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Toxicological Report No. S.0043781-18, October 2020
Toxicology Directorate

Comparative Serum Pharmacokinetics of Per- and Polyfluoroalkyl Substances (PFAS) in White-Footed Mice (*Peromyscus leucopus*), October 2017-July 2018

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Toxicology Directorate
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Specialty: 500C, Toxicity Test

ACKNOWLEDGEMENTS

Caroline Procell, Mark Way, Jarod Hanson and Rebecca Kilby were critical to conducting these experiments. Many thanks to scientists at 3M, including Sue Chang, Ryan Krisko, and Jeremy Zitzow for their analytical support and thorough review of this report. Thanks Carrie McDonough, Younjeong Choi, Sarah Choyke, and Chris Higgins from the Colorado School of Mines for their analytical support. Thanks to Emily Lent, Shane Hall, and Edna Pereira for assistance with statistical analysis. Finally, thanks to the Strategic Environmental Research and Development Program (SERDP) for their generous support (ER-2625).

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REPORT DOCUMENTATION PAGE					Form Approved OMB No. 0704-0188	
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.						
1. REPORT DATE (DD-MM-YYYY) 24/03/2021		2. REPORT TYPE Technical Report			3. DATES COVERED (From - To) October 2017 - July 2018	
4. TITLE AND SUBTITLE Comparative Serum Pharmacokinetics of Per- and Polyfluoroalkyl Substances (PFAS) in White-Footed Mice (<i>Peromyscus leucopus</i>)					5a. CONTRACT NUMBER	
					5b. GRANT NUMBER ER-2625	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Allison M. Narizzano Meredith E. Bohannon Michael J. Quinn, Jr.					5d. PROJECT NUMBER S.0043781-18	
					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Army Public Health Center Toxicology Directorate (MCHB-PH-TEV) 8252 Blackhawk Road, Building 5158 Aberdeen Proving Ground, MD 21010-5403					8. PERFORMING ORGANIZATION REPORT NUMBER S.0043781-18	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Strategic Environmental Research and Development Program (SERDP) 4800 Mark Center Drive Arlington, VA 22350-3605					10. SPONSOR/MONITOR'S ACRONYM(S) SERDP	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S) ER-2625	
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT Per- and polyfluoroalkyl substances (PFAS) are compounds manufactured for use in paints, cleaning agents, fire suppressants, non-stick cookware and food containers, and water-resistant products. Concerns about some perfluorooctyl-based PFAS, such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), stem from their ubiquitous presence in the environment, widespread reports of general toxicity in laboratory animals, and the resistance of these compounds to degradation. In the present study, five perfluoroalkyl substances and one polyfluoroalkyl substance were administered to white-footed mice (<i>Peromyscus leucopus</i>) to elucidate the accumulation of each chemical over 28 days of exposure. In general, similar serum levels at two time points depended on (i) the type of PFAS (<i>i.e.</i> , perfluoroalkyl sulfonic acids (PFSAs), perfluoroalkyl carboxylic acids (PFCAs), or polyfluorinated), (ii) continuous vs. bolus dosing, (iii) dose, and (iv) sometimes sex.						
15. SUBJECT TERMS PFAS, per- and polyfluoroalkyl substances, toxicity, peromyscus, subchronic, AFFF, aqueous film forming foam						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON	
a. REPORT	b. ABSTRACT	c. THIS PAGE			Allison M. Narizzano	
U	U	U	SAR	156	19b. TELEPHONE NUMBER (Include area code) DSN 584-3980, commercial 410-436-3980	

Study Title

Toxicological Report No. S.0043781-18
Protocol No. 67-17-04-01
Comparative Serum Pharmacokinetics of Per- and Polyfluoroalkyl Substances in the White-Footed Mouse (*Peromyscus leucopus*), October 2017-July 2018

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Study Completed On

March 24, 2021

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Laboratory Project ID

Protocol No. 67-17-04-01

Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The test article characterization (purity) was conducted by the manufacturer and it is not known if the testing was done in compliance with the above regulation.
2. Due to time and personnel constraints, the method of analysis for these compounds had not been developed prior to the study start in compliance with study protocol and modification requirements.
3. Animals were on the incorrect light cycle for the duration of the study. *Peromyscus* should be maintained on a 24-hour light/dark cycle with 16 hours of illumination and 8 hours of dark. Instead, they were maintained on a standard rodent light cycle with 12 hours of illumination and 12 hours of dark.

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COMMONLY USED TERMS

AFFF

Aqueous Film Forming Foam

ANOVA

Analysis of variance

ANCOVA

Analysis of covariance

APHC

U.S. Army Public Health Center

ATSDR

Agency for Toxic Substances and Disease Registry

CO₂

carbon dioxide

DOD

Department of Defense

CSM

Colorado School of Mines

°C

Degrees Celsius

°F

Degrees Fahrenheit

FTA(s)

Fluorotelomer alcohol(s)

FTS(s)

Fluorotelomer sulfonate(s)

HRMS

High-resolution mass spectrometry

LC/MS/MS

Liquid chromatography tandem mass spectrometry

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LD₅₀

Median lethal dose (*i.e.*, dose resulting in 50% mortality)

L/kg-day

Liters per kilogram per day

LOQ

limit of quantitation

mg/kg-day

milligrams per kilogram per day

mg/L

milligrams per liter

mg/mL

milligrams per milliliter

mL

milliliter

NIST

National Institute of Standards and Technology

NTP

National Toxicology Program

PFAS

Per- and polyfluoroalkyl substances

PFBS

Perfluorobutane sulfonate

PFCAs

Perfluoroalkyl carboxylic acids

PFEtCHxS

Perfluoroethylcyclohexane sulfonate

PFHxS

Perfluorohexane sulfonate

PFNA

Perfluorononanoic acid

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PFOA

Perfluorooctanoic acid

PFOS

Perfluorooctane sulfonate

PFSAs

Perfluoroalkyl sulfonic acids

TRV

Toxicity Reference Value

SD

Study Director

SERDP

Strategic Environmental Research and Development Program

uL

microliter

UPFOS

Unsaturated perfluorooctane sulfonate

USEPA

U.S. Environmental Protection Agency

XIC

Extracted Ion Chromatogram

3M

The 3M Company

6:2 FTS

6:2 fluorotelomer sulfonate

8:2 FTS

8:2 fluorotelomer sulfonate

10:2 FTS

10:2 fluorotelomer sulfonate

TOXICOLOGICAL REPORT NO. S.0043781-18
PROTOCOL NO. 67-17-04-01
COMPARATIVE SERUM PHARMACOKINETICS OF PER- AND POLYFLUOROALKYL
SUBSTANCES IN THE WHITE-FOOTED MOUSE (*PEROMYSCUS LEUCOPUS*),
OCTOBER 2017-JULY 2018

1 SUMMARY

1.1 Purpose

The PFAS are compounds manufactured for use in multiple products to include paints, cleaning agents, fire suppressants, nonstick cookware, food containers, and water-impermeable products in general. The PFAS are also key ingredients in highly efficient fire suppressant agents known as AFFFs, which have been used by the DOD since approximately 1970 for fire training and emergency response activities.

Increased concerns about some perfluorooctyl-based PFAS, such as PFOS and PFOA, stem from their ubiquitous presence in the environment, widespread reports of toxicity in laboratory animals, and the resistance of these compounds to degradation. The primary goal of the experiments reported herein was to assess bioaccumulation potential and establish a dose range with sufficient body burden for follow-up reproductive and developmental toxicity tests. Data from the reproductive and developmental toxicity tests will be used to inform human health risk assessments and derive wildlife TRVs, which are critical components of environmental risk assessments that help determine if the risk of environmental exposure is acceptable.

In this study, PFAS were administered to white-footed mice (*Peromyscus leucopus*) for 28 consecutive days via either drinking water *ab libitum* or daily oral gavage. Blood samples were collected every 7 days via facial/submandibular venipuncture and analyzed for the concentration of PFAS. At the end of the study, animals were necropsied and selected tissues were weighed. *Peromyscus* mice are the most abundant of the native North American mammals (Dewey and Dawson, 2001). Although many toxicity tests have been done with inbred and outbred rats and mice, no comprehensive test for any PFAS has been undertaken with a native mammalian species. Given that the serum elimination half-lives within this class of chemicals can vary greatly from hours to years based on species, sex, and dose, extrapolating between species is difficult and inaccurate. As such, wild mammalian-specific data are both novel and valuable for risk estimation in taxa that are directly site-specific.

1.2 Conclusions

In general, similar serum levels at two time points depended on (i) the type of PFAS (*i.e.*, PFASs, PFCAs, or polyfluorinated), (ii) continuous vs. bolus dosing, (iii) dose, and (iv) sometimes sex. As with previous studies, the liver appears to be the target organ of PFAS toxicity in *P. leucopus* based on hepatomegaly, with shorter-chain chemicals appearing less toxic than longer chain chemicals. This study provided information on the target organs of individual PFAS and the internal dosimetry of each compound. These data were used to

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determine dose levels and refine the design for follow-up reproductive and developmental toxicity studies with individual PFAS.

2 REFERENCES

See Appendix A for a listing of references.

3 AUTHORITY

This study was conducted with funding from the SERDP, Environmental Restoration Project No. ER-2625 (MIPR# W74RDV70043331). It was performed as part of a larger effort funded by SERDP entitled "Development of Toxicity Data to Support Toxicity Reference Values for Perfluorinated Compounds."

4 BACKGROUND

The PFAS are compounds manufactured for use in paints, cleaning agents, fire suppressants, non-stick cookware, food containers, and water-impermeable products, in general. The PFOS and PFOA, the two most commonly studied PFAS (Kotthoff et al. 2015; Cheng and Ng 2018), have attracted increased regulatory scrutiny because of their resistance to degradation, ability to bioaccumulate, and growing evidence of toxicity in animals. In 2016, the USEPA published combined lifetime drinking water Health Advisory values of 70 ng/L for PFOS and PFOA. However, these levels are often exceeded globally, specifically near military bases, chemical plants, and manufacturing facilities. Furthermore, various PFAS are detected in human serum, breast milk, and umbilical cord blood worldwide (ATSDR 2018).

Although many toxicity tests have been done with traditional laboratory rodent species, no comprehensive test for any PFAS has been undertaken with a native mammalian species. Inbred laboratory rodent strains are often differentially sensitive to toxicants and, therefore, less representative of toxicity for heterogeneous native species (Shorter et al. 2012). For example, white-footed mice exposed daily to TNT at the LD₅₀ for *Mus* exhibited no mortality (McCain and Ferguson 1998). Furthermore, the serum elimination half-lives within this class of chemicals can vary greatly from hours to years based on species, sex, and dose, thereby making extrapolation difficult and sometimes inaccurate. As such, wild mammalian-specific data are both novel and valuable for accurate risk assessment.

The primary goal of these experiments was to evaluate the serum pharmacokinetic profiles and target organs of six PFAS in white-footed mice (*Peromyscus leucopus*) during and after 28-day consecutive exposure. Serum samples were collected every 7 days via facial/submandibular venipuncture and were analyzed for the concentration of individual PFAS via LC/MS/MS. These valuable dose range data will be integrated into follow-up reproductive/developmental toxicity studies, which will be used to derive wildlife TRVs. These are critical components of environmental risk assessments that help determine what environmental exposures are acceptable.

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Table 1 identifies the dates of critical study events.

Table 1. Critical Study Events

Critical Event	Date of Event
Animal Use Protocol Approved	04/20/2017
In-Life Initiation	10/02/2017
In-Life Completion	07/19/2018
Experimental Completion	09/22/2020
Study Completion	03/24/2021

5 MATERIALS AND METHODS

5.1 Test Substances

The six test substances (see Table 2) were obtained from Sigma Aldrich, St. Louis, Missouri. The manufacturer conducted purity analyses for these compounds. The six test substances are—

- Heptadecafluorooctanesulfonic acid potassium salt (PFOS),
- Perfluorooctanoic acid (PFOA),
- Tridecafluorohexane-1-sulfonic acid potassium salt (PFHxS),
- Potassium nonafluoro-1-butanefulfonate (PFBS),
- Perfluorononanoic acid (PFNA), and
- Tridecafluorooctane-1-sulphonic acid (6:2 FTS).

Table 2. Test Substances

Chemical	Abbreviation	Molecular weight	CAS-No.	Purity
Heptadecafluorooctanesulfonic acid potassium salt	PFOS	538.22 g/mol	2795-39-3	≥98%
Perfluorooctanoic acid	PFOA	414.07 g/mol	335-67-1	>95%
Tridecafluorohexane-1-sulfonic acid potassium salt	PFHxS	438.20 g/mol	3871-99-6	≥98%
Potassium nonafluoro-1-butanefulfonate	PFBS	338.19 g/mol	29420-49-3	98%
Perfluorononanoic acid	PFNA	464.08 g/mol	375-95-1	97%
Tridecafluorooctane-1-sulphonic acid	6:2 FTS	428.17 g/mol	27619-97-2	98%

5.2 Animals and Housing Conditions*

* Animal use procedures were approved by the APHC Institutional Animal Care and Use Committee. Animal care and use was conducted according to *The Guide for the Care and Use of Laboratory Animals* and all applicable Federal and DOD regulations. The APHC Animal Care and Use Program is fully accredited by the AAALAC International.

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Adult male (n=196; 22.58 ± 0.27 g) and female (n=198; 20.49 ± 0.21 g) *Peromyscus leucopus* were obtained from the Peromyscus Genetic Stock Center at the University of South Carolina. Animals were obtained for Experiments 1 and 2 in one shipment and they were obtained for Experiment 3 in a separate shipment. Animals were acclimated to the facility for at least 5 days before initiation of dosing in Experiments 1 and 3; they were acclimated to the facility for 5 months before initiating dosing in Experiment 2. Experiments 1 and 2 were originally planned to be investigated concurrently; however, unexpected solubility issues with the test materials necessitated delaying the investigation of one chemical and proceeding with investigation of three chemicals. The study design for each Experiment is described in detail in Section 5.6 (*vide infra*).

The following were the age ranges for male and female mice at the initiation of each experiment:

- Experiment 1: males were between 133 and 222 days; females were between 136 and 238 days.
- Experiment 2: males were between 301 and 390 days; females were between 304 and 406 days.
- Experiment 3: males were between 343 and 392 days; females were between 301 and 392 days.

The large age range of the animals is partially due to the delay of Experiment 2. However other contributing factors included the availability of only one vendor for the *P. leucopus*, seasonal and sporadic breeding of the species, and smaller litter sizes (roughly 2.7 pups/litter) than traditional laboratory mice (roughly 8 pups/litter) (Schmidt and Hood 2012; Plumel et al. 2014).

Animals were assigned to a compound and dose group by a stratified random process using pre-study body weight data to ensure that body weights did not differ among groups (*i.e.*, chemical or dose level) within each experiment prior to initiation of dosing. Within each experiment, body weight did not differ among groups (*i.e.*, chemical or dose level) prior to initiation of dosing. A cage card uniquely identified each animal. Within each cage, animals were identified by a tail color.

All animals were housed in temperature-, relative humidity-, and light-controlled rooms. The target conditions of the rooms were 68–72°F and 30–70% humidity. An automatically controlled 12/12-hour light/dark cycle was maintained, with the dark period beginning at 1800 hours. A certified pesticide-free rodent chow (Harlan Teklad® 2016C Certified Rodent Diet) was available *ad libitum*. Depending on the experiment, animals were provided with filtered tap water or treated filtered tap water *ad libitum*. Animals were same sex group housed in suspended 7" wide x 10.5" tall x 5" deep plastic cages on a static, stainless steel rack. The APHC Institutional Animal Care and Use Committee approved the animal care and use procedures. Animal care and use was conducted according to "The Guide for the Care and Use of Laboratory Animals" and all applicable Federal and DOD regulations (NRC 2011). The APHC Animal Care and Use Program is fully accredited by the AAALAC International.

