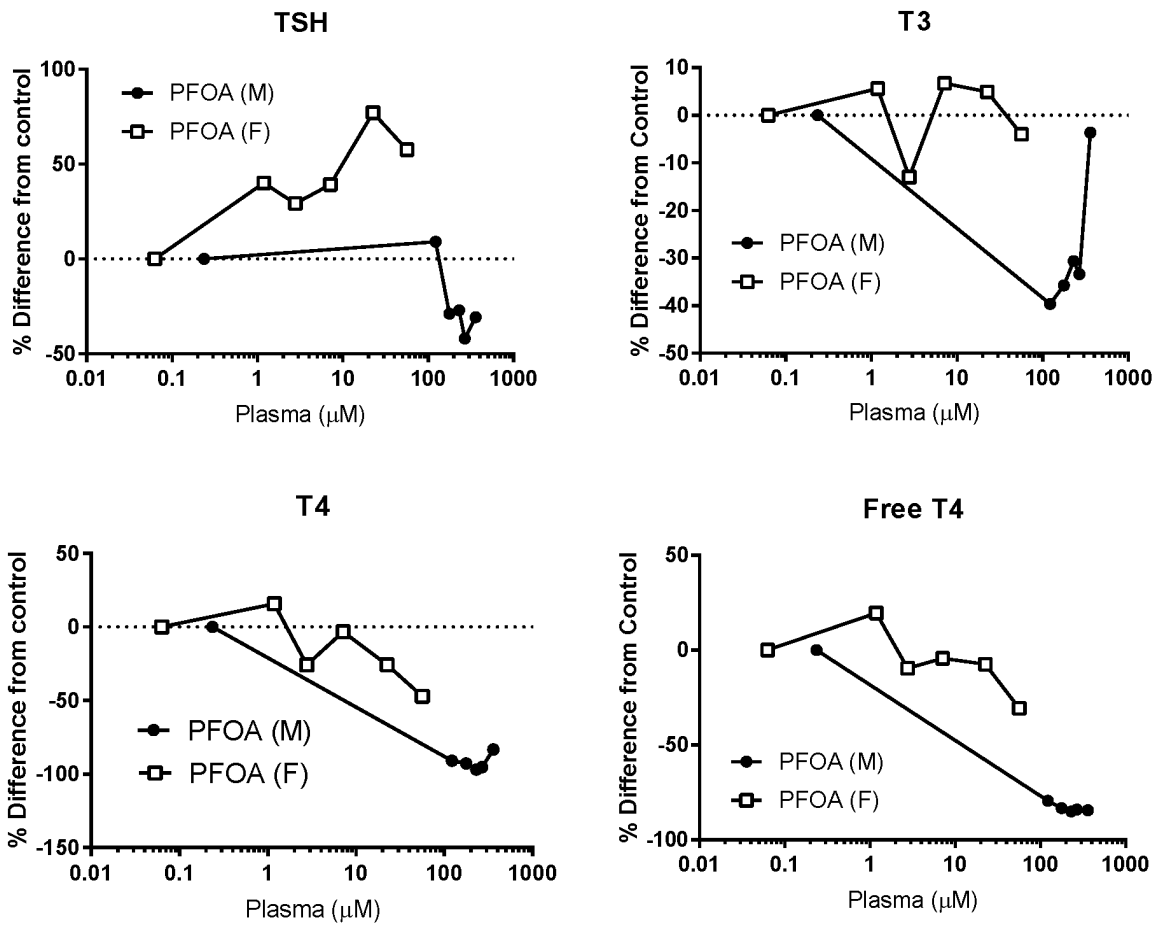


Figure 42. Comparison of Magnitude of Change for Thyroid Hormone Levels in Sprague Dawley Rats Administered Perfluorooctanoic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine.

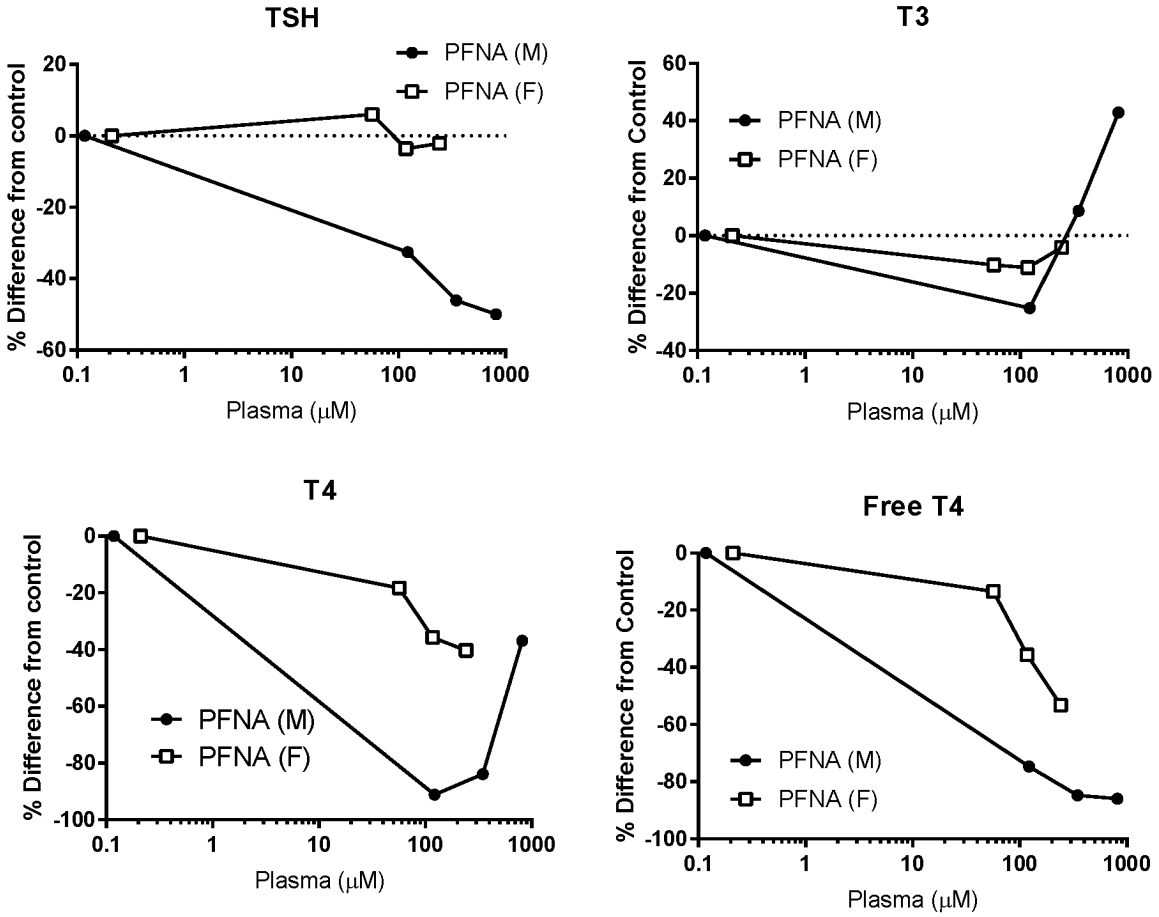


Figure 43. Comparison of Magnitude of Change for Thyroid Hormone Levels in Sprague Dawley Rats Administered Perfluorononanoic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine.

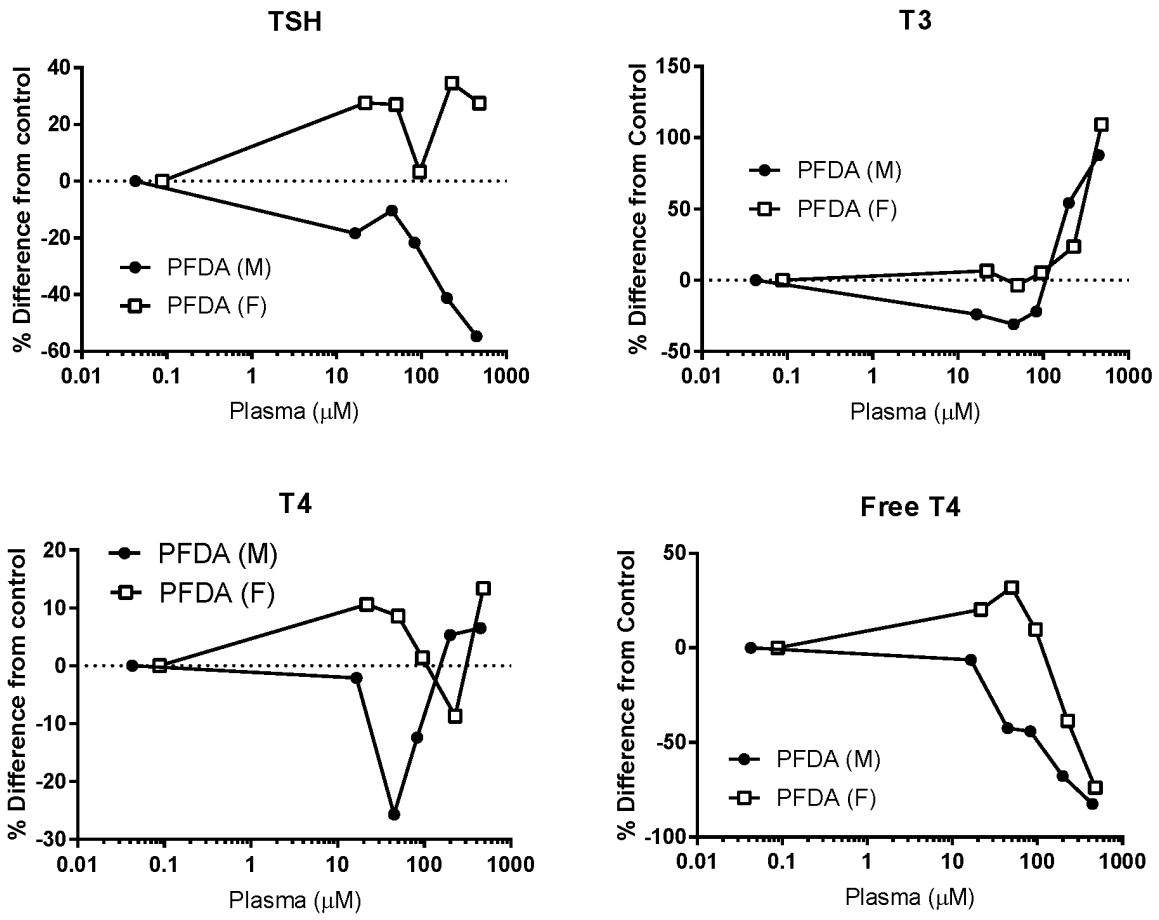


Figure 44. Comparison of Magnitude of Change for Thyroid Hormone Levels in Sprague Dawley Rats Administered Perfluorodecanoic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine.

Erratum

An error was identified in the NTP Toxicity Report on Perfluoroalkyl Carboxylates (TOX 97). The conclusion for bacterial mutagenicity for PFOA was equivocal and not negative for TA 98, as stated orginially in the report. This error has been corrected in the HTML and PDF versions of the abstract, summary table, results (page 92), and discussion (page 112) of this report.
[20 May 2020]

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Appendix A. **Reproductive Tissue Evaluations and Estrous Cycle Characterization**

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Table A-1. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Perfluorohexanoic Acid by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	<0.001	
Overall Tests	125 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	250 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	500 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	Overall	<0.001	
Extended Estrus	125 mg/kg/day vs. vehicle controls	0.006	–
Extended Estrus	250 mg/kg/day vs. vehicle controls	0.002	–
Extended Estrus	500 mg/kg/day vs. vehicle controls	0.022	–
Extended Diestrus	Overall	<0.001	
Extended Diestrus	125 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	250 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	500 mg/kg/day vs. vehicle controls	0.003	N
Extended Metestrus	Overall	1	
Extended Metestrus	125 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	250 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	500 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	125 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	250 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	500 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	0.998	
Skipped Estrus	125 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	250 mg/kg/day vs. vehicle controls	0.869	–
Skipped Estrus	500 mg/kg/day vs. vehicle controls	0.935	–
Skipped Diestrus	Overall	1	
Skipped Diestrus	125 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	250 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	500 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	125 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	250 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	500 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	125 mg/kg/day vs. vehicle controls	0.006	–
Extended Estrus	250 mg/kg/day vs. vehicle controls	0.002	–

Stage	Comparison	P Value	Trend[a]
Extended Estrus	500 mg/kg/day vs. vehicle controls	0.022	–
Extended Diestrus	125 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	250 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	500 mg/kg/day vs. vehicle controls	0.003	N

[a]N indicates that the dosed group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dosed group had more departures from normal cycling than did the vehicle control group. One-half the dose was administered twice daily. The overall tests consider all six types of departures from normal cycling. The overall comparison tests for equal transitional probabilities across all dose groups.

Table A-2. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Perfluorooctanoic Acid[a]

	Vehicle Control	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Number Weighed at Necropsy	10	10	10	10
Necropsy Body Wt. (g)	227 ± 5	219 ± 2	221 ± 3	215 ± 4
Proportion of Regular Cycling Females[b]	10/10	10/10	10/10	7/9
Estrous Cycle Length (days)	5.0 ± 0.09	4.9 ± 0.18	4.7 ± 0.15	4.8 ± 0.15
Estrous Stages (% of cycle)				
Diestrus	51.9	55.6	51.9	62.5
Proestrus	9.4	10.6	10.0	4.2
Estrus	36.9	29.4	33.8	32.6
Metestrus	1.3	4.4	4.4	0.7
Uncertain	0.6	0.0	0.0	0.0

[a]Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated that 100 mg/kg/day females had extended diestrus.
[b]Number of females with a regular cycle/number of females cycling.

Table A-3. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Perfluorooctanoic Acid by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	<0.001	
Overall Tests	25 mg/kg/day vs. vehicle controls	0.086	N
Overall Tests	50 mg/kg/day vs. vehicle controls	0.806	N
Overall Tests	100 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	Overall	0.086	
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.007	N
Extended Estrus	50 mg/kg/day vs. vehicle controls	0.767	N
Extended Estrus	100 mg/kg/day vs. vehicle controls	0.733	N
Extended Diestrus	Overall	<0.001	
Extended Diestrus	25 mg/kg/day vs. vehicle controls	0.191	–
Extended Diestrus	50 mg/kg/day vs. vehicle controls	0.943	–
Extended Diestrus	100 mg/kg/day vs. vehicle controls	<0.001	–
Extended Metestrus	Overall	1	
Extended Metestrus	25 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	50 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	100 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	50 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	100 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	1	
Skipped Estrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	50 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	100 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	Overall	1	
Skipped Diestrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	50 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	100 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	100 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.007	N
Extended Diestrus	100 mg/kg/day vs. vehicle controls	<0.001	–

[a]N indicates that the dosed group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dosed group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transitional probabilities across all dose groups.

Table A-4. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Perfluorononanoic Acid by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	<0.001	
Overall Tests	1.56 mg/kg/day vs. vehicle controls	0.001	N
Overall Tests	3.12 mg/kg/day vs. vehicle controls	0.001	N
Overall Tests	6.25 mg/kg/day vs. vehicle controls	0.026	N
Extended Estrus	Overall	0.312	
Extended Estrus	1.56 mg/kg/day vs. vehicle controls	0.624	N
Extended Estrus	3.12 mg/kg/day vs. vehicle controls	0.469	N
Extended Estrus	6.25 mg/kg/day vs. vehicle controls	0.098	N
Extended Diestrus	Overall	<0.001	
Extended Diestrus	1.56 mg/kg/day vs. vehicle controls	<0.001	N
Extended Diestrus	3.12 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	6.25 mg/kg/day vs. vehicle controls	0.192	–
Extended Metestrus	Overall	0.985	
Extended Metestrus	1.56 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	3.12 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	6.25 mg/kg/day vs. vehicle controls	0.604	–
Extended Proestrus	Overall	1	
Extended Proestrus	1.56 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	3.12 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	1	
Skipped Estrus	1.56 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	3.12 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	6.25 mg/kg/day vs. vehicle controls	0.935	–
Skipped Diestrus	Overall	1	
Skipped Diestrus	1.56 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	3.12 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	1.56 mg/kg/day vs. vehicle controls	0.001	N
Overall Tests	3.12 mg/kg/day vs. vehicle controls	0.001	N
Overall Tests	6.25 mg/kg/day vs. vehicle controls	0.026	N
Extended Diestrus	1.56 mg/kg/day vs. vehicle controls	<0.001	N
Extended Diestrus	3.12 mg/kg/day vs. vehicle controls	<0.001	–

[a]N indicates that the dosed group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dosed group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transitional probabilities across all dose groups.

Table A-5. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Perfluorodecanoic Acid by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	<0.001	
Overall Tests	0.625 mg/kg/day vs. vehicle controls	0.054	N
Overall Tests	1.25 mg/kg/day vs. vehicle controls	<0.001	N
Overall Tests	2.5 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	Overall	<0.001	
Extended Estrus	0.625 mg/kg/day vs. vehicle controls	0.21	N
Extended Estrus	1.25 mg/kg/day vs. vehicle controls	0.003	N
Extended Estrus	2.5 mg/kg/day vs. vehicle controls	<0.001	N
Extended Diestrus	Overall	<0.001	
Extended Diestrus	0.625 mg/kg/day vs. vehicle controls	0.065	–
Extended Diestrus	1.25 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	2.5 mg/kg/day vs. vehicle controls	<0.001	–
Extended Metestrus	Overall	1	
Extended Metestrus	0.625 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	1.25 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	2.5 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	0.625 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	1.25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	2.5 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	0.555	
Skipped Estrus	0.625 mg/kg/day vs. vehicle controls	0.973	N
Skipped Estrus	1.25 mg/kg/day vs. vehicle controls	0.307	N
Skipped Estrus	2.5 mg/kg/day vs. vehicle controls	0.307	N
Skipped Diestrus	Overall	1	
Skipped Diestrus	0.625 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	1.25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	2.5 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	1.25 mg/kg/day vs. vehicle controls	<0.001	N
Overall Tests	2.5 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	1.25 mg/kg/day vs. vehicle controls	0.003	N
Extended Estrus	2.5 mg/kg/day vs. vehicle controls	<0.001	N
Extended Diestrus	1.25 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	2.5 mg/kg/day vs. vehicle controls	<0.001	–

[a]N indicates that the dosed group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dosed group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transitional probabilities across all dose groups.

Table A-6. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Wyeth-14,643 by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	0.002	
Overall Tests	6.25 mg/kg/day vs. vehicle controls	0.085	–
Overall Tests	12.5 mg/kg/day vs. vehicle controls	<0.001	N
Overall Tests	25 mg/kg/day vs. vehicle controls	0.983	–
Extended Estrus	Overall	<0.001	
Extended Estrus	6.25 mg/kg/day vs. vehicle controls	0.032	N
Extended Estrus	12.5 mg/kg/day vs. vehicle controls	0.001	N
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.017	N
Extended Diestrus	Overall	<0.001	
Extended Diestrus	6.25 mg/kg/day vs. vehicle controls	0.005	–
Extended Diestrus	12.5 mg/kg/day vs. vehicle controls	0.001	–
Extended Diestrus	25 mg/kg/day vs. vehicle controls	0.009	–
Extended Metestrus	Overall	1	
Extended Metestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	12.5 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	12.5 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	0.572	
Skipped Estrus	6.25 mg/kg/day vs. vehicle controls	0.316	N
Skipped Estrus	12.5 mg/kg/day vs. vehicle controls	0.319	N
Skipped Estrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	Overall	1	
Skipped Diestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	12.5 mg/kg/day vs. vehicle controls	0.934	–
Skipped Diestrus	25 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	12.5 mg/kg/day vs. vehicle controls	<0.001	N
Extended Estrus	6.25 mg/kg/day vs. vehicle controls	0.032	N
Extended Estrus	12.5 mg/kg/day vs. vehicle controls	0.001	N
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.017	N
Extended Diestrus	6.25 mg/kg/day vs. vehicle controls	0.005	–
Extended Diestrus	12.5 mg/kg/day vs. vehicle controls	0.001	–
Extended Diestrus	25 mg/kg/day vs. vehicle controls	0.009	–

[a]N indicates that the dosed group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dosed group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transitional probabilities across all dose groups.

Figure A-1. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Perfluorohexanoic Acid

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus.
One-half the dose was administered twice daily.

Figure A-2. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Perfluorooctanoic Acid

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus.

DOSE (mg/kg)	ANIMAL																												
0	761	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D												
0	763												E	E	D	D	D	E	E	D	D	P	E	E	D	D	P	E	
0	765												E	E	D	D	D	E	E	D	D	D	D	D	D	D	D	D	
0	769											E	D	D	D	D	P	E	D	D	D	E	E	D	D	D	D		
0	766											D	E	E	D	D	D	E	E	D	D	D	E	D	D	D	D		
0	764										E	E	E	D	D	D	D	E	E	D	D	D	P	E	D	D			
0	762										D	D	E	E	D	D	P	E	E	D	D	D	E	E	D	D			
0	770									D	D	P	E	E	D	D	D	E	E	D	D	P	E	E	D				
0	767									D	D	D	E	E	D	D	D	E	E	D	D	D	D	D	D				
0	768				D	D	D	D	D	D	D	D	E	D	D	D	P	E	D										
1.56	776												E	E	D	D	D	E	E	D	D	D	E	E	D	D	D	P	
1.56	779												E	D	D	D	P	E	D	D	P	E	E	D	D	P	E	E	
1.56	772												E	D	D	D	P	E	D	D	P	E	E	D	D	D	D	D	
1.56	780												E	D	D	D	P	E	D	D	E	E	E	D	D	D	E	E	
1.56	771											P	E	D	D	D	P	E	D	D	D	E	E	D	D	D	D		
1.56	775											D	E	E	D	D	D	E	D	D	D	E	E	D	D	D	E		
1.56	773										D	P	E	D	D	D	P	E	D	D	D	E	E	D	D	P			
1.56	778										D	D	E	E	D	D	D	E	E	D	D	D	P	E	D	D			
1.56	774									D	D	P	E	D	D	D	P	E	D	D	D	E	E	D	D				
1.56	777				D	D	E	D	D	D	D	D	D	D	D	D	D	E	D										
3.12	781												E	D	D	D	P	E	D	D	P	E	E	D	D	D	P	E	
3.12	785												E	D	D	D	D	E	E	D	D	D	P	E	D	D	D	E	
3.12	783												E	D	D	D	D	E	E	D	D	D	P	E	D	D	D	D	
3.12	787												E	D	D	D	I	E	E	D	D	D	E	E	D	D	D	E	
3.12	782											D	E	E	D	D	P	E	E	D	D	P	E	E	D	D	P		
3.12	788										D	E	E	D	D	D	P	E	D	D	D	P	E	D	D	D			
3.12	786									D	D	D	P	E	D	D	D	E	E	D	D	D	P	E	D				
3.12	784									D	D	D	E	E	D	D	D	E	E	D	D	D	E	E	D				
3.12	789									D	D	D	D	E	D	D	P	E	D	D	D	E	E	D	D				
3.12	790		D	E	E	D	D	D	D	D	D	D	D	D	D	D	P	E											
6.25	791	D	D	D	P	E	E	D	D	D	M	M	D	D	D	D	D												
6.25	796	D	D	D	D	D	D	D	D	P	E	D	D	D	D	D	D												
6.25	800	D	D	D	D	D	D	D	D	D	D	D	D	D	D	P	E												
6.25	793										D	P	E	D	D	D	P	E	D	D	P	E	E	D	D	D			
6.25	798										D	D	E	E	D	D	P	E	E	D	D	D	D	D	D	D			
6.25	795									D	D	D	E	E	D	D	D	E	E	D	D	D	E	E	D				
6.25	797								D	D	D	D	E	D	D	D	D	E	D	D	D	D	D	D					
6.25	794							D	D	D	D	D	E	E	D	D	P	E	E	D	D	D	D						
6.25	792						D	D	D	D	D	D	D	E	D	D	D	E	E	D	D	D							
6.25	799				E	D	D	D	D	D	D	D	D	D	D	D	D	E	E										

Figure A-3. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Perfluorononanoic Acid

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus.