5.3 Quality Assurance

This study was conducted according to Good Laboratory Practices (CFR 2011). The APHC Quality Systems and Regulatory Compliance Office audited critical study phases. Appendix B provides the dates of these audits, the phases audited, and the dates the results were reported to the SD and Management.

5.4 Study Personnel

Appendix C lists the names of individuals contributing to the study performance.

5.5 Dose Selection

Dose selection was based on the ultimate objective of the study, which was to identify possible target organs of selected PFAS and the internal dosimetry of each compound. The intent was not to cause or detect toxicity, if possible. Doses were selected based on DeWitt *et al.* (2008) and Das *et al.* (2017).

5.6 Study Design

5.6.1 Experiment 1

Male and female white-footed mice were exposed to PFOA (9/sex/dose), PFHxS (9/sex/dose), and PFBS (8–9/sex/dose) via drinking water for 28 consecutive days. This experiment utilized a shared control group.

Dosing solutions were prepared by—

- Weighing the required amount of neat PFOA, PFHxS, and PFBS,
- Transferring the solid test material to a 1,000 mL volumetric flask,
- Adding approximately 900 mL of animal room drinking water,
- Stirring using a magnetic stir bar and stir plate until dissolved, and
- Adding water to the 1,000 mL mark.

Three drinking water dosing solutions (37.5, 75, and 150 mg/L) were used through the experiment. Using an assumed default water consumption rate of 0.003 L/kg-day and a default body weight of 0.0225 kg, dosing solutions corresponded to approximately 5, 10, and 20 mg/kg-day. Solutions were prepared weekly. Drinking water/dosing reservoirs were replaced completely every 4 days and reservoirs were changed every 2 weeks. Representative dosing solutions were verified via LC/MS/MS by Eurofins Eaton Analytical.

5.6.2. Experiment 2.1

Male and female white-footed mice were exposed to PFOS (8/sex/dose) via food treats 2 and 3 days, respectively. Food treats are a well-documented exposure type for toxicity

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testing with rodents, including flame retardant materials (Hays et al. 2002; Patisaul et al. 2009; McCaffrey et al. 2013).

Dosed treats were prepared by—

- Weighing the required amount of neat PFOS, and
- Adding the required amount of methanol to make a stock solution (18.5 mg/mL).

Stock solution was applied to Fruit Loops™ with a pipette (at variable volumes) to create dosed treats. Treats were dried for 24 hours to ensure methanol evaporated off prior to being administered to animals. Three doses, 2.5, 25, and 250 mg/kg-day were used through the experiment. Stock solution was prepared daily. Treats were prepared daily, 1 day prior to administration. Concentration of the stock solution was verified by the APHC Laboratory Sciences Directorate via LC/MS/MS.

5.6.2 Experiment 2.2

Male and female white-footed mice were exposed to PFOS (8–9/sex/dose) via oral gavage for 28 consecutive days. Male and female mice received their first four and three doses, respectively, via filtered tap water. Animals received their remaining doses via corn oil (*i.e.*, days 5 and 4 through 28 for males and females, respectively).

Dosing suspensions were prepared by—

- Weighing the required amount of neat PFOS,
- Transferring the solid test material to a polypropylene container,
- Measuring the appropriate volume of filtered tap water or corn oil using a graduated cylinder, and
- Adding the tap water or corn oil to the polypropylene container.

Three dosing solutions or suspensions, 0.25, 4.0, and 10.0 mg/mL, were used throughout the experiment, which corresponded to 2.5, 40, and 100 mg/kg-day. Solutions were prepared once and suspensions were prepared bi-weekly. Representative dosing solutions were verified via LC/MS/MS by Eurofins Eaton Analytical.

5.6.3 Experiment 3

Male and female white-footed mice were exposed to 6:2 FTS (9/sex/dose) or PFNA (9/sex/dose) via oral gavage for 28 consecutive days. Male and female white-footed mice were also exposed to PFHxS (6-9/sex/dose) via oral gavage for 28 consecutive days. Because PFHxS was administered to white-footed mice via drinking water in Experiment 1, fewer animals were used in Experiment 3. This experiment utilized a shared control group.

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Dosing solutions and suspensions were prepared by—

- Weighing the required amount of neat 6:2 FTS and PFNA,
- Adding methanol to make a stock solution (500 mg/mL),
- Adding the appropriate amount of stock solution to a 50 mL volumetric flask,
- Adding approximately 40 mL of animal room drinking water,
- Stirring using a magnetic stir bar and stir plate until dissolved, and
- Adding water to the 50 mL mark.

Solutions were prepared bi-weekly. Three dosing solutions, 0.25, 1.0, and 2.0 mg/mL, were prepared for 6:2 FTS, which corresponded to 2.5, 10, and 20 mg/kg-day. The 0.25 and 1.0 mg/mL 6:2 FTS dosing solutions were volumetrically adjusted such that they had the same volume of methanol as the 2.0 mg/mL 6:2 FTS dosing solution. Two dosing solutions, 0.25 and 1.0 mg/mL, were prepared for PFNA, which corresponded to 2.5 and 10 mg/kg-day. One dosing suspension, 2.0 mg/mL, was prepared for PFNA, which corresponded to 20 mg/kg-day. The 0.25 and 1.0 mg/mL PFNA dosing solutions were volumetrically adjusted such that they had the same volume of methanol as the 2.0 mg/mL PFNA dosing suspension. Given that the volume of methanol added to each dosing solution was 160 μ L and the total volume prepared was 40 mL, methanol represented approximately 0.4% of the solutions and was not expected to impact the study outcome, based on its LD₅₀ of 7300-10000 mg/kg in mice (Smith and Taylor 1982). Representative 6:2 FTS dosing solutions were verified via LC/MS/MS by 3M. Representative PFNA dosing solutions were verified via LC/MS/MS by Eurofins Eaton Analytical.

To determine occurrence of contaminants in the 6:2 FTS dosing solution, suspect screening analysis was performed by CSM via LC-QTOF-MS. The suspect screening analysis involved using the in-house PFAS XIC list which contained 1732 PFAS and in-house PFAS HRMS spectral library which contained 416 PFAS and 815 spectra. The level of confidence in identification was based on the following criteria: authentic standard verification, spectral library match (>70%), mass error (<5 ppm), retention time (>3.5 min) and isotope ratio difference (<20%).

Dosing solutions were prepared by—

- Weighing the required amount of neat PFHxS,
- Transferring the solid test material to a 50 mL volumetric flask,
- Adding approximately 40 mL of animal room drinking water,
- Stirring using a magnetic stir bar and stir plate until dissolved, and
- Adding water to the 50 mL mark.

Two dosing solutions, 1.0 and 2.0 mg/mL, were used through the experiment, which corresponded to 10 and 20 mg/kg-day. Representative PFHxS dosing solutions were verified via LC/MS/MS by Eurofins Eaton Analytical

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Because of the shared control group with 6:2 FTS and PFNA, the 1.0 and 2.0 mg/mL dosing solutions were volumetrically adjusted such that they had the same volume of methanol as the 2.0 mg/mL (6:2 FTS and PFNA) dosing solution and suspension. Again, given that methanol represented approximately 0.4% of the solutions, it was not expected to impact the study outcome, based on its LD₅₀ of 7300-10000 mg/kg (Smith and Taylor 1982)

5.7 Clinical Observations, Body Weight, Food Consumption

Animals were removed from their home cages and observed daily by study personnel for signs of toxicity, morbidity, and mortality in conjunction with dosing. Body weights were taken at the start of test substance administration, at least weekly thereafter, and at termination.

5.8 Blood Collection

The first day of dosing was designated day 0. Blood was collected via submandibular venipuncture from each animal on days 0, 7, 14, and 21 of test substance administration. Blood was collected in weigh boats free of additives, transferred to microcentrifuge tubes, allowed to clot at room temperature for 60 to 120 minutes, and centrifuged approximately 10 minutes at 600 x g two times. Serum was removed and frozen at -80°C.

5.9 Euthanasia, Trunk Blood Collection, Necropsy, and Organ Mass

After 28 days of dosing, surviving animals were rendered unconscious with carbon dioxide CO₂, euthanized via decapitation, and trunk blood was collected for serum analysis. Serum was removed and frozen at -80°C.

A full, detailed gross necropsy, including a careful examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents, was performed on all experimental animals following euthanasia. Brain, heart, kidneys, liver, ovaries, uterus, spleen, thymus, testes, and epididymides were removed, trimmed, and weighed. Testes and ovaries were weighed as pairs.

Full histopathological examination was not performed.

5.10 Sperm Analysis

Sperm analysis was only conducted for Experiment 3. The left epididymis from each male was removed during necropsy, weighed, placed in roughly 10 mL Gibco Medium 199 preheated at 37°C, minced using small scissors to release sperm into the medium, and incubated for 5 min at 37°C. Then, a small aliquot of the sperm dilution in M199 was loaded on a standard count chamber slide (Leja). Slides were immediately loaded into a Hamilton-Thorne IVOS Sperm Analysis System and sperm were counted using the IDENT program.

5.11 Serum Analysis

Serum analysis (Day 0, 21, and 28) was conducted at the CSM based on published methods (Reiner et al. 2009). Serum analysis (Day 28) was conducted at a second lab (3M) for inter-lab verification purposes based on published methods (Sundstrom et al. 2012; Ehresman et al. 2007; Olsen et al. 2009).

Non-targeted serum analysis (Day 28, high dose 6:2 FTS animals) was also conducted at the CSM to identify possible metabolites of 6:2 FTS. A Welsh t-test was used to prioritize features with a p-value < 0.4 and a log-fold change > 0.5 between the vehicle animal serum and high-dosed animal serum. Prioritized features were assessed based on a retention time of > 3.5 minutes and signal to noise of 10:1 in order to screen out for “noise.” Remaining features were screened against the in house PFAS XIC list and in-house PFAS HRMS spectral library and NIST HRMS spectral library. Features were identified based on spectral mass (>70%), mass error (< 5 ppm), retention time (> 3.5 minutes) and isotope ratio difference (<20%). The level of confidence in feature identification was based on the Schymanski Scale (Level 1 = confirmed structure identification, Level 2= probably structure with spectral library confirmation, Level 3= tentative structural identification, Level 4= unequivocal molecular formula, Level 5= exact mass only) (Schymanski et al. 2014).

5.12 Data Collection, Calculations, and Statistical Analyses

Experimental data generated during this study were recorded by hand and tabulated, summarized, and/or statistically analyzed using Minitab, SPSS, and GraphPad. Environmental data were automatically recorded using MetaSys Building Management System.

Data not normally distributed were log or arcsine transformed and retested for normality. If log or arcsine transformation did not normalize data, data were analyzed using the nonparametric, Kruskal-Wallis H test. Variance equality was determined by Levene’s test. Analyses were conducted for male and female mice separately.

Continuous data were analyzed using ANOVA with dose as a fixed factor. Absolute organ weight was analyzed by ANCOVA for brain, heart, kidneys, epididymides, spleen, testes, thymus, and uterus because these tissues demonstrate a non-linear relationship to body weight (Bailey et al. 2004). Dose group was used as the main effect and body weight at the end of the study as the covariate. Liver weights demonstrate a linear relationship to body weight and were analyzed as a ratio to body weight at the end of the study via ANOVA (Bailey et al. 2004). Ovaries and adrenal weights demonstrate a linear relationship to brain weight and were analyzed as a ratio to brain weight at the end of the study via ANOVA (Bailey et al. 2004). Weekly body weight data were analyzed using one-way ANOVA to determine dose effect. Repeated measures ANOVA were not used to avoid listwise deletions in SPSS. When significant main effects were observed ($p < 0.05$), appropriate post-hoc tests were used to compare dose groups to the control group (e.g., Tukey’s multiple comparison test or Sidak (for ANCOVA)).

The quality of the serum datasets varied by chemical due to the small and unequal sample sizes for some groups, missing samples, and a paucity (in some cases) of detections above the LOQ. For samples with chemical levels below the LOQ, the LOQ was used for analysis. To ensure normality, all data were $\log(x+1)$ transformed prior to analysis. Since the objective of the study was to determine if serum concentrations varied by sex, time, and/or dose, statistics excluded serum levels from vehicle control animals and all animals at day 0. Serum data (day 14 or 21 and 28, CSM) were analyzed using two-way repeated measures ANOVAs with sex and dose as fixed factors. If the interactions of within and between subject factors were significant, the effect of sex, time, and/or dose on the parameter was determined using a one-factor repeated measures ANOVA or a one-factor ANOVA. If tests of between subjects were non-significant, data from males and females were pooled. Serum data (day 28, 3M) were analyzed via two-way ANOVA with sex and dose as fixed factors. When significant main effects were observed ($p < 0.05$), appropriate post-hoc tests were used to compare dose groups to the control group (e.g., Tukey's multiple comparison test). If tests of between subjects were non-significant, data from males and females were pooled. If multiple ANOVAs were necessary, due to interactions, Type 1 error was corrected with a Bonferroni adjustment.

6 RESULTS

6.1 Experiment 1

6.1.1 Analytical Verification of Dosing Solutions

Comparison of the measured concentrations of PFOA, PFHxS, and PFBS in solution to the nominal concentrations of the solutions revealed ~78-100% accuracy for the solutions containing ≤ 75 mg/L PFOA, PFHxS, and PFBS at days 14 and 28 (Appendix D). Results were within the pre-established acceptance of 70-130% accuracy level. The analytically verified concentrations revealed that PFOA, PFHxS, and PFBS in the more concentrated solution (150 mg/L) at days 14 and 28 corresponded to ~60-66% of the nominal concentration (Appendix D). Thus, the doses administered in Experiment 1 were 37.5, 75, and 99 mg/L PFOA, 37.5, 75, and 98 mg/L PFHxS, and 37.5, 75, and 90 mg/L PFBS and will be referred to as such rather than the targeted concentrations (37.5, 75, and 150 mg/L).

Additional testing was conducted to determine if the PFOA, PFHxS, and PFBS partitioned at the water-air interface of undisturbed water bottles (Appendix D). For all three chemicals, concentrations of the lower dosing solutions at days 14 and 28 were similar and do not support the hypothesis that the chemicals partitioned at the water-air interface (Appendix D). However, the 150 mg/L solutions of PFOA, PFHxS, and PFBS were below the pre-established acceptance of 70% accuracy at days 14 and 28. This could be due to the chemicals adsorbing to the inner surfaces of the containers at higher concentrations, despite using the recommended plastic (polypropylene), especially given the length of holding time between mixing and analysis (Table 3). Note that data from Experiment 3 does not support this hypothesis (Appendix D), where dosing solutions were more concentrated (*i.e.*, mg/mL vs. mg/L) and also analyzed after lengthy holding times.

6.1.2 Clinical Observations and Body Weight

Clinical observations were limited to wounds, malocclusions, and hair loss. No compound-related mortality occurred, but some animals failed to recover from the submandibular venipuncture and died prior to 28 days of exposure. Body weight of males and females was unaffected by PFOA, PFHxS, and PFBS (Appendix E).