Figure A-4. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Perfluorodecanoic Acid

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus.

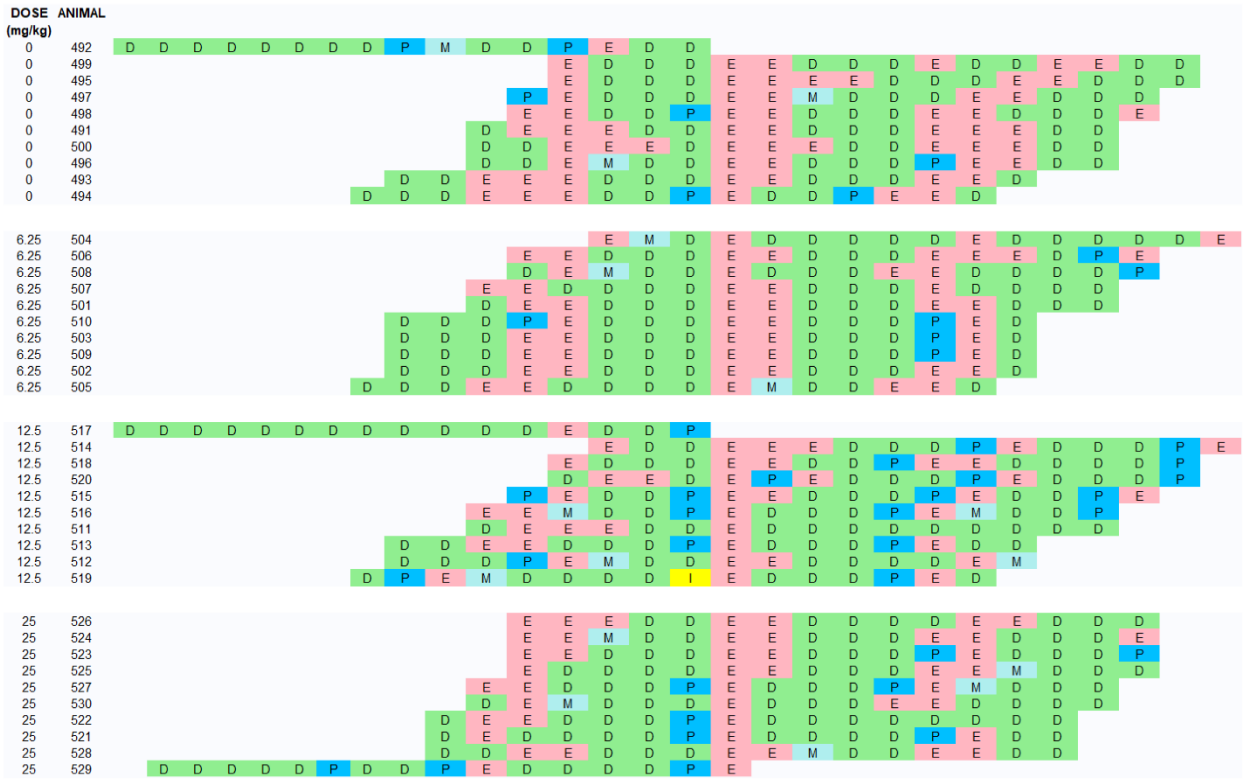

Figure A-5. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Wyeth-14,643

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus.

Appendix B. **Genetic Toxicology**

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Table B-1. Mutagenicity of Perfluorohexanoic Acid in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
TA100					
	0	44 ± 0	50 ± 3	41 ± 3	54 ± 2
	10	45 ± 5	44 ± 4		
	50	39 ± 3	35 ± 4		
	100	51 ± 3	59 ± 1	34 ± 3	57 ± 1
	500	50 ± 7	30 ± 4	40 ± 8	46 ± 7
	750	1 ± 0	19 ± 4		
	1,000			29 ± 3	54 ± 6
	5,000			33 ± 4	61 ± 4
	10,000			41 ± 3	56 ± 8
Trial Summary		Negative	Negative	Negative	Negative
Positive Control[b]		691 ± 52	616 ± 33	674 ± 23	885 ± 34
TA98					
	0	15 ± 2	15 ± 2	19 ± 3	17 ± 3
	10	12 ± 2	7 ± 0		
	50	9 ± 1	7 ± 2		
	100	15 ± 3	14 ± 2	15 ± 3	13 ± 1
	500	15 ± 2	12 ± 1	15 ± 1	15 ± 3
	750	10 ± 2	24 ± 15		
	1,000			21 ± 3	20 ± 2
	5,000			18 ± 2	17 ± 1
	10,000			13 ± 2	18 ± 2
Trial Summary		Negative	Negative	Negative	Negative
Positive Control		626 ± 8	449 ± 53	937 ± 50	910 ± 127
***Escherichia coli* WP2 *uvrA*/pKM101**					
	0	190 ± 6	188 ± 10	230 ± 13	125 ± 5
	100	209 ± 8	198 ± 2	240 ± 14	138 ± 1
	500	173 ± 16	144 ± 9	245 ± 6	134 ± 5
	1,000	135 ± 4	98 ± 7	256 ± 7	125 ± 6.3
	2,000	46 ± 11	61 ± 6		
	3,000	0 ± 0			
	5,000			246 ± 6	121 ± 11
	10,000			255 ± 22	131 ± 4
Trial Summary		Negative	Negative	Negative	Negative
Positive Control		736 ± 15	833 ± 54	786 ± 29	726 ± 14

[a]Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate was the solvent control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-o-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table B-2. Mutagenicity of Perfluorooctanoic Acid in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 10% Rat S9
TA100							
	0	44 ± 7	51 ± 5		49 ± 3	47 ± 3	54 ± 6
	100	44 ± 3	62 ± 2				60 ± 2
	250	50 ± 4	52 ± 5				64 ± 4
	500	50 ± 7	56 ± 2		53 ± 3	34 ± 5	68 ± 5
	750	26 ± 2	50 ± 6				
	1,000	27 ± 5	34 ± 5		54 ± 1	36 ± 4	53 ± 4
	2,000				54 ± 2	34 ± 5	32 ± 4
	3,500				45 ± 4	41 ± 5	
	5,000				36 ± 7	38 ± 1	
Trial Summary		Negative	Negative	–	Negative	Negative	Negative
Positive Control[b]		385 ± 4	377 ± 22	–	985 ± 39	648 ± 21	831 ± 93
TA98							
	0	14 ± 1	12 ± 1	21 ± 1	13 ± 1	26 ± 1	30 ± 2
	100	28 ± 0	17 ± 4				
	250	30 ± 5	25 ± 3				
	500	34 ± 2	18 ± 2	20 ± 4	48 ± 6	30 ± 3	33 ± 1
	750	34 ± 2	28 ± 8				
	1,000	24 ± 4	18 ± 0	20 ± 2	39 ± 3	24 ± 1	18 ± 1
	2,000			Toxic	42 ± 2	24 ± 6	20 ± 3
	3,500			Toxic	42 ± 4	22 ± 2	19 ± 1
	5,000			Toxic	41 ± 6	21 ± 2	17 ± 0
Trial Summary		Positive	Equivocal	Negative	Positive	Negative	Negative
Positive Control		170 ± 6	427 ± 4	874 ± 29	500 ± 28	826 ± 50	1,533 ± 29
***Escherichia coli* WP2 *uvrA*/pKM101**							
	0	178 ± 17	200 ± 13	164 ± 20	242 ± 9	246 ± 17	
	10			146 ± 9			
	25			131 ± 9			
	50			143 ± 5			
	100	206 ± 14	234 ± 4	148 ± 2			
	250	197 ± 9	208 ± 10	147 ± 10			
	500	183 ± 7	194 ± 7				
	750	149 ± 17	176 ± 9				
	1,000	101 ± 16	183 ± 4		242 ± 4	243 ± 9	

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 10% Rat S9
	2,500				252 ± 11	265 ± 7	
	5,000				234 ± 8	262 ± 8	
	7,500				246 ± 18	241 ± 14	
	10,000				214 ± 8	265 ± 6	
Trial Summary		Negative	Negative	Negative	Negative	Negative	–
Positive Control		768 ± 5	663 ± 46	2,139 ± 53	752 ± 7	713 ± 18	–

[a]Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate was the solvent control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-o-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table B-3. Mutagenicity of Perfluorononanoic Acid in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
TA100						
	0	61 ± 3	50 ± 4		73 ± 5	53 ± 3
	50	59 ± 6	55 ± 6			
	100	58 ± 7	52 ± 3		53 ± 3	50 ± 3
	200	62 ± 6	53 ± 1		52 ± 4	44 ± 1
	350	61 ± 6	53 ± 6			
	500	55 ± 6	47 ± 7		53 ± 4	51 ± 2
	750				68 ± 4	39 ± 1
	1,000				60 ± 10	55 ± 5
Trial Summary		Negative	Negative	–	Negative	Negative
Positive Control[b]		269 ± 3	211 ± 11	–	915 ± 29	719 ± 49
TA98						
	0	17 ± 3	18 ± 4		18 ± 3	28 ± 4
	50	10 ± 2	13 ± 1			
	100	13 ± 1	14 ± 2		18 ± 1	23 ± 5
	200	9 ± 1	12 ± 2		10 ± 1	22 ± 3
	350	10 ± 1	16 ± 2			
	500	2 ± 1	11 ± 1		9 ± 2	24 ± 2
	750				7 ± 1	22 ± 2
	1,000				8 ± 1	22 ± 1
Trial Summary		Negative	Negative	–	Negative	Negative
Positive Control		218 ± 23	516 ± 14	–	992 ± 27	790 ± 13

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
***Escherichia coli* WP2 *uvrA*/pKM101**						
	0	226 ± 9	233 ± 11	204 ± 6	248 ± 8	257 ± 5
	10			209 ± 7		
	50			153 ± 16		
	100			184 ± 4		
	500	235 ± 10	213 ± 11	175 ± 10		
	1,000	196 ± 13	188 ± 14	194 ± 9	285 ± 3	238 ± 10
	2,000	137 ± 3	143 ± 7			
	2,500				277 ± 4	256 ± 13
	3,500	73 ± 4	190 ± 84			
	5,000	5 ± 4	121 ± 51		276 ± 8	233 ± 24
	7,500				294 ± 16	218 ± 3
	10,000				296 ± 11	230 ± 34
Trial Summary		Negative	Negative	Negative	Negative	Negative
Positive Control		624 ± 8	554 ± 111	1,237 ± 37	765 ± 37	728 ± 92

[a]Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate was the solvent control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-o-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table B-4. Mutagenicity of Perfluorodecanoic Acid in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
TA100						
	0	61 ± 3	50 ± 4		73 ± 5	53 ± 3
	100	53 ± 2	49 ± 6			
	250	34 ± 5	43 ± 4			
	500	41 ± 3	41 ± 4			
	750	40 ± 2	42 ± 3			
	1,000	38 ± 6	41 ± 6		45 ± 3	34 ± 2
	2,500				47 ± 3	34 ± 6
	5,000				50 ± 9	38 ± 7
	7,500				46 ± 4	38 ± 4
	10,000				51 ± 10	35 ± 6
Trial Summary		Negative	Negative	–	Negative	Negative
Positive Control[b]		269 ± 3	211 ± 11	–	915 ± 29	719 ± 49
TA98						
	0	17 ± 3	18 ± 4	15 ± 1	18 ± 3	28 ± 4
	100	14 ± 2	18 ± 2	19 ± 1		
	250	11 ± 1	15 ± 2	13 ± 1		
	500	8 ± 2	17 ± 2	16 ± 1		
	750	15 ± 2	13 ± 3	13 ± 3		
	1,000	13 ± 1	16 ± 3	16 ± 1	19 ± 3	18 ± 2
	2,500				15 ± 3	17 ± 1
	5,000				14 ± 2	23 ± 1
	7,500				14 ± 2	13 ± 2
	10,000				13 ± 1	17 ± 2
Trial Summary		Negative	Negative	Negative	Negative	Negative
Positive Control		218 ± 23	516 ± 14	529 ± 57	992 ± 27	790 ± 13
***Escherichia coli* WP2 *uvrA*/pKM101**						
	0	226 ± 9	233 ± 11		248 ± 8	257 ± 5
	500	209 ± 22	190 ± 13			
	1,000	221 ± 11	166 ± 17		262 ± 9	249 ± 13
	2,000	149 ± 10	98 ± 14			
	2,500				270 ± 13	250 ± 14
	3,500	90 ± 15	61 ± 6			
	5,000	201 ± 66	137 ± 19		277 ± 16	209 ± 13

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
	7,500				264 ± 10	209 ± 11
	10,000				272 ± 8	233 ± 11
Trial Summary		Negative	Negative	–	Negative	Negative
Positive Control		624 ± 8	687 ± 34	–	765 ± 37	728 ± 92

[a]Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate was the solvent control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-o-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table B-5. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Exposure to Perfluorohexanoic Acid by Gavage for 28 Days[a]

	Dose (mg/kg/day)[b]	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[c]	P Value[d]	Micronucleated NCEs/1,000 NCEs[c]	P Value[d]	PCEs[c] (%)	P Value[d]
Male								
Vehicle Control[e]	0	5	0.57 ± 0.08		0.13 ± 0.02		1.06 ± 0.01	
PFHxA	62.6	5	0.51 ± 0.06	1.000	0.11 ± 0.01	1.000	1.02 ± 0.04	1.000
	125	5	0.80 ± 0.10	0.280	0.09 ± 0.02	1.000	0.74 ± 0.08	1.000
	250	5	0.45 ± 0.05	1.000	0.07 ± 0.02	1.000	1.19 ± 0.10	0.819
	500	5	0.58 ± 0.04	1.000	0.10 ± 0.03	1.000	3.79 ± 1.33	0.000
	1,000	5	1.04 ± 0.18	0.066	0.18 ± 0.04	1.000	15.12 ± 3.17	0.000
			p = 0.025[f]		p = 0.782		p ≤ 0.001	
Female								
Vehicle Control	0	5	0.68 ± 0.03		0.06 ± 0.01		0.76 ± 0.12	
PFHxA	62.6	5	0.75 ± 0.08	0.785	0.07 ± 0.01	1.000	0.70 ± 0.09	1.000
	125	5	0.56 ± 0.08	0.861	0.06 ± 0.02	1.000	0.91 ± 0.13	0.465
	250	5	0.48 ± 0.09	0.887	0.08 ± 0.03	1.000	1.30 ± 0.16	0.017
	500	5	0.40 ± 0.07	0.899	0.04 ± 0.00	1.000	2.57 ± 0.58	0.000
	1,000	5	0.86 ± 0.16	0.116	0.18 ± 0.06	0.377	5.36 ± 0.65	0.000
			p = 0.162		p = 0.450		p ≤ 0.001	

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte; PFHxA = perfluorohexanoic acid.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[121].
[b]One-half the dose was administered twice daily.
[c]Mean ± standard error.
[d]Pairwise comparison with the vehicle control group; dosed group values are significant at p ≤ 0.025 by Williams' or Dunn's test.
[e]The vehicle control was 2% Tween® 80 in deionized water.
[f]Dose-related trend; significant at p ≤ 0.025 by linear regression or Jonckheere's test.

Table B-6. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Exposure to Perfluorooctanoic Acid by Gavage for 28 Days[a]

	Dose (mg/kg/day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Male								
Vehicle Control[d]	0	5	0.42 ± 0.05		0.12 ± 0.01		1.02 ± 0.05	
PFOA	0.625	5	0.73 ± 0.05	0.046	0.07 ± 0.01	0.994	0.79 ± 0.04	0.147
	1.25	5	0.80 ± 0.15	0.043	0.09 ± 0.01	0.999	0.83 ± 0.04	0.175
	2.5	5	0.81 ± 0.10	0.014	0.07 ± 0.01	0.999	0.80 ± 0.06	0.186
	5	5	0.87 ± 0.06	0.001	0.07 ± 0.00	1.000	0.94 ± 0.15	0.190
	10	5	0.64 ± 0.09	0.282	0.06 ± 0.01	1.000	0.95 ± 0.08	0.192
			p = 0.025[e]		p = 0.998		p = 0.530	
Female								
Vehicle Control	0	5	0.65 ± 0.16		0.05 ± 0.01		0.97 ± 0.15	
PFOA	6.25	5	0.52 ± 0.05	0.708	0.04 ± 0.01	1.000	1.11 ± 0.07	1.000
	12.5	5	0.68 ± 0.06	0.792	0.03 ± 0.00	1.000	0.88 ± 0.09	1.000
	25	5	0.52 ± 0.12	0.824	0.02 ± 0.00	1.000	1.13 ± 0.14	1.000
	50	5	0.53 ± 0.083	0.839	0.03 ± 0.00	1.000	1.15 ± 0.24	1.000
	100	5	0.55 ± 0.07	0.851	0.04 ± 0.01	1.000	1.62 ± 0.17	0.089
			p = 0.740		p = 0.961		p = 0.048	

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte; PFOA = perfluorooctanoic acid.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[121].
[b]Mean ± standard error.
[c]Pairwise comparison with the vehicle control group; dosed group values are significant at $p \leq 0.025$ by Williams' or Dunn's test.
[d]The vehicle control was 2% Tween® 80 in deionized water.
[e]Dose-related trend; significant at $p \leq 0.025$ by linear regression or Jonckheere's test.

Table B-7. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Exposure to Perfluorononanoic Acid by Gavage for 28 Days[a]

	Dose (mg/kg/day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Male								
Vehicle Control[d]	0	5	0.99 ± 0.07		0.29 ± 0.05		1.08 ± 0.03	
PFNA	0.625	5	0.87 ± 0.10	0.614	0.26 ± 0.06	0.580	0.56 ± 0.03	0.000
	1.25	5	0.91 ± 0.24	0.703	0.27 ± 0.09	0.668	0.44 ± 0.05	0.000
	2.5	1	0.00 ± 0.00	–	0.00 ± 0.00	–	0.00 ± 0.00	–
			p = 0.642[e]		p = 0.624		p ≤ 0.001	
Female								
Vehicle Control	0	5	0.84 ± 0.17		0.14 ± 0.01		1.44 ± 0.24	
PFNA	1.56	5	0.42 ± 0.05	1.000	0.05 ± 0.01	0.999	1.38 ± 0.08	1.000
	3.12	5	0.46 ± 0.04	1.000	0.07 ± 0.01	1.000	1.11 ± 0.10	0.375
	6.25	5	0.72 ± 0.06	1.000	0.06 ± 0.01	1.000	1.26 ± 0.08	0.400
			p = 0.318		p = 0.991		p = 0.425	

NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte; PFNA=perfluorononanoic acid.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[121]. No data available for 5 or 10 mg/kg/day males or 12.5 or 25 mg/kg/day females due to high mortality.
[b]Mean ± standard error.
[c]Pairwise comparison with the vehicle control group when there were at least two samples; dosed group values are significant at p ≤ 0.025 by Williams' or Dunn's test.
[d]The vehicle control was 2% Tween® 80 in deionized water.
[e]Dose-related trend; significant at p ≤ 0.025 by linear regression or Jonckheere's test.

Table B-8. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Exposure to Perfluorodecanoic Acid by Gavage for 28 Days[a]

	Dose (mg/kg/day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Male								
Vehicle Control[d]	0	5	0.67 ± 0.11		0.09 ± 0.02		0.82 ± 0.13	
PFDA	0.156	5	0.65 ± 0.05	0.563	0.07 ± 0.02	0.641	0.79 ± 0.06	1.000
	0.312	5	0.64 ± 0.15	0.650	0.10 ± 0.03	0.714	0.83 ± 0.03	1.000
	0.625	5	0.62 ± 0.05	0.687	0.09 ± 0.02	0.749	0.72 ± 0.07	0.829
	1.25	5	0.78 ± 0.09	0.330	0.06 ± 0.01	0.768	0.28 ± 0.06	0.000
	2.5	3	1.17 ± 0.22	0.004	0.08 ± 0.01	0.798	0.13 ± 0.04	0.000
			p = 0.001[e]		p = 0.738		p ≤ 0.001	
Female								
Vehicle Control	0	5	0.50 ± 0.03		0.06 ± 0.01		1.06 ± 0.18	
PFDA	0.156	5	0.38 ± 0.05	0.689	0.05 ± 0.01	0.967	1.00 ± 0.09	1.000
	0.312	5	0.49 ± 0.11	0.618	0.04 ± 0.00	0.987	1.12 ± 0.10	1.000
	0.625	5	0.62 ± 0.04	0.292	0.03 ± 0.00	0.991	1.10 ± 0.05	1.000
	1.25	5	0.56 ± 0.14	0.303	0.04 ± 0.01	0.994	0.36 ± 0.03	0.000
			p = 0.106		p = 0.965		p ≤ 0.001	

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte; PFDA = perfluorodecanoic acid.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[121]. No data are available for 2.5 mg/kg/day females because no samples were received by ILS, Inc. from the study laboratory.
[b]Mean ± standard error.
[c]Pairwise comparison with the vehicle control group; dosed group values are significant at p ≤ 0.025 by Williams' or Dunn's test.
[d]The vehicle control was 2% Tween® 80 in deionized water.
[e]Dose-related trend; significant at p ≤ 0.025 by linear regression or Jonckheere's test.

Table B-9. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Exposure to Wyeth-14,643 by Gavage for 28 Days[a]

	Dose (mg/kg/day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Male								
Vehicle Control[d]		5	0.51 ± 0.09		0.08 ± 0.01		0.97 ± 0.05	
WY	6.25	5	0.80 ± 0.06	0.055	0.16 ± 0.04	0.131	0.76 ± 0.02	1.000
	12.5	5	0.86 ± 0.20	0.050	0.29 ± 0.13	0.032	1.18 ± 0.15	0.236
	25	5	0.79 ± 0.08	0.053	0.12 ± 0.03	0.465	1.08 ± 0.07	0.249
			p = 0.096[e]		p = 0.126		p = 0.110	
Female								
Vehicle Control		5	0.47 ± 0.09		0.04 ± 0.00		1.10 ± 0.09	
WY	6.25	5	0.73 ± 0.13	0.105	0.06 ± 0.03	0.056	0.95 ± 0.09	1.000
	12.5	5	0.58 ± 0.07	0.127	0.04 ± 0.01	0.889	1.29 ± 0.07	0.187
	25	5	0.62 ± 0.06	0.135	0.04 ± 0.01	0.946	1.40 ± 0.11	0.052
			p = 0.292		p = 0.446		p = 0.013	

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte; WY = Wyeth-14,643.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[121].
[b]Mean ± standard error.
[c]Pairwise comparison with the vehicle control group; dosed group values are significant at $p \leq 0.025$ by Williams' or Dunn's test.
[d]The vehicle control was 2% Tween® 80 in deionized water.
[e]Dose-related trend; significant at $p \leq 0.025$ by linear regression or Jonckheere's test.