6.1.3 Serum Analysis

6.1.3.1 PFOA

In a test of within-subjects effects, there was no significant effect of time on serum levels of PFOA (Wilks' Lambda = 0.980, $F_{1,41} = 0.844$, $p = 0.363$). The following interactions were also not significant: (i) time x sex x dose (Wilks' Lambda = 0.954, $F_{2,41} = 0.986$, $p = 0.382$), and (ii) time x dose (Wilks' Lambda = 0.996, $F_{2,41} = 0.077$, $p = 0.926$). There was, however, a significant time x sex interaction (Wilks' Lambda = 0.893, $F_{1,41} = 4.916$, $p = 0.032$) (Appendix F).

In separate tests of within-subjects effects, there was no main effect of time on serum levels of PFOA (males: Wilks' Lambda = 0.951, $F_{1,20} = 1.022$, $p = 0.324$; females: Wilks' Lambda = 0.832, $F_{1,21} = 4.241$, $p = 0.052$) and no significant time x dose interactions (males: Wilks' Lambda = 0.912, $F_{2,20} = 0.967$, $p = 0.397$; females: Wilks' Lambda = 0.979, $F_{2,21} = 0.229$, $p = 0.797$) (Appendix F). In separate tests of between-subjects effects, serum levels of PFOA were unaffected by dose in males ($F_{2,20} = 2.222$, $p = 0.134$) and were affected by dose in females ($F_{2,21} = 12.025$, $p < 0.001$). Data from 3M (day 28) exhibited the same trend (males: $F_{2,18} = 0.141$, $p = 0.869$; females: $F_{2,22} = 11.882$, $p < 0.001$).

6.1.3.2 PFHxS

In a test of within-subjects effects, there was no significant main effect of time on serum levels of PFHxS (Wilks' Lambda = 0.993, $F_{1,40} = 0.278$, $p = 0.601$). The following interactions were also not significant: (i) time x sex x dose (Wilks' Lambda = 0.999, $F_{2,40} = 0.016$, $p = 0.984$), (ii) time x sex (Wilks' Lambda = 0.945, $F_{1,40} = 2.340$, $p = 0.134$), and (iii) time x dose (Wilks' Lambda = 0.990, $F_{2,40} = 0.199$, $p = 0.821$) (Appendix F). In a test of between-subjects effects, serum levels of PFHxS were unaffected by (i) sex x dose interaction ($F_{2,40} = 2.431$, $p = 0.101$) and (ii) sex ($F_{1,40} = 0.776$, $p = 0.384$). However, they were significantly affected by dose ($F_{2,40} = 51.027$, $p < 0.001$) (Appendix F). A similar effect of dose was observed on data obtained at 3M on exposure day 28 ($F_{2,43} = 31.122$, $p < 0.001$).

6.1.3.3 PFBS

In a test of within-subjects effects, there was no significant main effect of time on serum levels of PFBS (Wilks' Lambda = 0.993, $F_{1,41} = 0.309$, $p = 0.582$). The following interactions were also not significant: (i) time x sex x dose (Wilks' Lambda = 0.894, $F_{2,41} =$

2.422, $p = 0.101$), (ii) time x sex (Wilks' Lambda = 0.997, $F_{1,41} = 0.137$, $p = 0.713$), and (iii) time x dose (Wilks' Lambda = 0.966, $F_{2,41} = 0.719$, $p = 0.493$) (Appendix F). In a test of between-subjects effects, serum levels of PFBS were unaffected by (i) sex x dose interaction ($F_{2,41} = 0.119$, $p = 0.888$), (ii) sex ($F_{1,41} = 1.618$, $p = 0.211$, and (iii) dose ($F_{2,41} = 1.116$, $p = 0.337$) (Appendix F). Data from 3M (day 28) exhibited the same trend ($F_{2,43} = 3.164$, $p = 0.052$).

6.1.4 Necropsy and Organ Weight

No PFOA, PFHXS, or PFBS-related gross observations or organ weight effects were noted in either sex during necropsy ($p > 0.05$) (Appendix G).

6.2 Experiment 2

6.2.1 Analytical Verification of Dosing Solutions

Comparison of the measured concentrations of PFOS in solution to the nominal concentrations of the solutions revealed ~74-91% accuracy for the solution containing 0.25 mg/mL PFOS at days 14 and 28 (Appendix D). Results were within the pre-established acceptance of 70-130% accuracy level. The analytically verified concentrations revealed that PFOS in the more concentrated solutions (4 and 10 mg/mL) at days 14 and 28 corresponded to ~24-27% and ~41-56% of the nominal concentration, respectively (Appendix D). Thus, the doses administered in Experiment 2 were 2.5, 20 and 40 mg/kg-day PFOS and will be referred to as such rather than the targeted doses (2.5, 40, and 100 mg/kg-day).

6.2.2 Clinical Observations and Body Weight

Clinical observations were limited to wounds, malocclusions, and hair loss. Experiment 2.1 was aborted after 3 days after the animals developed an aversion to PFOS-containing treats in a dose-dependent manner (*i.e.*, to high on day 1; medium and high on day 2; low, medium, and high on day 3). In Experiment 2.2, animals exposed to 20 and 40 mg/kg-day PFOS exhibited overt toxicity and died within 14 days. No PFOS-related mortality occurred in animals exposed to 2.5 mg/kg-day, but some animals failed to recover from submandibular venipuncture and died prior to 28 days of dosing.

Body weights of males were largely unaffected by PFOS, despite complete mortality in the two higher doses between days 0 and 14 (day 0: $F_{3,28} = 0.06$, $p = 0.979$; day 7: $F_{3,19} = 0.19$, $p = 0.899$; day 14: $F_{1,12} = 0.11$, $p = 0.743$; day 21: $F_{1,11} = 0.36$, $p = 0.560$) (Appendix E). Exposure to PFOS had an effect on male body weights on day 28 ($F_{1,10} = 5.21$, $p = 0.046$) because control animals lost close to 20% of their body weight between day 21 and 28 with no explanation. Body weights of females were unaffected by PFOS (day 0: $F_{3,30} = 0.07$, $p = 0.997$; day 7: $F_{2,18} = 0.28$, $p = 0.775$; day 14: $F_{1,14} = 0.00$, $p = 0.984$; day 21: $F_{1,12} = 0.02$, $p = 0.886$; day 28: $F_{1,12} = 0.23$, $p = 0.640$).

6.2.3 Serum Analysis

In a test of within-subjects effects, there was a significant main effect of time (Wilks' Lambda = 0.653, $F_{1,10} = 5.311$, $p = 0.044$) (Appendix F) on serum levels of PFOS. However, there were no significant time x sex interactions (Wilks' Lambda = 0.888, $F_{1,10} = 1.259$, $p = 0.288$). Between-subjects tests were not run, given that there was only one treatment group alive (2.5 mg/kg-day PFOS) at days 14 and 28 of exposure.

6.2.4 Necropsy and Organ Weight

Overt toxicity resultant in mortality precluded collection of organ weight data from animals exposed to 20 and 40 mg/kg-day PFOS. No PFOS-related gross observations were noted in either sex during necropsy. However, of the animals that survived 28 days of dosing, males exposed to 2.5 mg/kg-day PFOS exhibited increased liver weights ($F_{1,9} = 14.82$, $p = 0.004$) and decreased thymus weights ($F_{1,9} = 2.30$, $p = 0.030$) compared to controls (Appendix G). Females exposed to 2.5 mg/kg-day PFOS exhibited decreased uterine weights ($F_{1,11} = 8.08$, $p = 0.024$) compared to controls (Appendix G). All other results in both sexes were not statistically significant ($p > 0.05$).

6.3 Experiment 3

6.3.1 Analytical Verification of Dosing Solutions

Comparison of the measured concentrations of 6:2 FTS in solution to the nominal concentrations of the solutions revealed 72% accuracy for the solution containing 0.25 mg/mL 6:2 FTS at days 9 and 29 (Appendix D). Results were within the pre-established acceptance of 70-130% accuracy level. The analytically verified concentrations revealed that 6:2 FTS in the more concentrated solutions (1 and 2 mg/mL) at days 9 and 29 corresponded to ~58-65% and ~60-62% of the nominal concentration, respectively (Appendix D). Thus, the doses administered in Experiment 3 were 2.5, 6 and 12.5 mg/kg-day 6:2 FTS and will be referred to as such rather than the targeted doses (2.5, 10, and 20 mg/kg-day).

Additional targeted analysis of the 6:2 FTS dosing solution was performed using LC-QTOF-MS to identify contaminants in the highest concentration dosing solution (2 mg/mL). In the dosing solution, 4:2 FTS, 8:2 FTS, 10:2 FTS, 6:2 FTA, 8:2 FTA, 6:2 FTUA and 8:2 FTUA were identified and confirmed with analytical standards. 4:2 FTS (6.6 mg/mL) and 8:2 FTS (5.8 mg/mL) were detected at higher concentrations than 6:2 FTS (2.0 mg/mL, Appendix D). 10:2 FTS (0.8 mg/mL) was also detected, but the concentration was lower than 6:2 FTS. In addition, 6:2 FTA (0.18 mg/mL), 8:2 FTA (0.049 mg/mL), 6:2 FTUA (0.002 mg/mL) and 8:2 FTUA (0.118 mg/mL) were detected in the dosing solution but accounted for less than 10% of the 6:2 FTS concentration.

The suspect screening revealed additional long chain FTSs including 12:2 FTS, 14:2 FTS, 16:2 FTS and 18:2 FTS. These long chain FTSs were a level 2 confirmation with >90% spectral library match. Hydroxylated FTSs, including 1-OH-4:2 FTS and 1-OH-6:2 FTS,

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were also identified by suspect screening with a level 2 confirmation. Although there was no attempt to semi-quantify the compounds detected through suspect screening, the raw peak areas were less than 5% of the 6:2 FTS raw peak area.

Long chain PFCAs, including PFOA (2 ng/L), PFNA (50 ng/L), PFDA (2 ng/L) and PFUdA (18 ng/L), were detected at a low concentration in the animal room drinking water. Apart from PFOA and PFNA, PFCAs were not analyzed in the animal serum.

Comparison of the measured concentrations of PFNA in solution to the nominal concentrations of the solutions revealed ~44-59% accuracy at days 14 and 28 (Appendix D). All results were below the pre-established acceptance of 70-130% accuracy level. The measured doses of PFNA administered in Experiment 3 were 1, 6, and 10 mg/kg-day and, therefore, the measured doses will be referred to rather than the targeted concentrations of 5, 10, and 20 mg/kg-day.

Comparison of the measured concentrations of PFHxS in solution to the nominal concentrations of the solutions revealed ~69-74% accuracy. Average results (day 14 and 28) were within the pre-established acceptance of 70-130% accuracy level (Appendix D). As such, the measured doses of PFHxS will be reported as nominal: 10 and 20 mg/kg-day.

6.3.2 Clinical Observations and Body Weight

Clinical observations were limited to wounds, malocclusions, and hair loss. No compound-related mortality occurred, but some animals failed to recover from submandibular venipuncture and died prior to 28 days of exposure. Body weight of males and females was unaffected by 6:2 FTS, PFNA, and PFHxS (Appendix E).

6.3.3 Serum Analysis

6.3.3.1 6:2 FTS (targeted analysis)

In a test of within-subjects effects, there was a significant main effect of time on serum levels of 6:2 FTS (Wilks' Lambda = 0.030, $F_{1, 35} = 1122.245$, $p < 0.001$). In addition, the following interactions were not statistically significant: (i) time x sex x dose (Wilks' Lambda = 0.951, $F_{2, 35} = 0.899$, $p = 0.416$), and (ii) time x sex (Wilks' Lambda = 0.934, $F_{1, 35} = 2.462$, $p = 0.126$). However, there was a significant time x dose interaction (Wilks' Lambda = 0.443, $F_{2, 35} = 22.935$, $p < 0.001$) (Appendix F).

In separate tests of between-subjects effects, serum levels of 6:2 FTS were affected by dose at day 21 ($F_{2, 42} = 28.946$, $p < 0.001$) (Appendix F). Data from 3M (day 28) exhibited the same trend ($F_{2, 43} = 14.736$, $p < 0.001$, whereas CSM was unable to detect 6:2 FTS in serum at day 28).

6.3.3.2 6:2 FTS (non-targeted analysis)

A total of 5224 features were detected in more than five samples. A Welch t-test to screen for features with a p-value < 0.4 and a log-fold change > 0.5 yielded 1716 features. Further elimination of features with < 3.5 minutes retention time yielded 126 features. None of the 126 “real” peaks were (i) PFAS transformation products or (ii) appeared in the HRMS and NIST spectral libraries at significantly higher levels in the dosed vs. vehicle animals. Furthermore, no features matched to an in-house PFAS XIC list could be concluded to be metabolites of 6:2 FTS. Based on these results, 6:2 FTS is not thought to metabolize in *Peromyscus* serum.

Of the compounds that were discovered via non-targeted analysis, the most prominent were 8:2 FTS, 10:2 FTS, PFOS, and PFEtCHxS/UPFOS, however levels of these compounds are not quantified due to the nature of non-targeted analysis. Due to the chemical structures of these compounds, they are not likely 6:2 FTS metabolite candidates. Furthermore, 8:2 FTS and 10:2 FTS were confirmed to be present in the dosing solution, but not in the house water, indicating that they are impurities in the neat powder (see 6.3.1 and Appendix F). PFOS and PFEtCHxS/UPFOS were not found in the dosing solution or the house water, suggesting that they are present in the feed and/or enrichment, although this was not verified.

6.3.3.3 PFNA

In a test of within-subjects effects, there was no main effect of time (Wilks' Lambda = 0.982, $F_{1,46} = 0.849$, $p = 0.362$) on serum levels of PFNA. The following interactions were also not statistically significant: (i) time x sex x dose (Wilks' Lambda = 0.968, $F_{2,46} = 0.764$, $p = 0.471$), (ii) time x sex (Wilks' Lambda = 0.981, $F_{1,46} = 0.905$, $p = 0.346$), and (iii) time x dose (Wilks' Lambda = 0.983, $F_{2,46} = 0.404$, $p = 0.670$, Table 5-11, Figure 5-11). In a test of between-subjects effects, there was a significant (i) sex x dose interaction ($F_{2,46} = 5.959$, $p = 0.05$), (ii) sex ($F_{1,46} = 14.656$, $p < 0.001$), and (iii) a significant main effect of dose on serum levels of PFNA ($F_{2,46} = 77.237$, $p < 0.001$) (Appendix F).

In separate tests of between-subjects effects, serum levels of PFNA were affected by dose in males ($F_{2,23} = 15.408$, $p < 0.001$) and in females ($F_{2,23} = 106.943$, $p < 0.001$) (Appendix F). Data from 3M (day 28) exhibited the same trend (males: $F_{2,23} = 19.822$, $p < 0.001$; females: $F_{2,23} = 70.610$, $p < 0.001$).

6.3.3.4 PFHxS

In a test of within-subjects effects, there was a significant main effect of time (Wilks' Lambda = 0.255, $F_{1,22} = 64.335$, $p < 0.001$) on serum levels of PFHxS. There was also a time x sex interaction (Wilks' Lambda = 0.538, $F_{1,22} = 18.905$, $p < 0.001$) (Appendix F). However, the following interactions were not statistically significant: (i) time x sex x dose (Wilks' Lambda = 0.989, $F_{1,22} = 0.249$, $p = 0.623$) and (ii) time x dose (Wilks' Lambda = 0.992, $F_{1,22} = 0.176$, $p = 0.679$).

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In separate tests of within-subjects effects, there was a main effect of time on serum levels of PFHxS (males: Wilks' Lambda = 0.137, $F_{1,11} = 69.487$, $p < 0.001$; females: Wilks' Lambda = 0.595, $F_{1,11} = 7.502$, $p = 0.019$) (Appendix F). In addition, there was no significant time x dose interaction (males: Wilks' Lambda = 0.966, $F_{1,11} = 0.383$, $p = 0.548$; females: Wilks' Lambda = 1.000, $F_{1,11} = 0.004$, $p = 0.954$). In separate tests of between-subjects effects, serum levels of PFHxS were affected by dose in males (day 21: $F_{1,11} = 34.111$, $p < 0.001$; day 28: $F_{1,11} = 10.773$, $p = 0.007$) and were unaffected by dose in females (day 21: $F_{1,11} = 1.321$, $p = 0.275$; day 28: $F_{1,11} = 0.497$, $p = 0.495$). Data from 3M (day 28) exhibited the same trend (males: $F_{1,11} = 13.477$, $p = 0.004$; females: $F_{1,10} = 0.780$, $p = 0.398$).

6.3.4 Necropsy and Organ Weight

6.3.4.1 6:2 FTS

No 6:2 FTS-related gross observations or organ weight effects were noted in either sex during necropsy ($p > 0.05$) (Appendix G).

6.3.4.2 PFNA

No PFNA-related gross observations were noted in either sex during necropsy. However, exposure to PFNA had a significant effect on liver and kidney weights of males (liver: $F_{3,30} = 10.14$, $p < 0.001$; kidney: $F_{3,29} = 60.70$, $p < 0.001$) compared to controls (Appendix G). Exposure to PFNA had a significant effect on liver and ovarian weights of females (liver: $F_{3,30} = 24.61$, $p < 0.001$; ovaries: $F_{3,29} = 3.36$, $p = 0.032$) compared to controls. All other results in both sexes were not statistically significant ($p > 0.05$).

6.3.4.3 PFHxS

No PFHxS-related gross observations or organ weight effects were noted in either sex during necropsy ($p > 0.05$) (Appendix G).

6.3.5 Sperm Analysis

Total sperm count ranged from 4.5 to 91.25 million and did not differ between 6:2 FTS-treated and control groups (Appendix H). Total sperm count ranged from 0.6 to 70.3 million and did not differ between PFNA-treated and control groups. Total sperm count ranged from 2.7 to 84.2 million and did not differ between PFHxS-treated and control groups.

6.4 Summary of Results

Table 3 summarizes the results from Experiments 1-3.

Table 3. Summary of Results from 28-day Experiments with PFAS

Chemical	Type of PFAS	Exposure Type	Body weight	Organ weight	Serum	
					Sex differences	At two time points
PFOA	PFCA	drinking water	unaffected	unaffected	↑ [PFOA] in F than M	21 = 28
PFNA	PFCA	oral gavage	unaffected	↑ liver ↑ kidney (M) ↓ ovarian (F)	↑ [PFNA] in F than M	21 = 28
PFBS	PFSA	drinking water	unaffected	unaffected	no	21 = 28
PFHxS	PFSA	drinking water	unaffected	unaffected	no	21 = 28
PFHxS	PFSA	oral gavage	unaffected	unaffected	↑ [PFHxS] in M than F	↓ between 21 and 28
PFOS	PFSA	oral gavage	unaffected	↑ liver (M) ↑ thymus (M) ↓ uterine (F)	no	↑ between 14 and 28
6:2 FTS	Polyfluorinated	oral gavage	unaffected	unaffected	no	↓ between 21 and 28

Note: Males (M), females (F).

7 DISCUSSION

In the present study, male and female white-footed mice were exposed to six PFAS via drinking water and/or oral gavage. These chemicals were selected to include different chain lengths (C4-C9), two functional groups (*i.e.*, carboxylic and sulfonic acid), and one polyfluorinated substance (as there is a paucity of research on polyfluorinated substances).

In the first experiment, animals were exposed to PFOA, PFHxS, and PFBS via drinking water. Subsequently, compounds were administered via oral gavage based on a lack of effects observed on lymphoid organ and body weights at doses that were reported to have caused effects in C57BL/6 mice (Dewitt et al. 2008). This was perhaps due to the possibility that the compounds may have partitioned at the top of the water column instead of remaining homogeneously mixed throughout the dosing solutions, which inadvertently altered exposure concentration. One chemical, PFHxS, was delivered via both drinking water and oral gavage as a direct comparison. Concentration verification of representative samples did not support the hypothesis that PFAS partitioned at the top of the water. Instead, data revealed that

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homogenous, accurate solutions at the two lower doses (37.5 and 75 mg/mL) and lower-than-anticipated concentrations at the higher dose (target 150 mg/mL), was likely due to the solubility limit of each chemical.

In general, within the limit of the study designs described herein, similar serum levels at two time points depended on (i) the type of PFAS (*i.e.*, PFSA, PFCAs, or polyfluorinated), (ii) continuous vs. bolus dosing, (iii) dose, and (iv) sometimes sex (Table 3). Specifically, serum concentrations were comparable in males and females exposed to PFHxS and PFBS via drinking water and were stable between days 21 and 28 of exposure.

Additionally, serum concentrations were comparable in males and females exposed to 2.5 mg/kg-day PFOS via oral gavage, though they increased between days 14 and 28 of exposure. This increase is likely due to the low dose of 2.5 mg/kg-day PFOS, given that saturation occurs more slowly at lower doses. Serum concentrations beyond day 0 were not measured in animals exposed to 20 and 40 mg/kg-day PFOS due to overt toxicity and mortality within 14 days. This was likely due to the cumulative dose reaching the oral LD₅₀ threshold (~250 mg/kg) (OECD 1984).

Finally, serum concentrations were comparable in males and females exposed to 6:2 FTS via oral gavage, which suggests either upregulation of clearance mechanisms or biotransformation, since concentrations drastically decreased between day 21 and 28 of exposure. Non-targeted serum analysis via LC-QTOF-MS yielded no likely metabolites (Wang et al. 2011). Furthermore, fluorotelomer-based compounds are known to degrade and form PFCAs (Butt et al. 2014; Zhang et al. 2016; Hoke et al. 2015). Additionally, 6:2 FTS is itself a metabolite of other fluorotelomer thioether amido sulfonates (Harding-Marjanovic et al. 2015). Finally, *in vitro* studies indicate that 6:2 FTS may not be readily metabolized (ECHA 2019; Hoke et al. 2015). Taken together, the decrease in 6:2 FTS serum levels between days 21 and 28 of exposure in this study are likely due to upregulation of clearance rather than biotransformation.

In contrast, female *P. leucopus* exposed to PFOA and PFNA had higher serum levels than males. This was unexpected, since PFOA was detected at similar levels in plasma, liver, and kidneys of male and female mice (Lou et al. 2009). Furthermore, PFOA and PFNA had higher renal clearance in female than male rats (Ohmori et al. 2003). Nevertheless, both males and females had similar serum concentrations at days 21 and 28 of exposure.

It is important to note that there was a marked difference in accumulation between animals exposed to PFHxS via drinking water and oral gavage. Specifically, while serum concentrations were comparable in males and females exposed to PFHxS via drinking water, males exposed via oral gavage had higher serum PFHxS concentrations than females exposed to the same doses and via the same method on days 21 and 28 of exposure. Results suggest that differences in absorption, distribution, metabolism and excretion from exposure types (*i.e.*, continuous vs. bolus) may play a role in accumulation and/or elimination of PFHxS.

In general, the elimination half-life of PFHxS is approximately 1 month in mice and 4 months in monkeys depending on sex (Sundstrom et al. 2012). However, in rats exposed to PFHxS, the differences in elimination half-life are dramatic (*i.e.*, 2 days in females and 29 days in males).

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Therefore, the results obtained in this study are of particular interest following PFHxS administration via oral gavage where males demonstrated a drastic decrease in serum concentrations between days 21 and 28 of exposure. Analyzing serum data from both sexes at 7 and 14 days of exposure could elucidate if a similar pattern of rapid clearance occurred in females prior to day 21 of exposure to PFHxS. Altogether, the data support that accumulation of PFAS is complex and illustrates the importance of conducting toxicity testing with PFAS in both sexes.

Given that the goal of this project was to compare serum levels over time and not serum-elimination half-lives, it is somewhat difficult to compare the results in this report to what is widely discussed in the literature. Furthermore, merely generalizing serum-elimination half-lives is challenging. For example, the serum elimination half-life of PFOA is (i) 22 days in male mice vs. 16 days in female mice, (ii) 5.63 days in male rats vs. 0.08 days in female rats, and (iii) 21 days in male monkeys vs. 33 days in female monkeys (Butenhoff et al. 2004; Ohmori et al. 2003; Lou et al. 2009). However, given that male and female *P. leucopus* received the same doses, similar accumulation could be a surrogate for similar elimination. As such, the results obtained during this project with PFOA, PFHxS (via drinking water), and PFOS are somewhat surprising, given the variable sex-dependent half-lives for these chemicals in mice, rats, and monkeys. In contrast, studies with rats and monkeys exposed to PFBS suggest similar rates of elimination for males and females, including faster rates (4–4.5 h in rats, 83–93 h in monkeys), especially when compared to other PFAS (Olsen et al. 2009; Lau et al. 2020).

Consistent with work in experimental animals, liver weights were increased in animals exposed to PFOS (8C) and PFNA (9C) compared to controls. Interestingly, this finding was not observed with PFOA at the doses tested, which is inconsistent with previous studies in mice, rats, and monkeys (Butenhoff et al. 2002; Das et al. 2017; Griffith and Long 1980; Kennedy 1987). Also consistent with work in experimental animals, PFHxS (6C) and PFBS (4) appear to be less toxic than PFOS in *P. leucopus*, based on a lack of change in liver weights of exposed animals compared to controls (NTP 2019; Chang et al. 2018; Butenhoff et al. 2009).

8 CONCLUSIONS

In general, similar serum levels at two time points depended on (i) the type of PFAS (*i.e.*, perfluoroalkyl sulfonic acids (PFSA), perfluoroalkyl carboxylic acids (PFCA), or polyfluorinated), (ii) continuous vs. bolus dosing, (iii) dose, and (iv) sometimes sex. Exceptions to this generalization include PFOA and PFNA where females had higher serum levels than males and 6:2 FTS and PFHxS, where serum levels drastically decreased between days 21 and 28 of exposure, likely due to upregulation of clearance. As such, future work involving the pharmacokinetics of these four chemicals in *P. leucopus* would be valuable.

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9 POINT OF CONTACT

Questions pertaining to this report should be referred to Allison Narizzano at DSN 584-3980, commercial 410-436-3980, or by e-mail: usarmy.apg.medcom-aphc.mbx.tox-info@mail.mil.

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Appendix A

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Appendix B

Quality Assurance Statement

FOR: Toxicology Study No. S.0043781.18. Protocol No. 67-17-04-01; Comparative Serum Pharmacokinetics of Per- and Polyfluoroalkyl Substances (PFAS) in White-Footed Mice (*Peromyscus leucopus*), October 2017-October 2018 the following critical phases were inspected/audited Quality Systems and Regulatory Compliance Office (QSARC):

Study Specific Critical Phase Inspected/Audited	Date Inspected/Audited:	Date Reported to Management/SD:
Study Protocol Good Laboratory Practice Standards and Animal Care Review	03/07/2017	03/07/2017
Test System – Facilities, Husbandry, Feed and Water Supply and Veterinary Care	10/06/2017	10/16/2017
Test System – Receipt, Identification and Enrichment Procedures	10/06/2017	10/16/2017
Maintenance and Calibration of Equipment and Good Documentation Practice	10/06/2017	10/16/2017
Test Article – Dose Selection and Administration of the Test Substance via Drinking Water	10/26/2017	11/03/2017
Test Article – Facilities, Control, Handling, Mixing and Transfer to Administration Containers	10/26/2017	11/03/2017
Test System – Identification, Body Weight and Food/Water Consumption and Post Procedure Observations	10/26/2017	11/06/2017
Test System – Bio-sample Collection: Facial/Submandibular Venipuncture Procedures	10/26/2017	11/06/2017
Compliance with Study Protocol Modification Procedures	10/26/2017	11/06/2017
Necropsy Study Personnel Qualifications, Training Records Review and Good Documentation Procedures	11/01/2017	11/21/2017
Male Mice Necropsies – Anesthesia/Analgesia/Tranquilization and Blood Sample Collection Procedures	11/01/2017	11/09/2017
Male Mice Necropsies – Euthanasia and Gross Necropsy, Organ Weight and Tissue Prep Procedures	11/01/2017	11/09/2017
Female Mice – Decapitation procedures, serum collection procedure, and sub-study endpoint criteria	11/09/2017	11/17/2017
Female Mice– Histopathology organ/tissue collection and tissue weighing procedures	11/09/2017	11/17/2017

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Study Specific Critical Phase Inspected/Audited	Date Inspected/Audited:	Date Reported to Management/SD:
Sub-Study Endpoint Criteria Compliance	04/18/2018	05/04/2018
Necropsy Procedures, Maintenance and Calibration of Equipment and Good Documentation Procedures	04/18/2018	05/04/2018
TOX Special Medical Waste Collection and Disposal Procedure Compliance	04/18/2018	05/04/2018
Final Study Animal In-Life Endpoint Criteria Compliance	07/20/2018	07/25/2018
Study Raw Data Good Laboratory Practice Standard Review	09/11/2019	09/16/2019
Final Study Good Laboratory Practice Standard Report Review	09/14/2020	09/16/2020

Note 1: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.

Note 2: This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and the procedures followed.

Note 3: In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.

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Quality Assurance Specialist, QSARC

Appendix C

Archives and Study Personnel

C-1 ARCHIVES

All raw data, documentation, records, protocol, and a copy of the final report generated as a result of this study will be archived in room 1026, building E-2100, APHC, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Records on animal receipt, diet, and facility environmental parameters will be archived by the Veterinary Support Office for a minimum of five (5) years following submission of the final report to the Sponsor.

Some ancillary records pertaining to this study, such as instrument maintenance logs, animal room observation logs, etc., will not be archived until those logbooks have been completed. Once complete they will be archived in room 1026, building E-2100, APHC. Wet tissues, histology slides, and paraffin blocks are stored in building E-5158.

C-2 PERSONNEL

Management: Mark Johnson, Ph.D., D.A.B.T., Director, Toxicology Directorate; Michael J. Quinn, Ph.D., Chief, Health Effects Division (HEF); Mr. Arthur J. O'Neill, Chief, Toxicity Evaluation Division (TEV).

Study Director: Allison M. Narizzano, Biologist, TEV.

Quality Assurance: Michael P. Kefauver, Quality Assurance Specialist, Quality System and Regulatory Compliance.

Veterinary Support and Animal Care: Michael R. Bonhage, DVM, COL, VC; Rebecca Kilby, Animal Health Technician.

In-Life Support: Taryn K. Brown, Biological Science Technician, TEV; Theresa L. Hanna, Biological Science Technician, TEV; Jarod M. Hanson, Executive Officer, TOX; Allison M. Narizzano, Biologist, TEV; Caroline L. Procell, ORISE Fellow, HEF; Michael J. Quinn, Biologist, HEF; Thomas E. Sussan, Biologist, HEF; Mark R. Way, Biologist, TEV.

Necropsy: Theresa L. Hanna, Biological Science Technician, TEV; Keith A. Koistinen, Toxicologic Pathologist, TOX-PATH; Emily May Lent, Toxicologist, TEV; Allison M. Narizzano, Biologist, TEV; Shannon Y. Pilcher, ORISE Fellow, TOX-PATH; Caroline L. Procell, ORISE Fellow, HEF; Alicia A. Shiflett, Lab Manager, TOX-PATH; Mark R. Way, Biologist, TEV.

Archivist: Lee C.B. Crouse, Biologist, TEV.