Appendix C. Chemical Characterization and Dose Formulation Studies

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C.1. Procurement and Characterization

C.1.1. Perfluoroalkyl Carboxylates and Wyeth-14,643

Perfluorohexanoic acid (PFHxA) was obtained from Matrix Scientific (Columbia, SC) in one lot (Q02G); perfluorooctanoic acid (PFOA) was obtained from Sigma-Aldrich (St. Louis, MO) in one lot (03427TH); perfluorononanoic acid (PFNA) was obtained from Oakwood Products, Inc. (West Columbia, SC) in one lot (D11G); perfluorodecanoic acid (PFDA) was obtained from Sigma-Aldrich in one lot (01820LE); and Wyeth-14,643 (WY) was obtained from Chem Syn Laboratories (Lenexa, KS) in one lot (91-314-72-07/91-314-100-33A); all lots were used in the 28-day gavage studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory and the study laboratory at Battelle (Columbus, OH). Reports on analyses performed in support of the perfluoroalkyl carboxylate and WY studies are on file at the National Institute of Environmental Health Sciences.

Lot Q02G (PFHxA), a clear, colorless liquid; lot 03427TH (PFOA), white crystals; lot D11G (PFNA), a white, crystalline powder; lot 01820LE (PFDA), white, transparent wet crystals; and lot 91-314-72-07/91-314-100-33A (WY), a white powder, were identified using Fourier transform infrared (FTIR) spectroscopy, mass spectrometry (MS) (perfluoroalkyl carboxylates only), and proton (WY), carbon-13, and fluorine-19 (perfluoroalkyl carboxylates only) nuclear magnetic resonance (NMR) spectroscopy. The Advanced Chemistry Development (ACD) spectral prediction program was used to predict carbon-13 NMR spectra for each test article and proton NMR spectra for WY. Annotated fluorine-fluorine correlated spectroscopy (COSY) two-dimensional data were used to identify the fluorine-fluorine coupling within the perfluoroalkyl carboxylates by the observation of cross-peaks in each fluorine-19 NMR spectrum. For the WY molecule, COSY was used to identify proton-proton coupling, a distortionless enhancement by polarization transfer carbon-13 spectrum series was constructed to determine the types of carbon (methine, methylene, and methyl), and a proton-carbon-13 heteronuclear multiple quantum coherence two-dimensional spectrum was acquired to identify directly bonded protons and carbons. Spectra were in good agreement with the proposed structure of each test chemical, with ACD computer-predicted spectra, and with expected COSY-identified proton-proton and fluorine-fluorine coupling. Observed chemical shifts were in good agreement with those expected and reported for each test article. Representative IR spectra of lots Q02G (PFHxA), 03427TH (PFOA), D11G (PFNA), 01820LE (PFDA), and 91-314-72-07/91-314-100-33A (WY) are presented in Figure C-1, Figure C-2, Figure C-3, Figure C-4, and Figure C-5, respectively.

For the perfluoroalkyl carboxylates, the purities of lots Q02G, 03427TH, D11G, and 01820LE were determined by high-performance liquid chromatography (HPLC)/ion chromatography (IC) with suppressed conductivity (SC) detection (all lots except 01820LE), and gas chromatography (GC) with flame ionization detection (FID) and electron capture detection (ECD); in addition, GC/MS was used to confirm the identity of each perfluoroalkyl carboxylate test article and identify impurities within each lot. Additional GC/FID, GC/ECD, and GC/MS analyses were performed on lot D11G to screen it for selected volatile solvents. All GC analyses were performed on derivatized (methylated) aliquots of each perfluoroalkyl carboxylate. Differential scanning calorimetry (DSC) was used to determine the purities of lots 03427TH and D11G using a Perkin-Elmer (Shelton, CT) Diamond differential scanning calorimeter scanning 1°C per minute over the ranges of 25° to 65°C, and 50° to 85°C, respectively. In addition, Karl Fischer titration for lots Q02G and 03427TH was performed by Prevalere Life Sciences, Inc.

(Whitesboro, NY) and similar analysis for lot D11G was performed by Galbraith Laboratories, Inc. (Knoxville, TN).

For WY, the purity of lot 91-314-72-07/91-314-100-33A was determined by HPLC with ultraviolet light (UV) detection and DSC as described for the perfluoroalkyl carboxylates over a range of 135° to 175°C.

For lot Q02G (PFHxA), Karl Fischer titration indicated 0.47% water. HPLC/IC/SC analysis using System A (Table C-1) indicated one major peak that was 99.6% of the total peak area and two reportable (areas ≥0.1% of the total peak area) impurities with a combined area of 0.37% of the total peak area. GC/FID analysis using System A (Table C-2) indicated one major peak (99.1%) and five reportable impurities with a combined area of 0.95% of the total peak area. GC/ECD analysis using System A showed that the impurities were likely fluorinated compounds. GC/MS analysis using System A confirmed the identity of the test article and indicated that one of the impurities representing 0.33% of the total was an isomer of PFHxA. The overall purity of lot Q02G was determined to be greater than 99%.

For lot 03427TH (PFOA), Karl Fischer titration indicated 0.24% water. DSC indicated an average purity of 98.96%. HPLC/IC/SC analysis using System B (Table C-1) indicated one major peak that was 98.8% of the total peak area and three reportable impurities with a combined area of 1.2% of the total peak area. GC/FID analysis using System A (Table C-2) indicated one major peak (98.3%) and four reportable impurities with a combined area of 1.66% of the total peak area. GC/ECD analysis using System A showed that the impurities were likely fluorinated compounds. GC/MS analysis using System A confirmed the identity of the test article and indicated that two of the impurities representing 1.04% of the total were isomers of PFOA. The overall purity of lot 03427TH was determined to be greater than 98%.

For lot D11G (PFNA), Karl Fischer titration indicated 0.29% water. DSC indicated an average purity of 99.5%. HPLC/IC/SC analysis using system B (Table C-1) indicated one major peak that was 98.5% of the total peak area and two reportable impurities with a combined area of 1.5% of the total peak area. GC/FID analysis using System A (Table C-2) indicated a purity of 100%. GC/ECD analysis using System A indicated a purity of 99.0% with six reportable impurities. GC/MS analysis using System A confirmed the identity of the test article and indicated the presence of perfluoroheptanoic acid as an impurity. GC/FID analysis using System B showed the presence of low concentrations of the nonhalogenated solvents (≤0.007%) hexane, diethyl ether, and acetone. GC/ECD analysis using System B showed the presence of low concentrations of the halogenated solvents (≤0.0009%) chloroform and carbon tetrachloride. The overall purity of lot D11G was determined to be greater than 98%.

For lot 01820LE (PFDA), GC/FID analysis using System A (Table C-2) indicated one major peak (97.2%) and 10 reportable impurities with a combined area of 2.8% of the total peak area. GC/ECD analysis using System A showed that the impurities were likely fluorinated compounds. GC/MS analysis using System A confirmed the identity of the test article and indicated that one of the impurities representing 0.59% of the total was an isomer of PFDA. The overall purity of lot 01820LE was determined to be greater than 97%.

For lot 91-314-72-07/91-314-100-33A (WY), HPLC/UV analysis using System C (Table C-1) indicated one major peak (99.4%) and two reportable impurities with a combined area of 0.64%

of the total peak area. DSC indicated an average purity of 94.3%; this lower calculated purity compared to that shown by HPLC was considered an indication of thermal decomposition rather than impurity of the bulk chemical. The overall purity of lot 91-314-72-07/91-314-100-33A was determined to be greater than 99%.

Stability studies of lots Q02G (PFHxA) and D11G (PFNA) of the bulk chemicals were performed by the analytical chemistry laboratory using HPLC/IC/SC System A (Table C-1) and GC/ECD System A (Table C-2), respectively. Stability was confirmed for bulk chemical samples stored in sealed amber glass bottles at temperatures up to 60°C for at least 14 days. To ensure stability, all of the bulk chemicals were stored at room temperature in sealed opaque plastic containers (lots Q02G and D11G) or sealed amber glass bottles (lots 03427TH, 01820LE, and 91-314-72-07/91-314-100-33A. Reanalyses of the bulk chemicals were performed by the study laboratory on January 5, 2012 (lot Q02G), January 12, 2012 (lot 03427TH), January 10, 2012 (lot D11G), January 16, 2012 (lot 01820LE), and January 10, 2012 (lot 91-314-72-07/91-314-100-33A) (prior to the gavage studies) using GC/ECD System D (Table C-2) for PFHxA, GC/ECD System A for PFOA, PFNA, and PFDA, and HPLC/UV System C (Table C-1) for WY. No degradation of the bulk chemicals was detected.

C.1.2. Tween® 80

Tween 80 (polysorbate 80) was obtained from Spectrum Laboratory Products, Inc. (Gardena, CA), and was used at a 2% concentration as the vehicle in the 28-day gavage studies. The vehicle was prepared by mixing the appropriate amount of Tween 80 with deionized water in a calibrated carboy and stirring with an overhead stirrer until all the Tween 80 was dissolved.

C.2. Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing PFHxA, PFOA, PFNA, PFDA, or WY with 2% Tween 80 in deionized water to give the required concentrations (Table C-3). Each formulation pH was adjusted to between 6 and 8. The dose formulations were stored at room (PFHxA, PFOA, PFNA, and PFDA) or refrigerated (WY) temperatures in amber glass bottles sealed with Teflon®-lined lids for no more than 43 days.

Homogeneity studies of the 6.26 and 100 mg/mL dose formulations of PFHxA and a stability study of the 6.26 mg/mL dose formulation were performed by the analytical chemistry laboratory using GC/ECD System D (Table C-2). Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.05 mg/mL formulation and the 20 mg/mL dose formulation of PFOA and a stability study of a 0.05 mg/mL formulation were performed by the analytical chemistry laboratory using GC/ECD System D (Table C-2). These studies were performed on lot 02702EE (Sigma-Aldrich) which was not used in the animal studies. In addition, homogeneity and stability of a 0.05 mg/mL formulation of lot 03427TH were assessed by the analytical chemistry laboratory using the same GC/ECD system. Homogeneity was confirmed, and stability was confirmed for at least 42 days for formulations stored in amber glass bottles with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.12 mg/mL formulation and the 5 mg/mL dose formulation of PFNA and a stability study of a 0.12 mg/mL formulation were performed by the analytical chemistry laboratory using GC/ECD System A (Table C-2). Homogeneity was confirmed, and stability was confirmed for at least 43 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.04 mg/mL formulation and the 0.5 mg/mL dose formulation of PFDA and a stability study of a 0.04 mg/mL formulation were performed by the analytical chemistry laboratory using GC/ECD System A (Table C-2). An additional stability study of the 0.0312 mg/mL dose formulation was performed by the study laboratory using the same GC/ECD system. Homogeneity was confirmed, and stability was confirmed for at least 42 (analytical chemistry laboratory) or 45 (study laboratory) days for formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of the 1.26 and 5 mg/mL dose formulations of WY and a stability study of the 1.26 mg/mL dose formulation were performed by the analytical chemistry laboratory using HPLC/UV System C (Table C-1). Homogeneity was confirmed, and stability was confirmed for at least 42 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at refrigerated temperature and for at least 3 hours under simulated animal room conditions.