Appendix D

Analytical Chemistry – Dosing Solutions

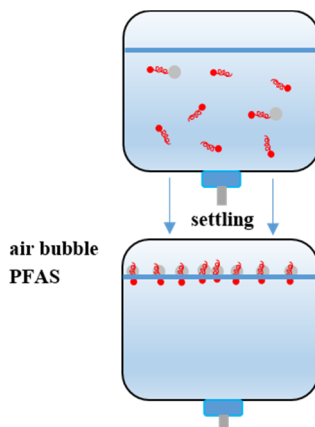
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Table D-1. Mean Analytical Concentrations of PFOA, PFHxS, and PFBS in Water

	Conc. (mg/L)	Measured (mg/L) Day 14	Accuracy (%) Day 14	Measured (mg/L) Day 28	Accuracy (%) Day 28	Average (mg/L)	Average Accuracy (%)	Dose (mg/kg- day)
P F O A	0	2.08e ⁻⁶	--	3.05e ⁻⁴	--	1.5e ⁻⁴	--	0
	37.5	37.3	99.5	34.1	90.9	36	95.2	5
	75	72.4	96.5	67.7	90.3	70	93.4	10
	150	98.9	65.9	98.8	65.9	99	65.9	13
P F H x S	0	6.64e ⁻⁵	--	4.83e ⁻⁵	--	5.7e ⁻⁵	--	0
	37.5	34.1	90.9	33.8	90.1	34	90.5	5
	75	66.7	88.9	66.1	88.1	66	88.5	10
	150	94.8	63.2	102	68.0	98	65.6	13
P F B S	0	7.12e ⁻⁶	--	9.56e ⁻⁵	--	5.1e ⁻⁵	--	0
	37.5	30.5	81.3	33.3	88.8	32	85.1	5
	75	58.4	77.9	NS	NS	58	77.9	10
	150	91.0	60.7	89.5	59.7	90	60.2	12

Dosing solutions were prepared and shipped immediately to Eurofins Eaton Analytical for initial sample verification. Samples for stability verification were held at APHC for 4 days and sent to Eurofins. Day 14: Samples were held for 2 weeks prior to being extracted (controls) or diluted (treated) and analyzed. Day 28: Samples were held another 2 weeks prior to being extracted (controls) or diluted (treated) and analyzed. NS = no sample.

Figure D-1. Diagram of PFAS Separating out of Water and Partitioning at the Water-Air Interface



PFOA, PFHxS, and PFBS may be separating out of the water and partitioning at the water-air interface, represented by the solid line (adapted from (Meng et al. 2017)).

Table D-2. Mean Analytical Concentrations of PFOS in Water

	Conc. (mg/mL)	Measured (mg/mL) Day 14	Accuracy (%) Day 14	Measured (mg/mL) Day 28	Accuracy (%) Day 28	Average (mg/mL)	Average Accuracy (%)	Dose (mg/kg- day)
P F O S	0	<2.10e ⁻⁹	--	6.37e ⁻⁹	--	--	--	0
	0.25	0.23	91.2	0.14	57.2	0.2	74.2	2.5
	4	1.09	27.3	3.37	84.3	2.2	55.8	20
	10	2.45	24.5	5.76	57.6	4.1	41.1	40

Dosing solutions were prepared and shipped immediately to Eurofins Eaton Analytical for initial sample verification. Samples for stability verification were held at APHC for 2 weeks and sent to Eurofins. Day 14: Samples were held for 2 weeks prior to being extracted (controls) or diluted (treated) and analyzed. Day 28: Samples were held another 2 weeks prior to being extracted (controls) or diluted (treated) and analyzed.

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Table D-3. Mean Analytical Concentrations of 6:2 FTS in Water

	Conc. (mg/mL)	Measured (mg/mL) Day 9	Accuracy (%) Day 9	Measured (mg/mL) Day 29	Accuracy (%) Day 29	Average (mg/mL)	Average Accuracy (%)	Dose (mg/kg- day)
6 : 2 F T S	0	<LOQ	--	<LOQ	--	<LOQ	--	0
	0.25	0.18	72.0	0.18	72.0	0.18	72.0	2.5
	1	0.58	58.0	0.62	62.0	0.60	60.0	6
	2	1.30	65.0	1.20	60.0	1.25	62.5	12.5

Dosing solutions were prepared and shipped immediately to 3M. Samples were held for 1 week prior to being diluted and analyzed. Samples for stability verification were held at 3M for 3 weeks prior to being diluted and analyzed.

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Table D-4. Target Analysis of 6:2 FTS in Dosing Solution (2 mg/mL) and House Water

Compound	Molecular weight (kg/mol)	Group Name	Day 0 (ng/L)	Day 14 (ng/L)	House water
3:3 FTCA	241.01	X:3 FTCA	ND	ND	ND
5:3 FTCA	341.00	X:3 FTCA	ND	ND	ND
7:3 FTCA	441.00	X:3 FTCA	ND	ND	ND
6:2 FTCA	376.99	X:2 FTCA	179383	196647	ND
8:2 FTCA	476.98	X:2 FTCA	48559	43011	ND
10:2 FTCA	576.97	X:2 FTCA	ND	ND	ND
PFBA	212.98	PFCA	ND	ND	ND
PFBA MRM	212.98	PFCA	ND	ND	ND
PFPeA	262.98	PFCA	< MDL	ND	ND
PFHxA	312.97	PFCA	46791	34787	ND
PFHpA	362.97	PFCA	6932	6070	ND
PFOA	412.97	PFCA	ND	ND	2
PFNA	462.96	PFCA	20738	21066	52
PFDA	512.96	PFCA	ND	ND	2
PFUdA	562.96	PFCA	ND	ND	19
PFDoA	612.95	PFCA	ND	ND	1
PFTTrDA	662.95	PFCA	ND	ND	2
PFTeDA	712.95	PFCA	ND	ND	ND
PFHxDA	812.94	PFCA	ND	ND	ND
PFODA	912.93	PFCA	ND	ND	ND
FOSA	497.95	FASA	ND	ND	ND
MeFOSA	511.96	MeFASA	ND	ND	ND
EtFOSA	525.98	EtFASA	ND	ND	ND
FOSAA	555.95	FASAA	ND	ND	ND
MeFOSAA	569.97	MeFASAA	ND	ND	ND
EtFOSAA	583.98	EtFASAA	ND	ND	ND
4:2 FTS	326.97	X:2 FTS	6612000	6238500	ND
6:2 FTS	426.97	X:2 FTS	2000100	1953600	< MDL
8:2 FTS	526.96	X:2 FTS	5895600	4452900	ND
10:2 FTS	626.96	X:2 FTS	855300	415680	ND
6:2 UFTCA	356.98	X:2 UFTCA	2108.58	2067.81	ND
8:2 UFTCA	456.97	X:2 UFTCA	118498	123231.10	ND
10:2 UFTCA	556.97	X:2 UFTCA	ND	ND	ND
PFPPrS	248.95	PFAS	ND	ND	ND
PFBS	298.94	PFAS	< MDL	< MDL	< MDL
PFPeS	348.94	PFAS	ND	ND	ND
PFHxS	398.94	PFAS	< MDL	< MDL	< MDL
PFHpS	448.94	PFAS	ND	ND	ND
PFOS	498.93	PFAS	< MDL	< MDL	< MDL
PFNS	548.93	PFAS	ND	ND	ND
PFDS	598.92	PFAS	ND	ND	ND
PFDoS	698.92	PFAS	ND	ND	ND
PFEtCHxS	460.93	CHxS	ND	ND	ND
ADONA	376.97	ADONA	ND	ND	ND
Cl-PFOS	514.90	Cl-PFAS	ND	ND	ND
Cl-O-PFNS	530.90	Cl-O-PFAS	ND	ND	ND
Cl-O-PFUdS	630.89	Cl-O-PFAS	ND	ND	ND

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Dosing solution (2 mg/mL) was prepared and shipped immediately to CSM. Samples were held for eight weeks prior to being diluted at 600x (dosing solution) or 1x (house water) and analyzed. ND = not detected, < MDL = attained value was below the method detection limit

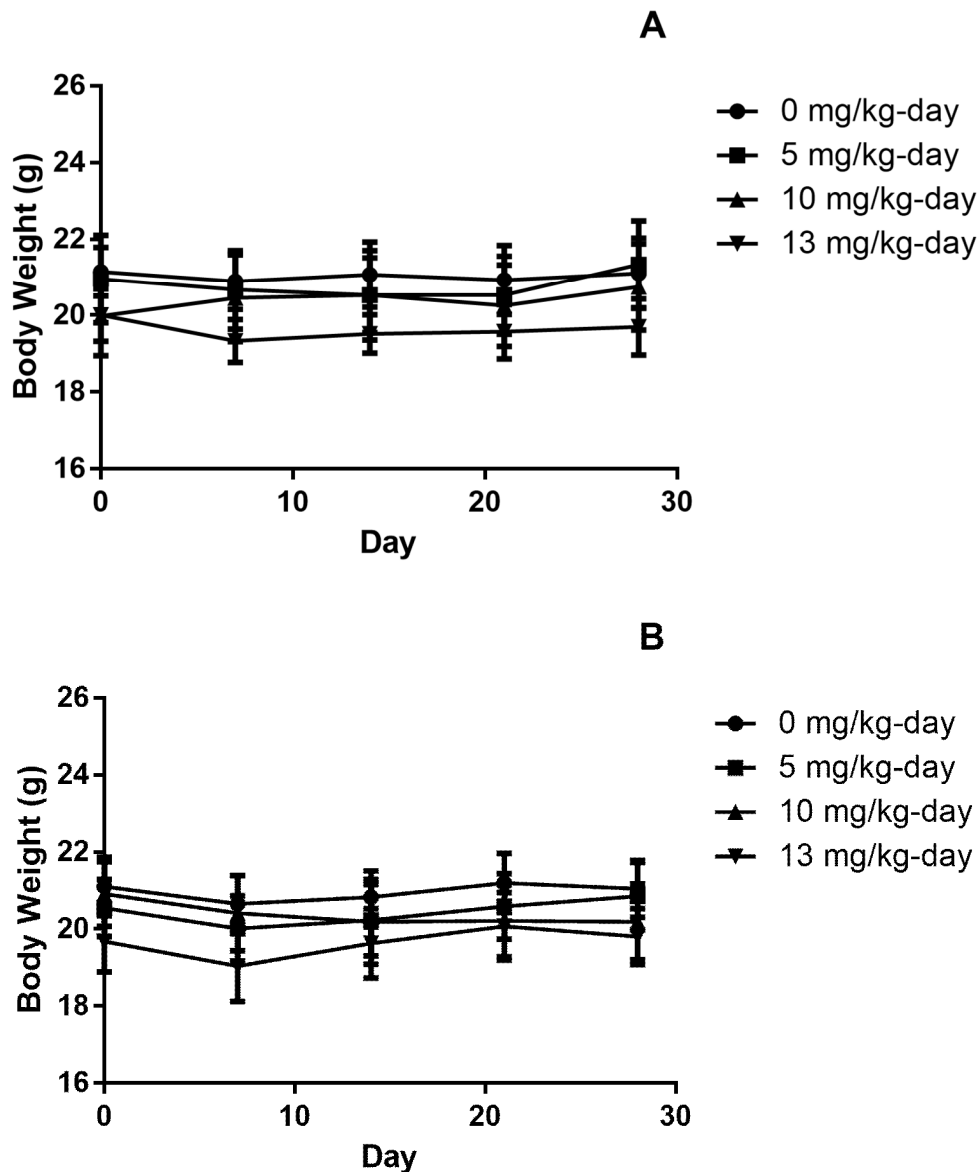
Table D-5. Mean Analytical Concentrations of PFNA and PFHxS in Water

	Conc. (mg/mL)	Measured (mg/mL) Day 14	Accuracy (%) Day 14	Measured (mg/mL) Day 28	Accuracy (%) Day 28	Average (mg/mL)	Average Accuracy (%)	Dose (mg/kg- day)
P F N A	0	5.05e ⁻⁹	--	2.05e ⁻⁸	--	1.3e ⁻⁸	--	0
	0.25	0.11	45.2	0.14	57.6	0.13	51.4	1
	1	0.52	52.3	0.66	65.6	0.59	59.0	6
	2	0.88	43.8	1.08	54.0	0.98	49.0	10
P F H x S	0	7.64e ⁻⁹	--	2.96e ⁻⁹	--	5.3e ⁻⁹	--	0
	1	0.69	69.3	0.75	75.2	0.72	72.3	10
	2	1.41	70.5	1.56	78.0	1.5	74.3	20

Dosing solutions were prepared and shipped immediately to Eurofins Eaton Analytical for initial sample verification. Samples were held for 2 weeks prior to being extracted (controls) or diluted (treated) and analyzed. Samples for stability verification were held at APHC for 2 weeks and sent to Eurofins. Samples were held another 2 weeks prior to being extracted (controls) or diluted (treated) and analyzed.

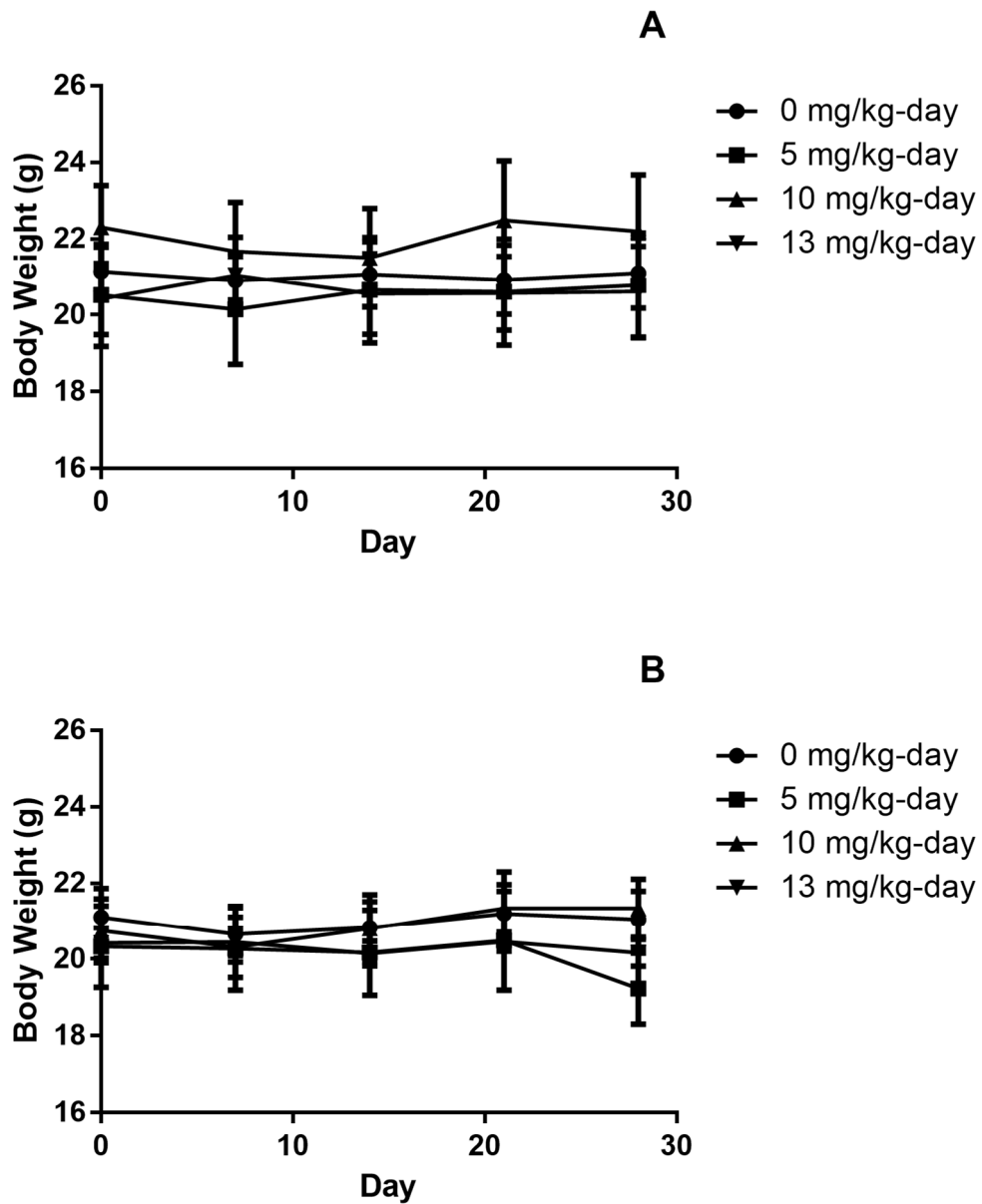
Appendix E
Body Weights

Figure E-1. Body Weights of White-Footed Mice Exposed to PFOA for 28 Days via Drinking Water