Analyses of the dose formulations for the 28-day studies of PFHxA, PFOA, PFNA, and PFDA were conducted once for each study by the study laboratory using GC/ECD Systems D, D, A, and A, respectively (Table C-2). HPLC/UV System C (Table C-1) was used to conduct similar analyses for the 28-day study of WY. All dose formulations for all five chemicals were within 10% of the target concentrations (Table C-4). Animal room samples of these dose formulations were also analyzed; with the exceptions of two of 10 samples for the PFOA study, all animal room samples for all five chemicals were within 10% of the target concentrations.

Table C-1. High-Performance Liquid Chromatography Systems Used in the 28-day Gavage Studies of Perfluoroalkyl Carboxylates and Wyeth-14,643[a]

Detection System	Column	Solvent System
System A		
Suppressed Conductivity	IonPac™ NS1, 150 mm × 4 mm, 5 µm particle size (Dionex Corporation, Sunnyvale, CA)	A) 0.1 M NaOH in water, B) 70% isopropanol in water, and C) water (4% A:28% B:68% C), isocratic; flow rate 0.6 mL/minute
System B		
Suppressed Conductivity	IonPac™ NS1, 150 mm × 4 mm, 5 µm particle size (Dionex Corporation)	A) 0.1 M NaOH in water, B) 70% isopropanol in water, and C) water; linear gradients from 1% A: 30% B: 69% C for 3 minutes, to 1% A: 43% B: 56% C in 14 minutes, held for 3 minutes, then to 1% A: 30% B: 69% C in 1 minute, held for 14 minutes; flow rate 0.6 mL/minute
System C		
Ultraviolet (254 nm) Light	Inertsil® ODS-2, 150 mm × 4.6 mm, 5 µm particle size (Phenomenex, Torrance, CA)	A) 7.5 mM heptanesulfonic acid (pH 3.4) in water:methanol (32:68), isocratic; flow rate 1 mL/minute

[a]The high-performance liquid chromatographs were manufactured by Hitachi High-Technologies Science America, Inc. (Tokyo, Japan) (Systems A and B) or Agilent Technologies, Inc. (Santa Clara, CA) or Waters Corporation (Milford, MA) (System C).

Table C-2. Gas Chromatography Systems Used in the 28-day Gavage Studies of Perfluoroalkyl Carboxylates and Wyeth-14,643[a]

Detection System	Column	Carrier Gas	Oven Temperature Program
System A			
Flame Ionization, Electron Capture, or Mass Spectrometry with Electron Ionization	Rtx®-5, 30 m × 0.32 mm, 1.0 or 0.5 µm film (Restek, Bellefonte, PA)	Helium at ~1 mL/minute	70°C for 2 minutes, then 7°C/minute to 240°C, held for 4 minutes
System B			
Flame Ionization or Electron Capture	Rtx®-624, 30 m × 0.53 mm, 3.0 µm film (Restek)	Helium at ~5 mL/minute	35°C for 14 minutes, then 15°C/minute to 40°C, held for 3 minutes, then 15°C/minute to 240°C, held for 2 minutes
System C			
Mass Spectrometry with Electron Ionization	Rtx® 624, 30 m × 0.32 mm, 1.8 µm film (Restek)	Helium at ~2 mL/minute	35°C for 14 minutes, then 15°C/minute to 40°C, held for 3 minutes, then 15°C/minute to 240°C, held for 2 minutes

Detection System	Column	Carrier Gas	Oven Temperature Program
System D			
Electron Capture	Rtx®-5, 30 m × 0.32 mm, 1.0 or 0.5 µm film (Restek)	Helium at ~1 mL/minute	70°C for 3 minutes, then 5°C/minute to 120°C, then 15°C/minute to 240°C, held for 4 minutes

[a]The gas chromatographs and mass spectrometer were manufactured by Agilent Technologies, Inc. (Palo Alto, CA).

Table C-3. Preparation and Storage of Dose Formulations in the 28-day Gavage Studies of Perfluoroalkyl Carboxylates and Wyeth-14,643

Perfluorohexanoic Acid	Perfluorooctanoic Acid	Perfluorononanoic Acid	Perfluorodecanoic Acid	Wyeth-14,643
Preparation				
The appropriate amount of test article was weighed and quantitatively transferred into a calibrated mixing container containing a stir bar, and approximately 80% of the final total vehicle volume of 2% Tween® 80 in deionized water was added to the mixing container; the formulation was stirred on a stir plate to wet the test article and then sonicated for 15 minutes. After sonication, the formulation was stirred on a stir plate for approximately 15 minutes. The pH was measured, and if necessary, it was adjusted to between 6 and 8 with 10 N, and/or 1 N, and/or 0.1 N NaOH; the formulation was then diluted to final volume with vehicle and stirred for an additional 15 minutes.	Same as perfluorohexanoic acid except the mixture was not stirred for 15 minutes following the 15-minute sonication.	Same as PFHxA	Same as PFHxA	The appropriate amount of Wyeth-14,643 was weighed and quantitatively transferred into a calibrated mixing container containing a stir bar, approximately 90% of the final total vehicle volume of 2% Tween 80 in deionized water was added to the mixing container, and the formulation was stirred on a stir plate and/or sonicated for approximately 15 minutes. If necessary, the pH was adjusted to between 6 and 8 with 10 N, and/or 1 N and/or 0.1 N NaOH; the formulation was then diluted to final volume with vehicle and stirred and/or sonicated for an additional 15 minutes.
Chemical Lot Number				
Q02G	03427TH	D11G	01820LE	91-314-72-07/91-314-100-33A
Maximum Storage Time				
38 days	43 days	43 days	42 days	39 days

Perfluorohexanoic Acid	Perfluorooctanoic Acid	Perfluorononanoic Acid	Perfluorodecanoic Acid	Wyeth-14,643
Storage Conditions				
Stored in amber glass bottles sealed with Teflon®-lined lids at 25°C	Stored in amber glass bottles sealed with Teflon-lined lids at 25°C	Stored in amber glass bottles sealed with Teflon-lined lids at 25°C	Stored in amber glass bottles sealed with Teflon-lined lids at 25°C	Stored in amber glass bottles sealed with Teflon-lined lids at 5°C
Study Laboratory				
Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)

Table C-4. Results of Analyses of Dose Formulations Administered to Rats in the 28-day Gavage Studies of Perfluoroalkyl Carboxylates and Wyeth-14,643

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Perfluorohexanoic Acid				
January 9, 2012	January 11, 2012	6.26	6.36	+2
		12.5	13.2	+6
		25	25.8	+3
		50	50.6	+1
		100	103	+3
	February 17, 2012[b]	6.26	6.29	+1
		12.5	13.0	+4
		25	25.4	+2
		50	49.6	−1
		100	99.0	−1
Perfluorooctanoic Acid				
January 17, 2012	January 20, 2012	0.125	0.122	−2
		0.25	0.269	+8
		0.5	0.547	+9
		1	1.03	+3
		1.25	1.37	+10
		2	2.19	+10
		2.5	2.62	+5
		5	5.48	+10
		10	10.4	+4
		20	19.6	−2
	February 29, 2012[b]	0.125	0.137	+10
		0.25	0.293	+17
		0.5	0.578	+16
		1	1.06	+6
		1.25	1.30	+4
		2	2.06	+3
		2.5	2.53	+1
		5	5.47	+9
		10	10.6	+6
		20	20.6	+3

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Perfluorononanoic Acid				
January 19, 2012	January 23, 2012	0.125	0.120	-4
		0.25	0.260	+4
		0.312	0.309	−1
		0.5	0.501	0
		0.624	0.635	+2
		1	1.06	+6
		1.25	1.28	+2
		2	2.04	+2
		2.5	2.56	+2
		5	5.08	+2
	March 2, 2012[b]	0.125	0.123	-2
		0.25	0.264	+6
		0.312	0.320	+3
		0.5	0.505	+1
		0.624	0.625	0
		1	1.00	0
		1.25	1.30	+4
		2	2.07	+4
		2.5	2.57	+3
		5	5.01	0
Perfluorodecanoic Acid				
January 25, 2012	January 26, 2012	0.0312	0.0317	+2
		0.0624	0.0630	+1
		0.125	0.127	+2
		0.25	0.261	+4
		0.5	0.486	−3
	March 10, 2012[b]	0.0312	0.0306	−2
		0.0624	0.0608	−3
		0.125	0.124	−1
		0.25	0.253	+1
		0.5	0.502	0
Wyeth-14,643				
January 16, 2012	January 18, 2012	1.25	1.24	−1
		2.5	2.48	−1

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
		5	4.95	−1
	March 1, 2012[b]	1.25	1.25	0
		2.5	2.49	0
		5	4.94	−1

[a]Results of duplicate analyses. Dosing volume for all studies = 5 mL/kg/day. One-half the perfluorohexanoic acid formulations were administered twice per day; perfluorooctanoic acid, perfluorononanoic acid, perfluorodecanoic acid, and Wyeth-14,643 formulations were administered once per day. For the perfluorohexanoic study, 6.26 mg/mL = 62.6 mg/kg/day, 12.5 mg/mL = 125 mg/kg/day, 25 mg/mL = 250 mg/kg/day, 50 mg/mL = 500 mg/kg/day, and 100 mg/mL = 1,000 mg/kg/day. For males in the perfluorooctanoic study, 0.125 mg/mL = 0.625 mg/kg/day, 0.25 mg/mL = 1.25 mg/kg/day, 0.5 mg/mL = 2.5 mg/kg/day, 1 mg/mL = 5 mg/kg/day and 2 mg/mL = 10 mg/kg/day. For females in the perfluorooctanoic study, 1.25 mg/mL = 6.25 mg/kg/day, 2.5 mg/mL = 12.5 mg/kg/day, 5 mg/mL = 25 mg/kg/day, 10 mg/mL = 50 mg/kg/day, and 20 mg/mL = 100 mg/kg/day. For males in the perfluorononanoic study, 0.125 mg/mL = 0.625 mg/kg/day, 0.25 mg/mL = 1.25 mg/kg/day, 0.5 mg/L = 2.5 mg/kg/day, 1 mg/mL = 5 mg/kg/day, and 2 mg/mL = 10 mg/kg/day. For females in the perfluorononanoic study, 0.312 mg/mL = 1.56 mg/kg/day, 0.624 mg/mL = 3.12 mg/kg/day, 1.25 mg/mL = 6.25 mg/kg/day, 2.5 mg/mL = 12.5 mg/kg/day, and 5 mg/mL = 25 mg/kg/day. For the perfluorodecanoic study, 0.0312 mg/mL = 0.156 mg/kg/day, 0.0624 mg/mL = 0.312 mg/kg/day, 0.125 mg/mL = 0.625 mg/kg/day, 0.25 mg/mL = 1.25 mg/kg/day, and 0.5 mg/mL = 2.5 mg/kg/day. For the Wyeth-14,643 study, 1.25 mg/mL = 6.25 mg/kg/day, 2.5 mg/mL = 12.5 mg/kg/day, and 5 mg/mL = 25 mg/kg/day.
[b]Animal room samples.

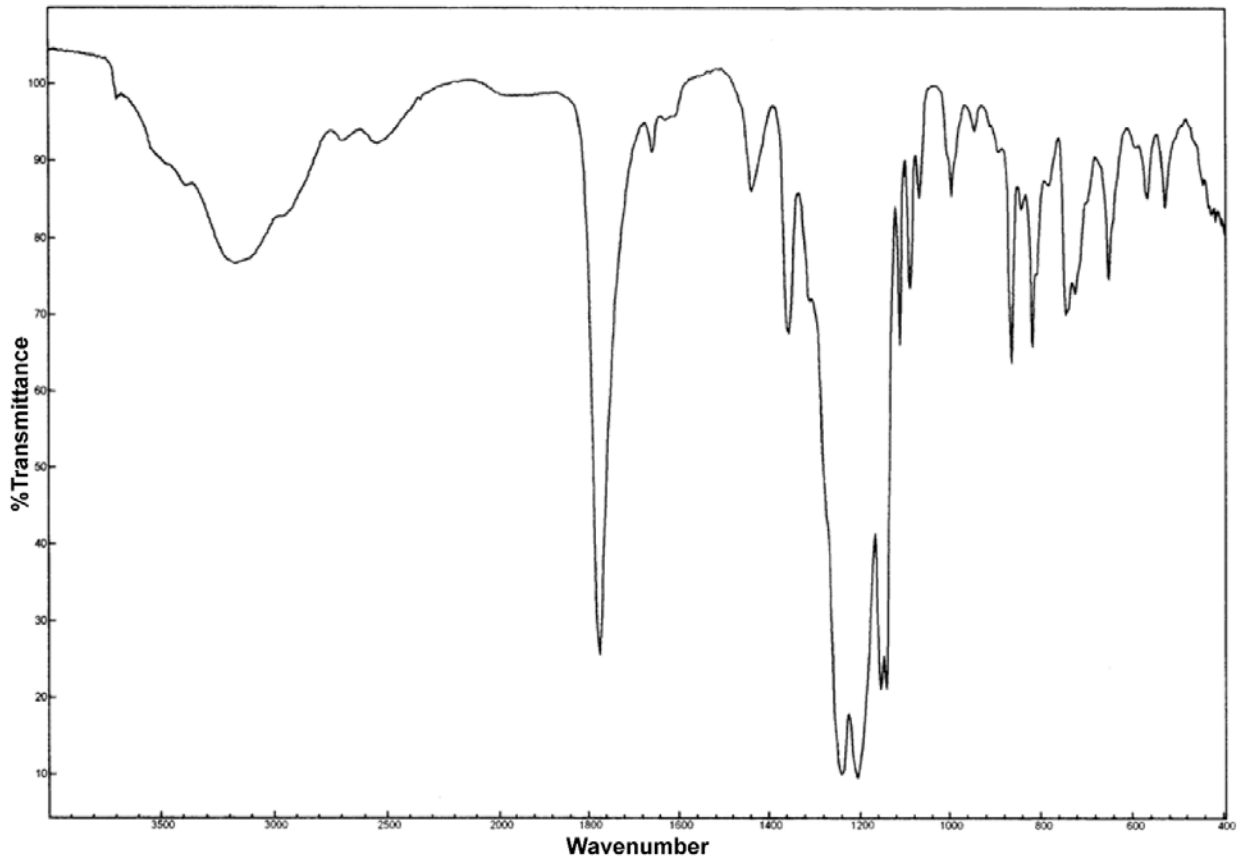

Figure C-1. Fourier Transform Infrared Absorption Spectrum of Perfluorohexanoic Acid

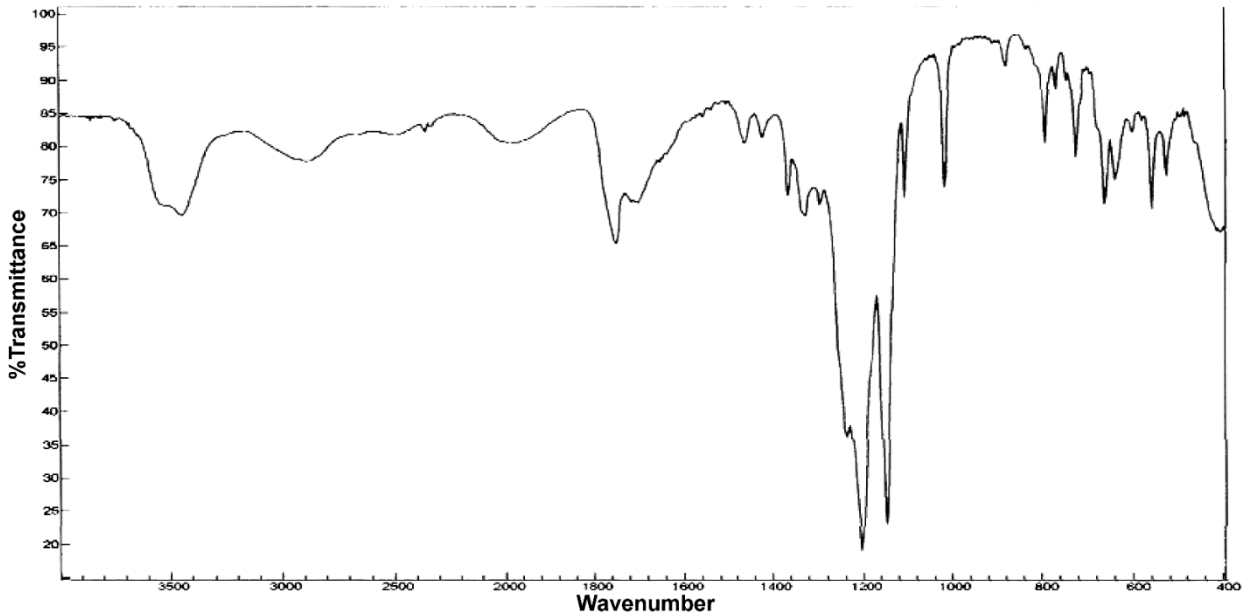

Figure C-2. Fourier Transform Infrared Absorption Spectrum of Perfluorooctanoic Acid

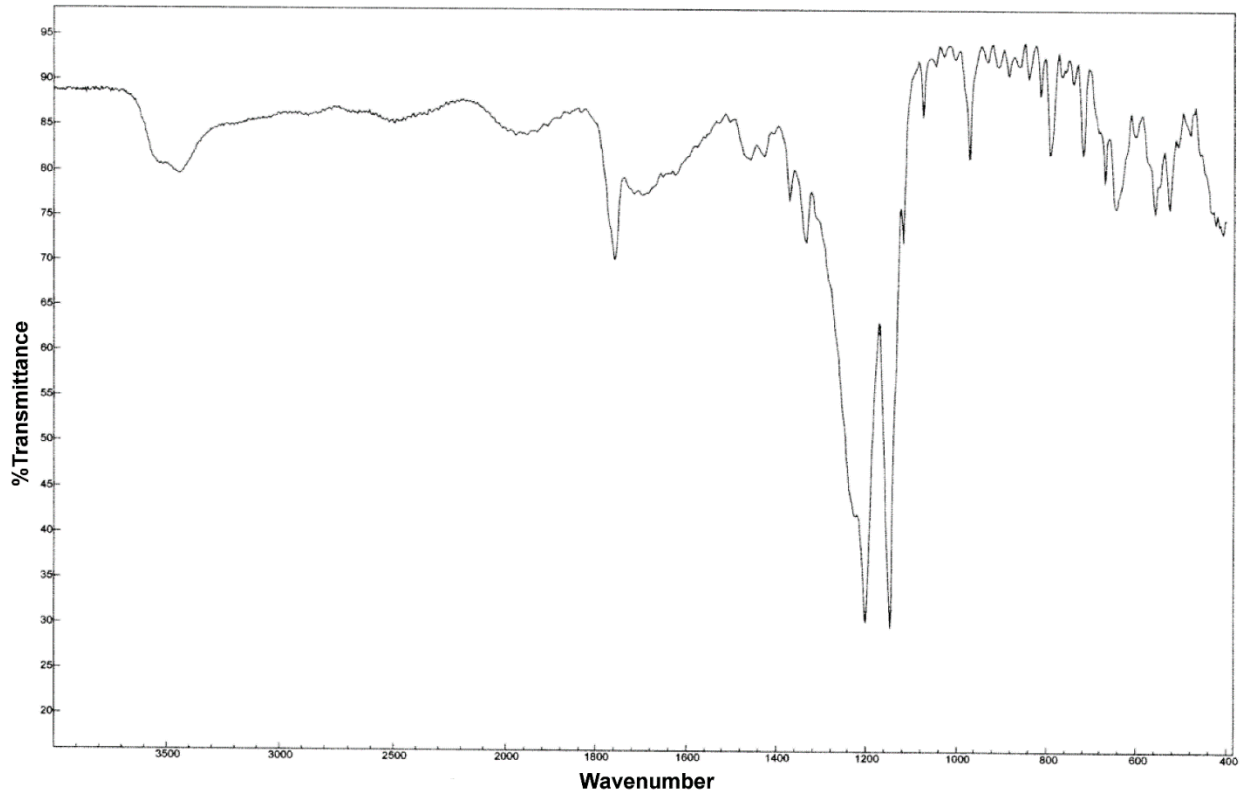

Figure C-3. Fourier Transform Infrared Absorption Spectrum of Perfluorononanoic Acid

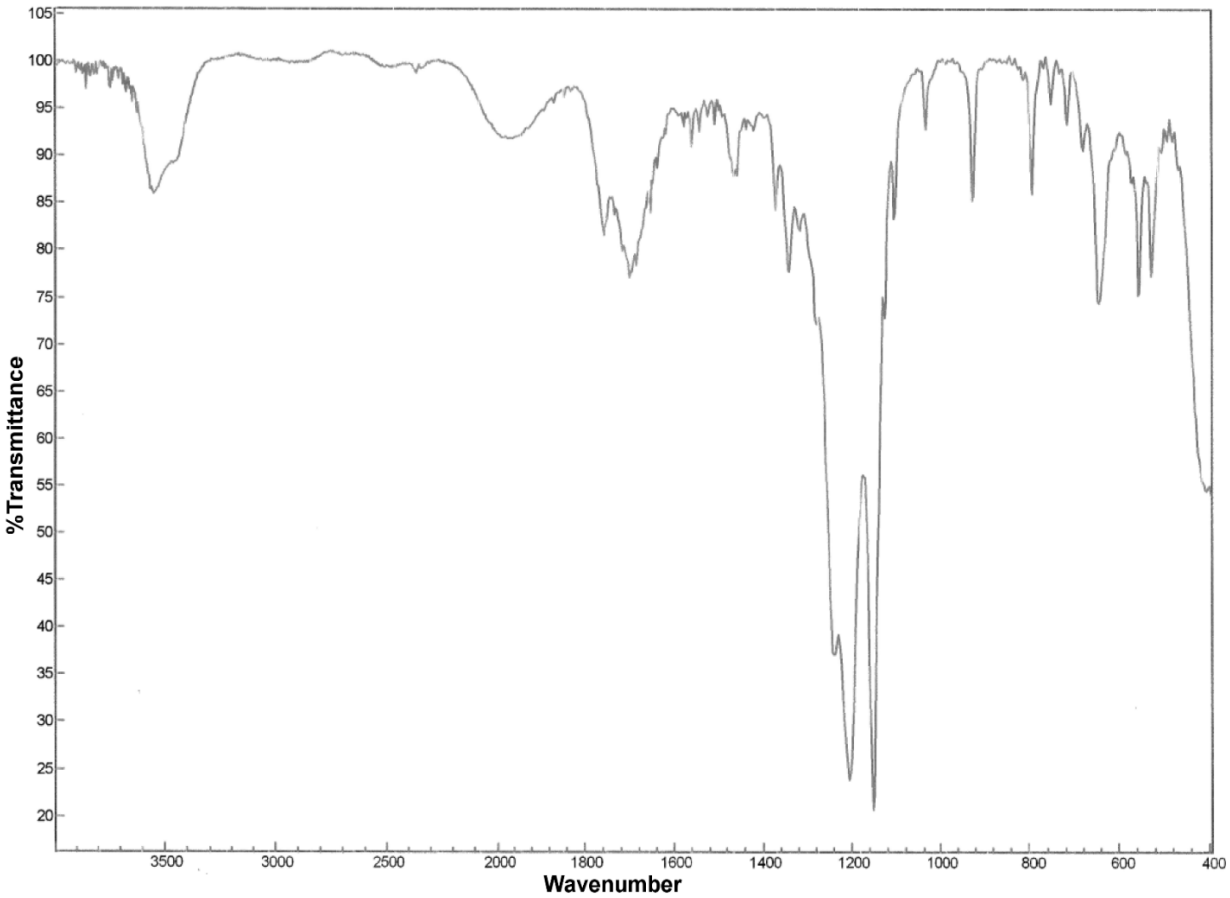

Figure C-4. Fourier Transform Infrared Absorption Spectrum of Perfluorodecanoic Acid