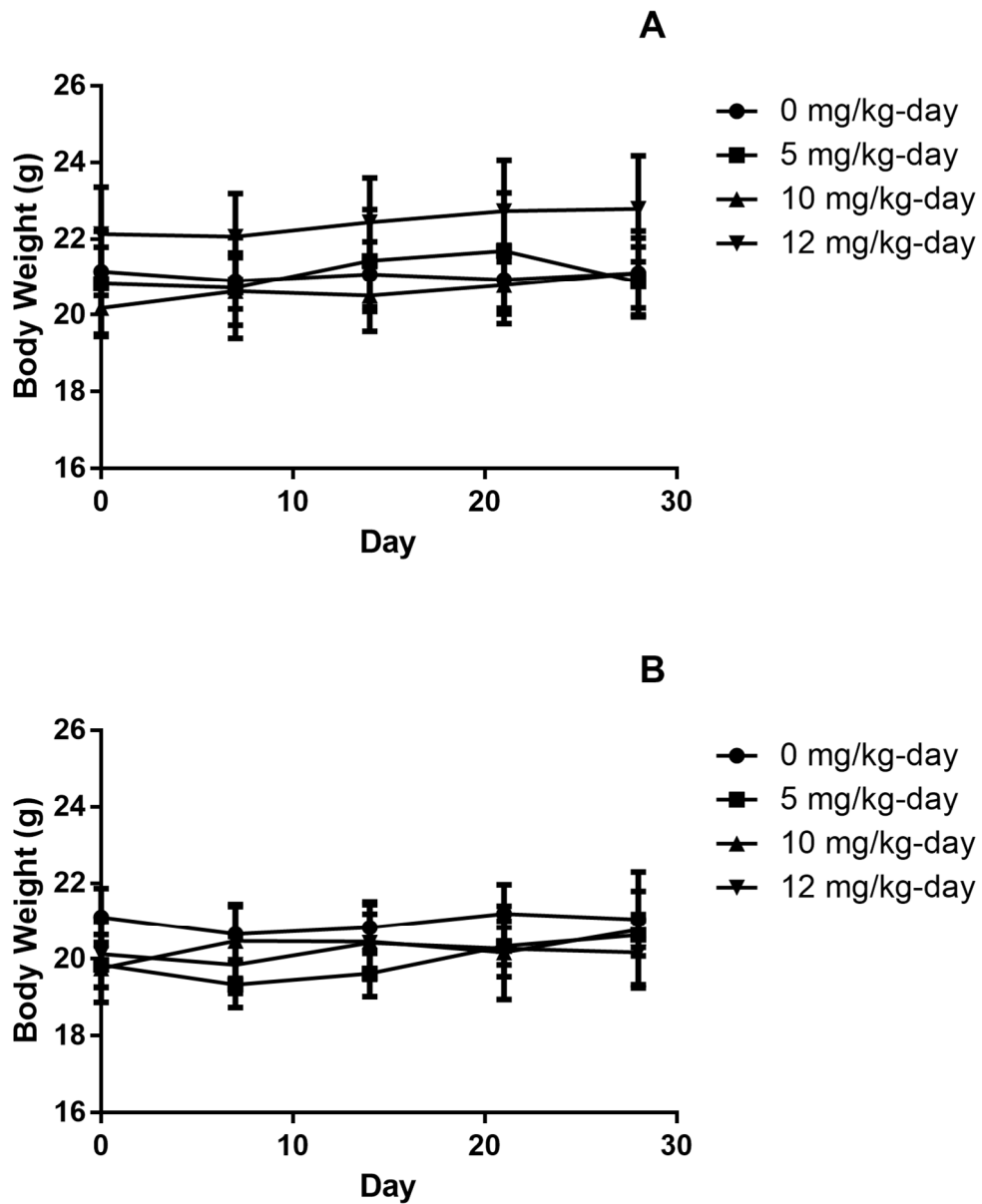
Mean ($\pm$ SEMs) weight (g) of male (A, $n=7-9/\text{dose}$) and female (B, $n=8-9/\text{dose}$) white-footed mice exposed to PFOA via drinking water. All results were not statistically significant ($p > 0.05$).

Figure E-2. Body Weights of White-Footed Mice Exposed to PFHxS for 28 Days via Drinking Water



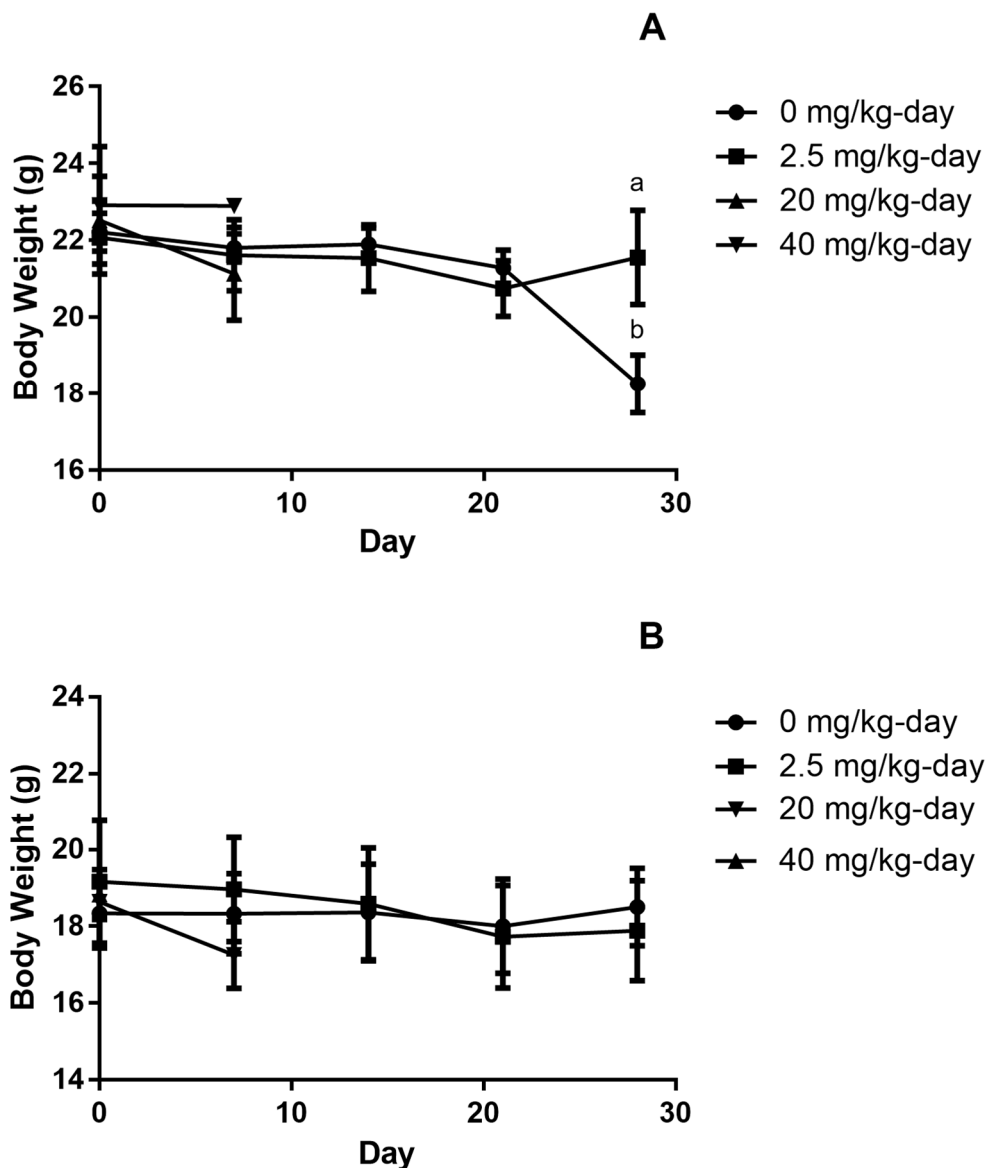
Mean ($\pm$ SEMs) weight (g) of male (A, $n=8-9/\text{dose}$) and female (B, $n=8-9/\text{dose}$) white-footed mice exposed to PFHxS via drinking water. All results were not statistically significant ($p > 0.05$).

Figure E-3. Body Weights of White-Footed Mice Exposed to PFBS for 28 Days via Drinking Water



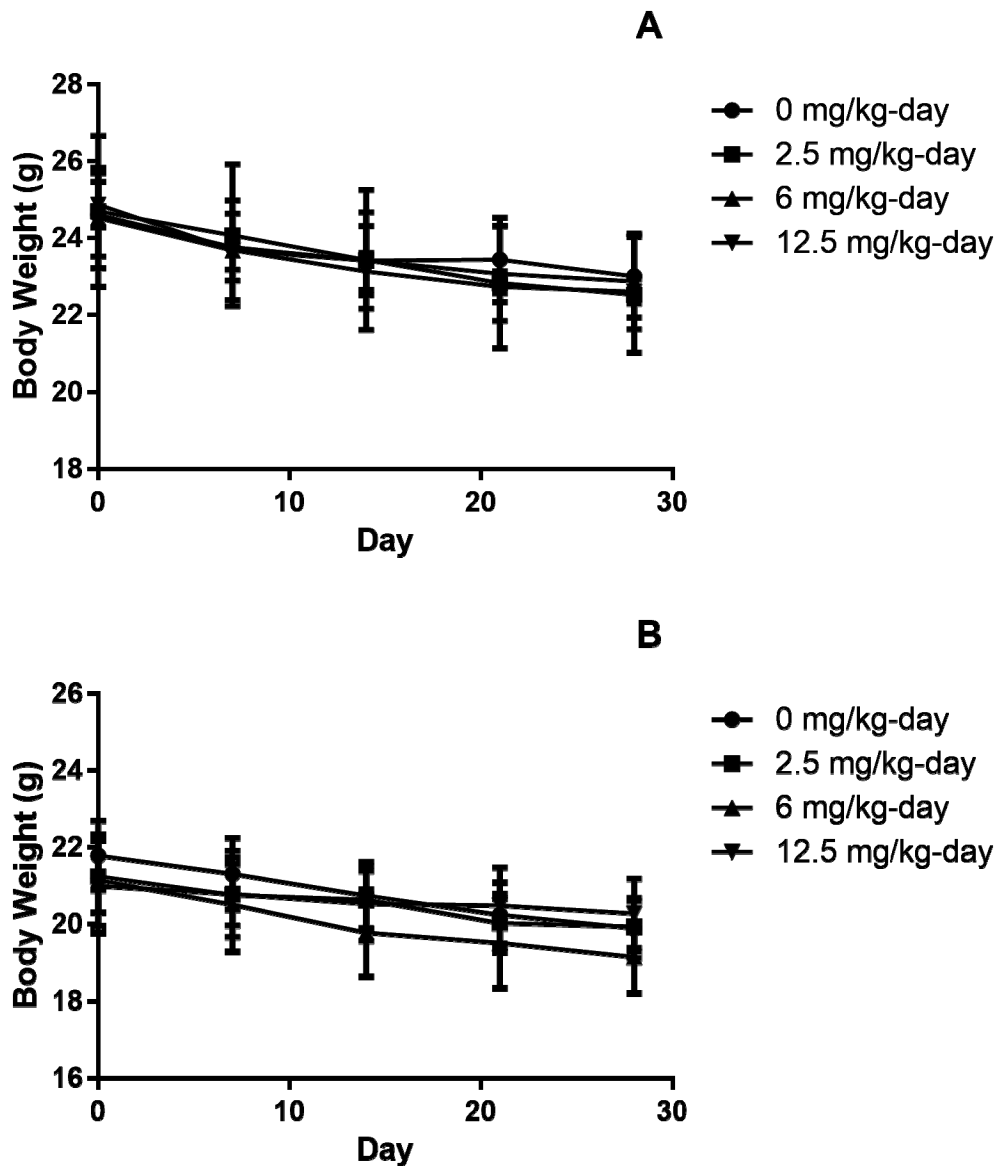
Mean ($\pm$ SEMs) weight (g) of male (A, $n=7-9/\text{dose}$) and female (B, $n=6-9/\text{dose}$) white-footed mice exposed to PFBS via drinking water. All results were not statistically significant ($p > 0.05$).

Figure E-4. Body Weights of White-Footed Mice Exposed to PFOS for 28 Days via Oral Gavage



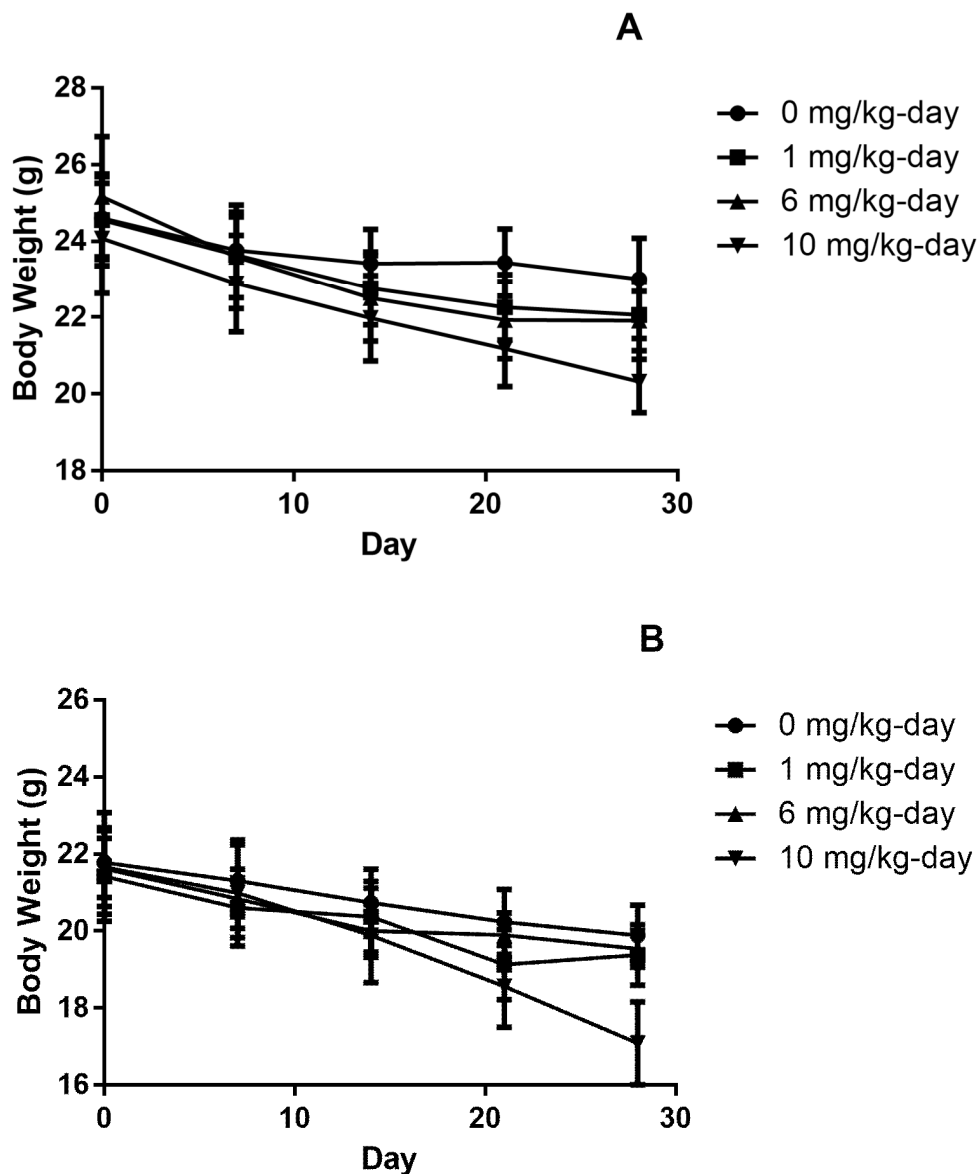
Mean ($\pm$ SEMs) weight (g) of male (A, $n=6-8$ /dose) and female (B, $n=6-9$ /dose) white-footed mice exposed to PFOS via oral gavage. Results in males (days 0-21) and females were not statistically significant ($p > 0.05$). PFOS had a significant effect on male body weights on day 28 ($p = 0.046$) with treated animals weighing significantly more than control animals.

Figure E-5. Body Weights of White-Footed Mice Exposed to 6:2 FTS for 28 Days via Oral Gavage



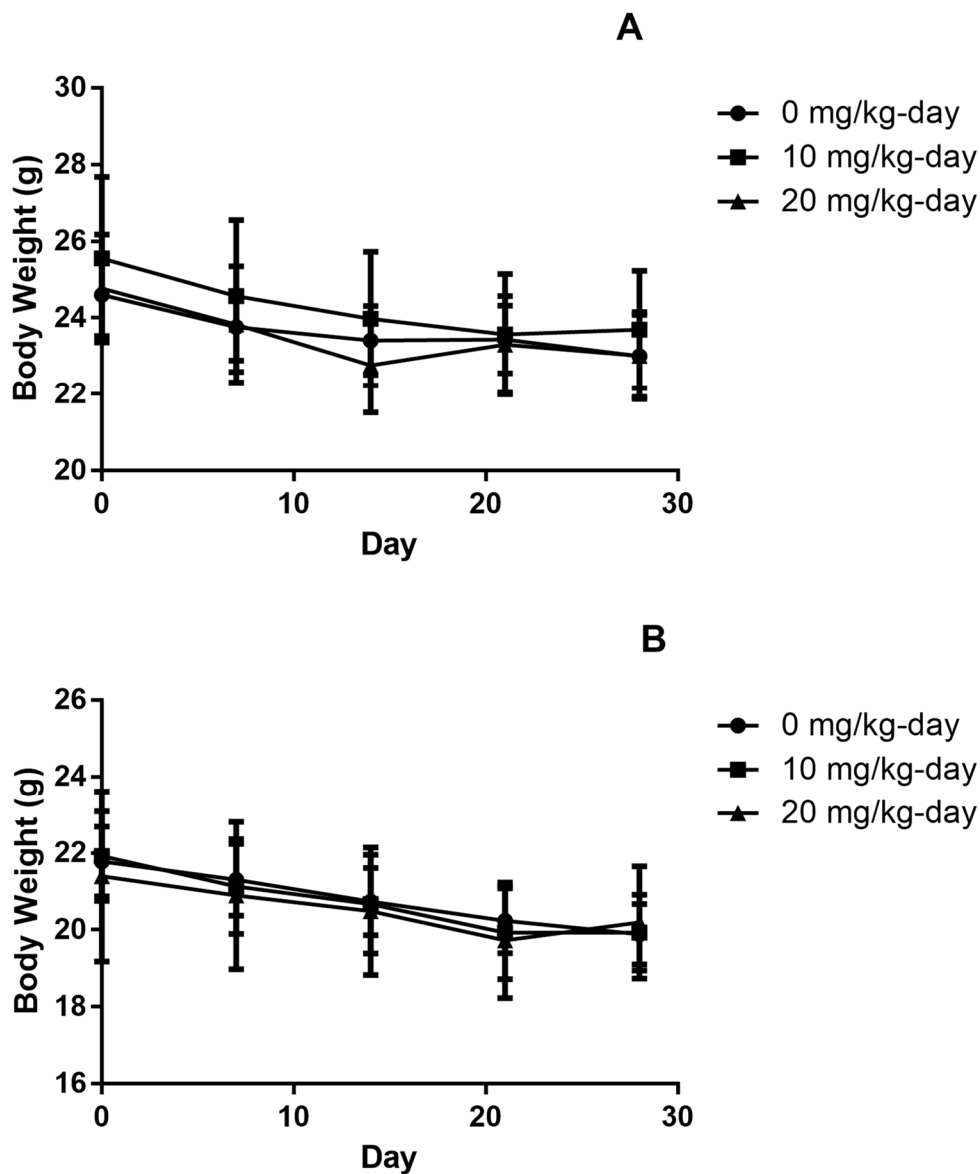
Mean ($\pm$ SEMs) weight (g) of male (A, $n=8-9/\text{dose}$) and female (B, $n=6-9/\text{dose}$) white-footed mice exposed to 6:2 FTS via oral gavage. All results were not statistically significant ($p > 0.05$).

Figure E-6. Body Weights of White-Footed Mice Exposed to PFNA for 28 Days via Oral Gavage



Mean ($\pm$ SEMs) weight (g) of male (A, $n=8-9/\text{dose}$) and female (B, $n=8-9/\text{dose}$) white-footed mice exposed to PFNA via oral gavage. All results were not statistically significant ($p > 0.05$).

Figure E-7. Body Weights of White-Footed Mice Exposed to PFHxS for 28 Days via Oral Gavage



Mean ($\pm$ SEMs) weight (g) of male (A, $n=6-9$ /dose) and female (B, $n=6-9$ /dose) white-footed mice exposed to PFHxS via oral gavage. All results were not statistically significant ($p > 0.05$).

Appendix F

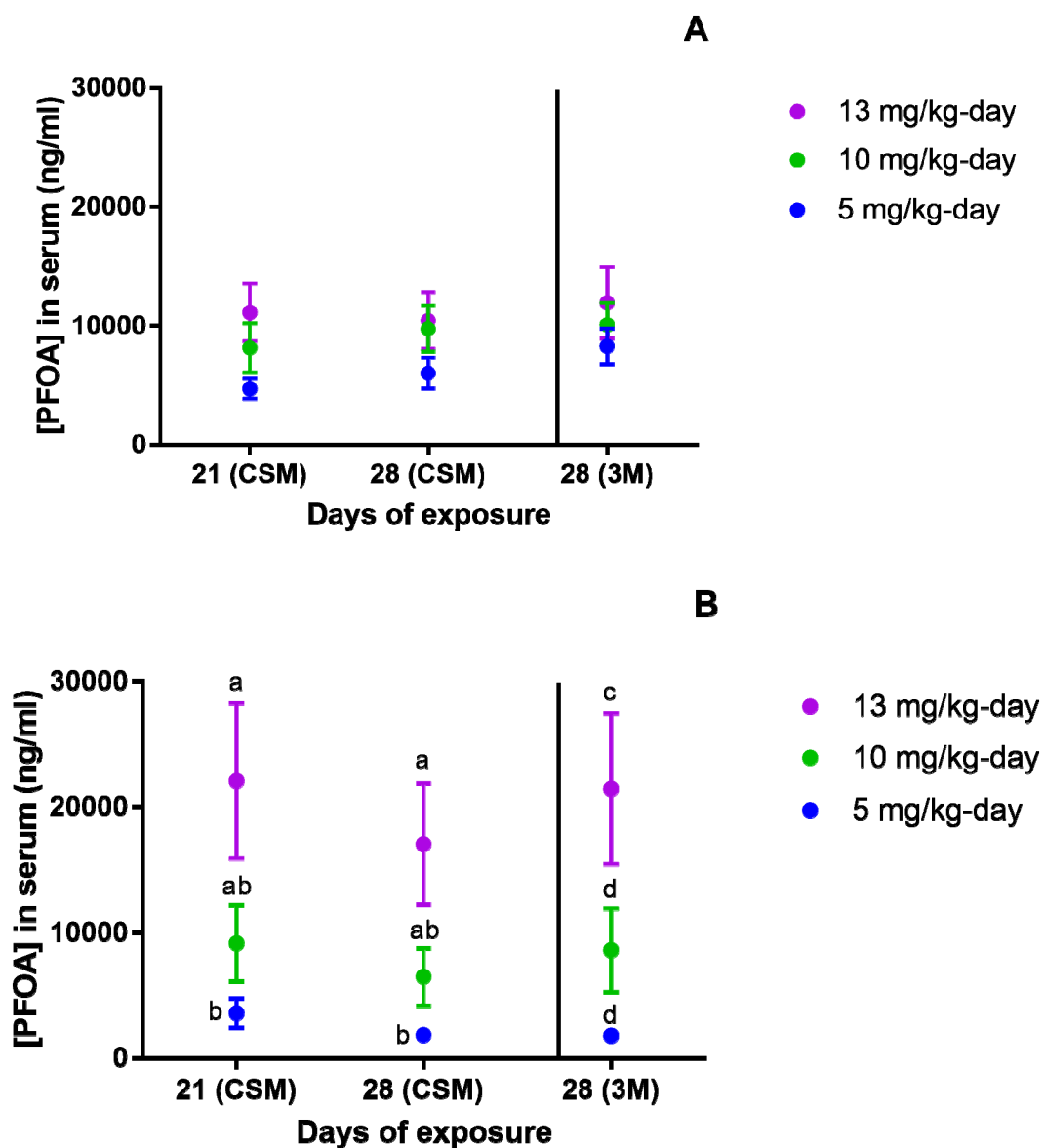
Analytical Chemistry – Serum

Table F-1. PFOA in Serum of White-Footed Mice Exposed to PFOA via Drinking Water

Conc (mg/L)	Dose (mg/kg-day)	Sex	PFOA in serum (ng/mL)			
			Colorado School of Mines			3M
			Day 0	Day 21	Day 28	Day 28*
0	0	Male	179 ± 179 (n=3)	97 ± 97 (n=3)	0 ± 0 (n=2) <LOQ (n=1)	<LOQ (n=3)
37.5	5	Male	106 ± 81 (n=7) <LOQ (n=2)	4677 ± 871 ^a (n=9)	6019 ± 1312 ^a (n=8) NS (n=1)	8266 ± 1494 ^a (n=7) NS (n=1) IS (n=1)
75	10	Male	148 ± 148 (n=7) <LOQ (n=1) NS (n=1)	8148 ± 2073 ^a (n=7) NS (n=2)	9740 ± 1936 ^a (n=7) NS (n=2)	10082 ± 1807 ^a (n=6) NS (n=2) IS (n=1)
99	13	Male	55 ± 36 (n=8) NS (n=1)	11113 ± 2424 ^a (n=8) NS (n=1)	10433 ± 2415 ^a (n=8) NS (n=1)	11906 ± 3010 ^a (n=8) NS (n=1)
0	0	Female	0 ± 0 (n=2) <LOQ (n=1)	0 ± 0 (n=2) <LOQ (n=1)	0 ± 0 (n=2) NS (n=1)	10 (n=1) <LOQ (n=1) NS (n=1)
37.5	5	Female	0 ± 0 (n=5) <LOQ (n=4)	3614 ± 1170 ^b (n=9)	1866 ± 349 ^b (n=9)	1812 ± 288 ^d (n=9)
75	10	Female	28 ± 28 (n=9)	9172 ± 3061 ^{ab} (n=7) NS (n=2)	6482 ± 2278 ^{ab} (n=8) NS (n=1)	8600 ± 3334 ^d (n=8) IS (n=1)
99	13	Female	0 ± 0 (n=5) <LOQ (n=4)	22069 ± 6182 ^a (n=8) NS (n=1)	17063 ± 4800 ^a (n=8) NS (n=1)	21441 ± 5998 ^c (n=8) NS (n=1)

Mean (± SEMs) PFOA in serum (ng/ml) of animals exposed to PFOA via drinking water. This experiment utilized a shared control group (n=9/sex) with PFHxS and PFBS; therefore, three control animals/sex were analyzed for PFOA. Data from day 0, 21, and 28 were generated at CSM and LOQ was 157.4 ng/mL. Data from day 28* were generated at 3M and LOQ was 1 ng/mL unless a sample was diluted, in which case the effective LOQ = working LOQ x dilution factor. Given the different methods, day 28 data from CSM and 3M are not comparable. Within each column, means that do not share a letter are different ($p < 0.05$) by Tukey HSD. Across timepoints (CSM day 21 and 28), means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$). NS = no sample, IS = insufficient sample.

Figure F-1. PFOA in Serum of White-Footed Mice Exposed to PFOA via Drinking Water



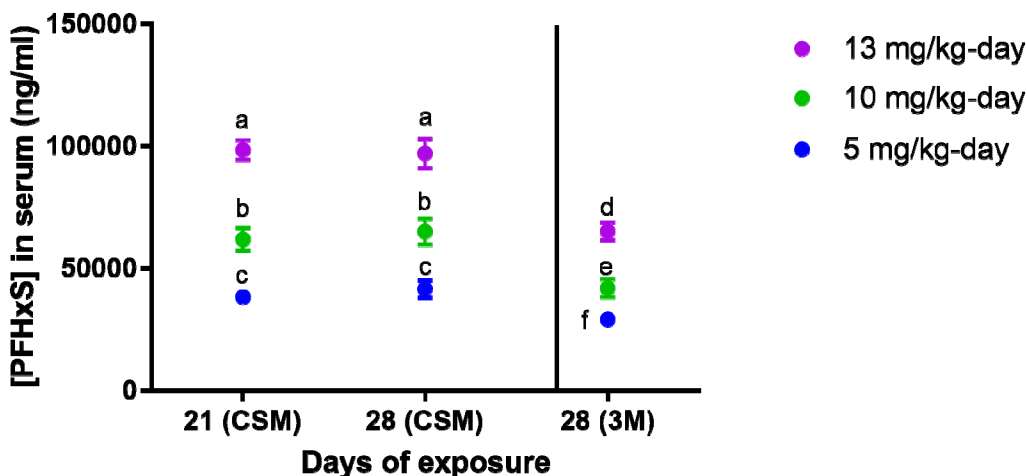
Mean ($\pm$ SEMs) [PFOA] in male (A, $n=2-9$) and female (B, $n=3-9$) white-footed mice exposed to PFOA via drinking water. Data from day 21 and 28 were generated at CSM and analyzed via repeated measures ANOVA to determine whether serum levels were similar at multiple time points. Data from day 28 were also generated at 3M, but were not compared to day 28 CSM data, due to differences in analytical methods. Means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$).