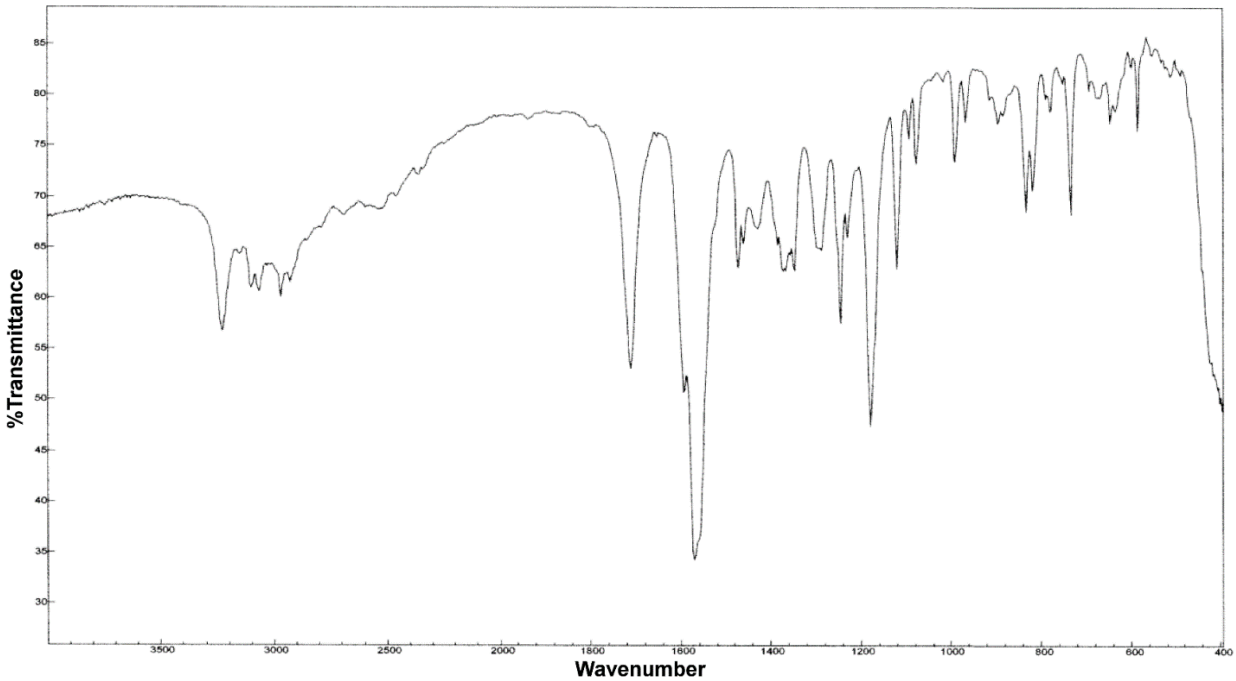

Figure C-5. Fourier Transform Infrared Absorption Spectrum of Wyeth-14,643

Appendix D. **Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Rat and Mouse Ration**

Tables

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Table D-1. Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	22.26
Ground #2 Yellow Shelled Corn	22.18
Wheat Middlings	15.0
Oat Hulls	8.5
Alfalfa Meal (Dehydrated, 17% Protein)	7.5
Purified Cellulose	5.5
Soybean Meal (49% Protein)	5.0
Fish Meal (60% Protein)	4.0
Corn Oil (without Preservatives)	3.0
Soy Oil (without Preservatives)	3.0
Dried Brewer's Yeast	1.0
Calcium Carbonate (USP)	0.9
Vitamin Premix[a]	0.5
Mineral Premix[b]	0.5
Calcium Phosphate, Dibasic (USP)	0.4
Sodium Chloride	0.3
Choline Chloride (70% Choline)	0.26
Methionine	0.2

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table D-2. Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl Acetate	100 IU	–
Niacin	23 mg	–
Folic Acid	1.1 mg	–
d-Pantothenic Acid	10 mg	*d*-Calcium pantothenate
Riboflavin	3.3 mg	–
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	–
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	*d*-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a]Per kg of finished product.

Table D-3. Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	15.47 ± 1.05	14.1–16.5	3
Crude fat (% by weight)	8.7 ± 0.10	8.6–8.8	3
Crude fiber (% by weight)	9.73 ± 0.44	9.25–10.1	3
Ash (% by weight)	4.96 ± 0.14	4.80–5.04	3
Amino Acids (% of total diet)			
Arginine	0.802 ± 0.075	0.67–0.97	28
Cystine	0.220 ± 0.022	0.15–0.25	28
Glycine	0.703 ± 0.038	0.62–0.80	28
Histidine	0.342 ± 0.071	0.27–0.68	28
Isoleucine	0.549 ± 0.041	0.43–0.66	28
Leucine	1.097 ± 0.064	0.96–1.24	28
Lysine	0.700 ± 0.106	0.31–0.86	28
Methionine	0.410 ± 0.042	0.26–0.49	28
Phenylalanine	0.623 ± 0.047	0.47–0.72	28
Threonine	0.512 ± 0.042	0.43–0.61	28
Tryptophan	0.155 ± 0.027	0.11–0.20	28
Tyrosine	0.420 ± 0.066	0.28–0.54	28
Valine	0.666 ± 0.040	0.55–0.73	28
Essential Fatty Acids (% of total diet)			
Linoleic	3.88 ± 0.455	1.89–4.55	28
Linolenic	0.30 ± 0.065	0.007–0.368	28
Vitamins			
Vitamin A (IU/kg)	3,073 ± 49	2,520–3,450	3
Vitamin D (IU/kg)	1,000[a]	–	–
α-Tocopherol (ppm)	2,543 ± 13,044	27.0–69,100	28
Thiamine (ppm)[b]	7.53 ± 0.51	7.1–8.1	3
Riboflavin (ppm)	8.06 ± 2.83	4.20–17.50	28
Niacin (ppm)	78.6 ± 8.26	66.4–98.2	28
Pantothenic acid (ppm)	26.6 ± 11.22	17.4–81.0	28
Pyridoxine (ppm)[b]	9.78 ± 2.08	6.44–14.3	28
Folic acid (ppm)	1.58 ± 0.44	1.15–3.27	28
Biotin (ppm)	0.32 ± 0.09	0.20–0.704	28
Vitamin B_{12} (ppb)	50.6 ± 35.5	18.3–174.0	28
Choline (ppm)[b]	2,615 ± 635	1,160–3,790	28

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Minerals			
Calcium (%)	0.921 ± 0.021	0.906–0.945	3
Phosphorus (%)	0.564 ± 0.017	0.549–0.582	3
Potassium (%)	0.667 ± 0.030	0.626–0.733	28
Chloride (%)	0.393 ± 0.045	0.300–0.517	28
Sodium (%)	0.197 ± 0.026	0.160–0.283	28
Magnesium (%)	0.217 ± 0.055	0.185–0.490	28
Sulfur (%)	0.170 ± 0.029	0.116–0.209	14
Iron (ppm)	191.6 ± 36.8	135–311	28
Manganese (ppm)	50.1 ± 9.59	21.0–73.1	28
Zinc (ppm)	57.4 ± 26.0	43.3–184.0	28
Copper (ppm)	7.53 ± 2.53	3.21–16.3	28
Iodine (ppm)	0.531 ± 0.201	0.158–0.972	28
Chromium (ppm)	0.684 ± 0.258	0.330–1.380	27
Cobalt (ppm)	0.225 ± 0.154	0.086–0.864	26

[a]From formulation.
[b]As hydrochloride (thiamine and pyridoxine) or chloride (choline).

Table D-4. Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.1993 ± 0.047	0.16–0.25	3
Cadmium (ppm)	0.0507 ± 0.006	0.050–0.051	3
Lead (ppm)	0.072 ± 0.012	0.06–0.08	3
Mercury (ppm)	<0.02	–	3
Selenium (ppm)	0.122 ± 0.081	0.029–0.177	3
Aflatoxins (ppb)	<5.00	–	3
Nitrate Nitrogen (ppm)[c]	12.27 ± 3.4	10.0–16.2	3
Nitrite Nitrogen (ppm)[c]	0.61	–	3
BHA (ppm)[d]	<1.0	–	3
BHT (ppm)[d]	<1.0	–	3
Aerobic Plate Count (CFU/g)	<10.0	–	3
Coliform (MPN/g)	3.0	–	3
Escherichia coli (MPN/g)	<10.0	–	3
Salmonella (MPN/g)	Negative	–	3
Total Nitrosoamines (ppb)[e]	10.7 ± 6.8	5.0–18.2	3
N-Nitrosodimethylamine (ppb)[e]	0.8 ± 0.7	0–1.3	3
N-Nitrosopyrrolidine (ppb)[e]	10 ± 6.2	5.0–17.0	3
Pesticides (ppm)			
α-BHC	<0.01	–	3
β-BHC	<0.02	–	3
γ-BHC	<0.01	–	3
δ-BHC	<0.01	–	3
Heptachlor	<0.01	–	3
Aldrin	<0.01	–	3
Heptachlor Epoxide	<0.01	–	3
DDE	<0.01	–	3
DDD	<0.01	–	3
DDT	<0.01	–	3
HCB	<0.01	–	3
Mirex	<0.01	–	3
Methoxychlor	<0.05	–	3
Dieldrin	<0.01	–	3
Endrin	<0.01	–	3

	Mean ± Standard Deviation[b]	Range	Number of Samples
Telodrin	<0.01	−	3
Chlordane	<0.05	−	3
Toxaphene	<0.10	−	3
Estimated PCBs	<0.20	−	3
Ronnel	<0.01	−	3
Ethion	<0.02	−	3
Trithion	<0.05	−	3
Diazinon	<0.10	−	3
Methyl Chlorpyrifos	0.06 ± 0.033	0.02–0.08	3
Methyl Parathion	<0.02	−	3
Ethyl Parathion	<0.02	−	3
Malathion	0.034 ± 0.016	0.02–0.051	3
Endosulfan I	<0.01	−	3
Endosulfan II	<0.01	−	3
Endosulfan Sulfate	<0.03	−	3

[a]All samples were irradiated. CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride.
[b]For values less than the limit of detection, the detection limit is given as the mean.
[c]Sources of contamination: alfalfa, grains, and fish meal.
[d]Sources of contamination: soy oil and fish meal.
[e]All values were corrected for percent recovery.

Appendix E. **Sentinel Animal Program**

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E.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

For the toxicity studies of four perfluoroalkyl carboxylates, blood samples from the sentinel animals were collected, allowed to clot, and the serum was separated. All samples were processed appropriately with serology performed by the Research Animal Diagnostic Laboratory (RADIL, currently IDEXX BioResearch), University of Missouri, Columbia, MO, for determination of the presence of pathogens. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Blood was collected from five rats per sex.

Table E-1. Laboratory Methods and Agents Tested for in the Sentinel Animal Program

Method and Test	Time of Collection
Multiplex Fluorescent Immunoassay	
Mycoplasma pulmonis	End of quarantine
PVM (Pneumonia Virus of Mice)	End of quarantine
RCV/SDA (Rat Coronavirus/Sialodacryoadenitis Virus)	End of quarantine
RMV (Rat Minute Virus)	End of quarantine
RPV (Rat Parvovirus)	End of quarantine
RTV (Rat Theilovirus)	End of quarantine
Sendai	End of quarantine
TMEV (Theiler's Murine Encephalomyelitis Virus)	End of quarantine
Toolan's H-1	End of quarantine

E.2. Results

All test results were negative.

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