Table F-2. PFHxS in Serum of White-Footed Mice Exposed to PFHxS via Drinking Water

Conc (mg/L)	Dose (mg/kg-day)	Sex	PFHxS in serum (ng/mL)			
			Colorado School of Mines			3M
			Day 0	Day 21	Day 28	Day 28*
0	0	Both	0 ± 0 (n=5) <LOQ (n=1)	0 (n=3) <LOQ (n=3)	0 (n=2) <LOQ (n=4)	42 ± 19 (n=5) <LOQ (n=1)
37.5	5	Both	0 ± 0 (n=12) <LOQ (n=6)	38309 ± 2648 ^c (n=18)	41520 ± 3490 ^c (n=16) NS (n=2)	29173 ± 2168 ^f (n=15) NS (n=2) IS (n=1)
75	10	Both	0 ± 0 (n=12) <LOQ (n=6)	61961 ± 4701 ^b (n=15) NS (n=3)	65030 ± 5374 ^b (n=15) NS (n=3)	41847 ± 3700 ^e (n=15) NS (n=3)
98	13	Both	0 ± 0 (n=13) <LOQ (n=4) NS (n=1)	98495 ± 4085 ^a (n=17) NS (n=1)	97023 ± 6081 ^a (n=16) NS (n=2)	65131 ± 3737 ^d (n=16) NS (n=2)

Mean (± SEMs) PFHxS in serum (ng/ml) of animals exposed to PFHxS via drinking water. This experiment utilized a shared control group (n=9/sex) with PFOA and PFBS; therefore, three control animals/sex were analyzed for PFHxS. Data from day 0, 21, and 28 were generated at CSM and LOQ was 525 ng/mL. Data from day 28* were generated at 3M and LOQ was 1 ng/mL unless a sample was diluted, in which case the effective LOQ = working LOQ x dilution factor. Given the different methods, day 28 data from CSM and 3M are not comparable. Within each column, means that do not share a letter are different (p < 0.05) by Tukey HSD. Across timepoints (CSM day 21 and 28), means that do not share a letter are different (p < 0.05) by Tukey HSD. All other results were not statistically significant (p > 0.05). NS = no sample, IS = insufficient sample.

Figure F-2. PFHxS in Serum of White-Footed Mice Exposed to PFHxS via Drinking Water



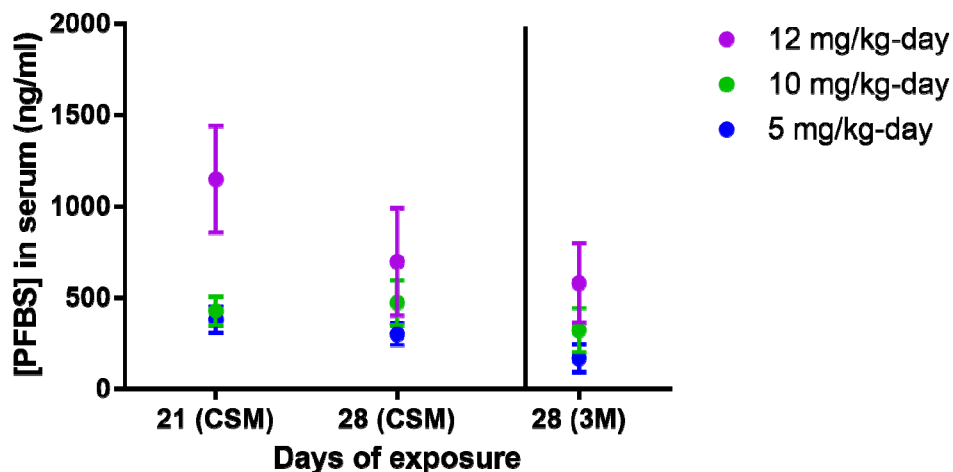
Mean ($\pm$ SEMs) [PFHxS] in serum of white-footed mice ($n=6-18/\text{dose}$) exposed to PFHxS via drinking water. Since there were no sex differences, data from male and female mice are presented together. Data from day 21 and 28 were generated at CSM and analyzed via repeated measures ANOVA to determine whether serum levels were similar at multiple time points. Data from day 28 were also generated at 3M, but were not compared to day 28 CSM data, due to differences in analytical methods. Means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$).

Table F-3. PFBS in Serum of White-Footed Mice Exposed to PFBS via Drinking Water

Conc (mg/L)	Dose (mg/kg-day)	Sex	PFBS in serum (ng/mL)			
			Colorado School of Mines			3M
			Day 0	Day 21	Day 28	Day 28*
0	0	Both	0 ± 0 (n=5) NS (n=1)	0 ± 0 (n=5) NS (n=1)	0 ± 0 (n=4) <LOQ (n=1) NS (n=1)	25 ± 3 (n=2) <LOQ (n=3) NS (n=1)
37.5	5	Both	0 ± 0 (n=15) <LOQ (n=2) NS (n=1)	514 ± 142 ^a (n=7) <LOQ (n=8) NS (n=3)	552 ± 552 ^a (n=2) <LOQ (n=13) NS (n=3)	169 ± 76 ^b (n=15) NS (n=3)
75	10	Both	0 ± 0 (n=14) NS (n=3)	537 ± 120 ^a (n=9) <LOQ (n=6) NS (n=2)	890 ± 309 ^a (n=5) <LOQ (n=10) NS (n=2)	322 ± 122 ^b (n=15) NS (n=2)
90	12	Both	0 ± 0 (n=16) NS (n=2)	1713 ± 396 ^a (n=11) <LOQ (n=7)	1085 ± 535 ^a (n=9) <LOQ (n=8) NS (n=1)	581 ± 218 ^b (n=17) NS (n=1)

Mean (± SEMs) PFBS in serum (ng/ml) of animals exposed to PFBS via drinking water. This experiment utilized a shared control group (n=9/sex) with PFOA and PFHxS; therefore, three control animals/sex were analyzed for PFBS. Data from day 0, 21, and 28 were generated at (CSM and LOQ was 262.5 ng/mL. Data from day 28* were generated at 3M and LOQ was 1 ng/mL unless a sample was diluted, in which case the effective LOQ = working LOQ x dilution factor. Given the different methods, day 28 data from CSM and 3M are not comparable. Within each column, means that do not share a letter are different (p < 0.05). Across timepoints (CSM day 21 and 28), means that do not share a letter are different (p < 0.05). All results were not statistically significant (p > 0.05). NS = no sample.

Figure F-3. PFBS in Serum of White-Footed Mice Exposed to PFBS via Drinking Water



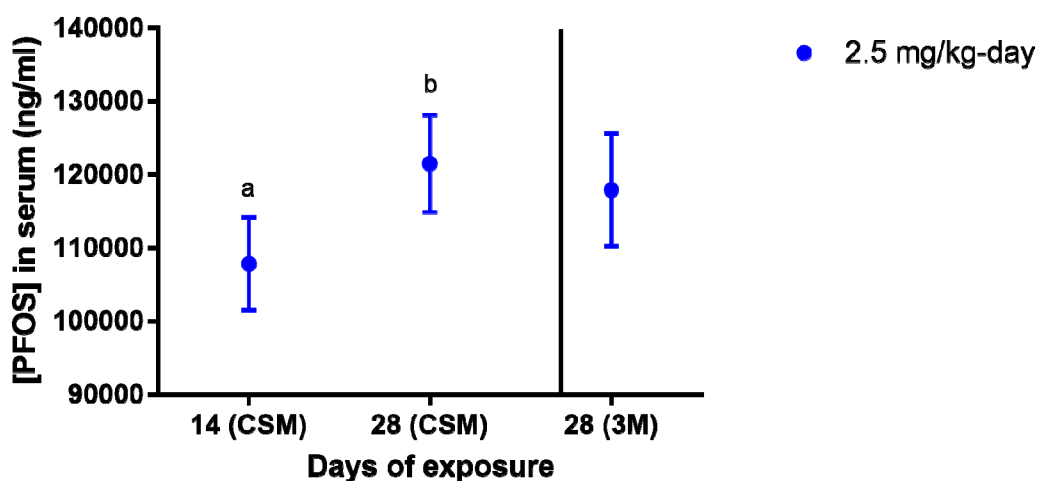
Mean ($\pm$ SEMs) [PFBS] in serum of white-footed mice ($n=5-18/\text{dose}$) exposed to PFBS via drinking water. Since there were no sex differences, data from male and female mice are presented together. Data from day 21 and 28 were generated at CSM and analyzed via repeated measures ANOVA to determine whether serum levels were similar at multiple time points. Data from day 28 were also generated at 3M, but were not compared to day 28 CSM data, due to differences in analytical methods. All results were not statistically significant ($p > 0.05$).

Table F-4. PFOS in Serum of White-Footed Mice Exposed to PFOS via Oral Gavage

Dose (mg/kg-day)	Sex	PFOS in serum (ng/mL)			
		Colorado School of Mines			3M
		Day 0	Day 14	Day 28	Day 28*
0	Both	181 ± 181 (n=10) <LOQ (n=3)	1759 ± 274 (n=10) <LOQ (n=3)	2660 ± 476 (n=10) <LOQ (n=3)	2003 ± 453 (n=10) <LOQ (n=3)
2.5	Both	202 ± 89 (n=12) <LOQ (n=4) NS (n=1)	107858 ± 6323 ^a (n=17)	121537 ± 6647 ^b (n=12) NS (n=5)	117950 ± 7659 (n=12) NS (n=5)

Mean (± SEMs) PFOS in serum (ng/ml) of animals exposed to PFOS via oral gavage. Data from day 0, 14, and 28 were generated at CSM and LOQ was 262.5 ng/mL. Data from day 28* were generated at 3M and LOQ was 1 ng/mL unless a sample was diluted, in which case the effective LOQ = working LOQ x dilution factor. Given the different methods, day 28 data from CSM and 3M are not comparable. Across timepoints (CSM day 14 and 28), means that do not share a letter are different ($p < 0.05$). NS = no sample.

Figure F-4. PFOS in Serum of White-Footed Mice Exposed to PFOS via Oral Gavage



Mean (± SEMs) [PFOS] in serum white-footed mice (n=11-17/dose) exposed to PFOS via oral gavage. Since there were no sex differences, data from male and female mice are presented together. Data from day 14 and 28 were generated at CSM and analyzed via repeated measures ANOVA to determine whether serum levels were similar at multiple time points. Data from day 28 were also generated at 3M, but were not compared to day 28 CSM data, due to

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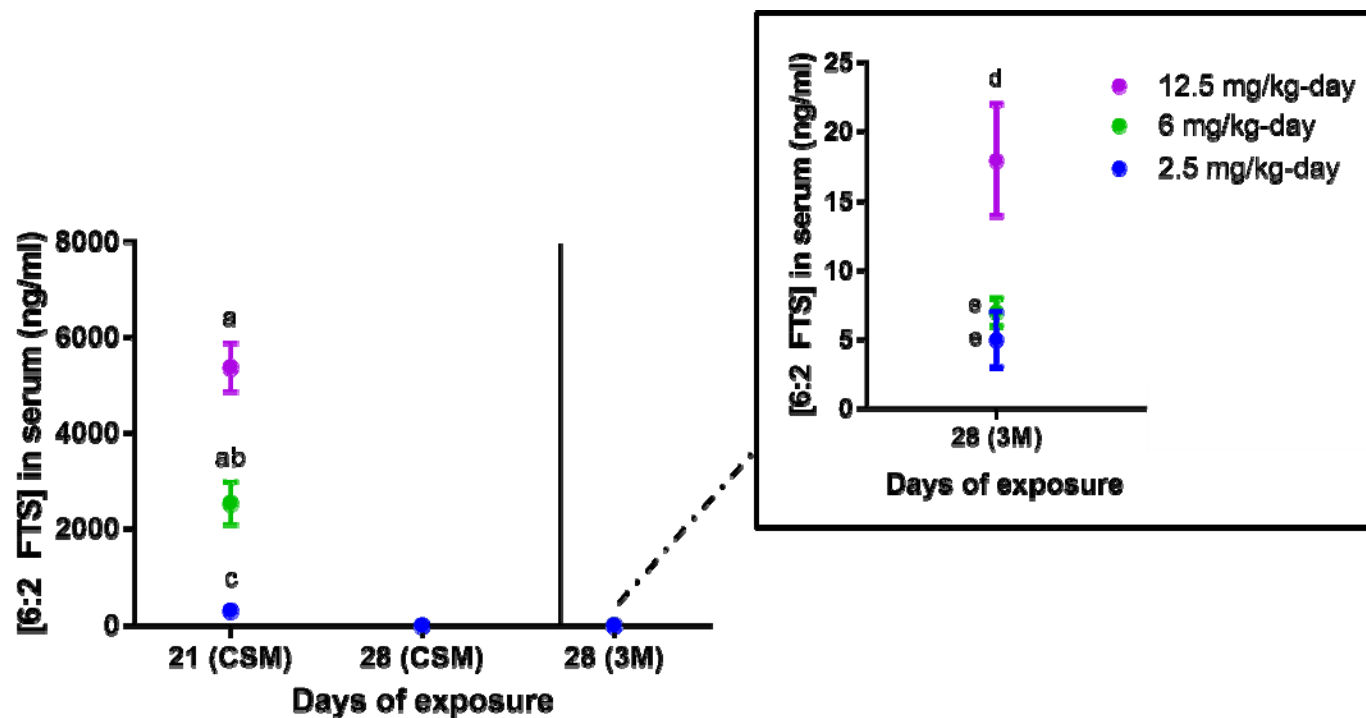
differences in analytical methods. Means that do not share a letter are different ($p < 0.05$) by Tukey HSD.

Table F-5. 6:2 FTS in Serum of White-Footed Mice Exposed to 6:2 FTS via Oral Gavage

Dose (mg/kg-day)	Sex	6:2 FTS in serum (ng/mL)			
		Colorado School of Mines			3M
		Day 0	Day 21	Day 28	Day 28*
0	Both	0 ± 0 (n=4) <LOQ (n=1) NS (n=1)	86 ± 86 (n=5) <LOQ (n=1)	60 ± 60 (n=6)	<LOQ (n=6)
2.5	Both	347 ± 246 (n=17)	340 ± 70 (n=10) ^c <LOQ (n=5) NS (n=2)	0 ± 0 (n=14) NS (n=3)	6 ± 3 (n=11) ^e <LOQ (n=3) NS (n=3)
6	Both	124 ± 94 (n=17) <LOQ (n=1)	2713 ± 446 ^{ab} (n=13) <LOQ (n=1) NS (n=4)	0 ± 0 (n=14) NS (n=4)	8 ± 1 (n=13) ^e <LOQ (n=2) NS (n=2) IS (n=1)
12.5	Both	309 ± 309 (n=14) <LOQ (n=3) NS (n=1)	5378 ± 505 ^a (n=16) NS (n=2)	0 ± 0 (n=16) NS (n=2)	18 ± 4 (n=17) ^d NS (n=1)

Mean (± SEMs) 6:2 FTS in serum (ng/ml) of animals exposed to 6:2 FTS via oral gavage. This experiment utilized a shared control group (n=9/sex) with PFNA and PFHxS; therefore, three control animals/sex were analyzed for 6:2 FTS. Data from day 0, 21, and 28 were generated at CSM and LOQ was 262.5 ng/mL. Data from day 28* were generated at 3M and LOQ was 1 ng/mL unless a sample was diluted, in which case the effective LOQ = working LOQ x dilution factor. Given the different methods, day 28 data from CSM and 3M are not comparable. Within each column, means that do not share a letter are different (p < 0.05) by Tukey HSD. Across timepoints (CSM day 21 and 28), means that do not share a letter are different (p < 0.05) by Tukey HSD. All other results were not statistically significant (p > 0.05). NS = no sample, IS = insufficient sample.

Figure F-5. 6:2 FTS in Serum of White-Footed Mice Exposed to 6:2 FTS via Oral Gavage



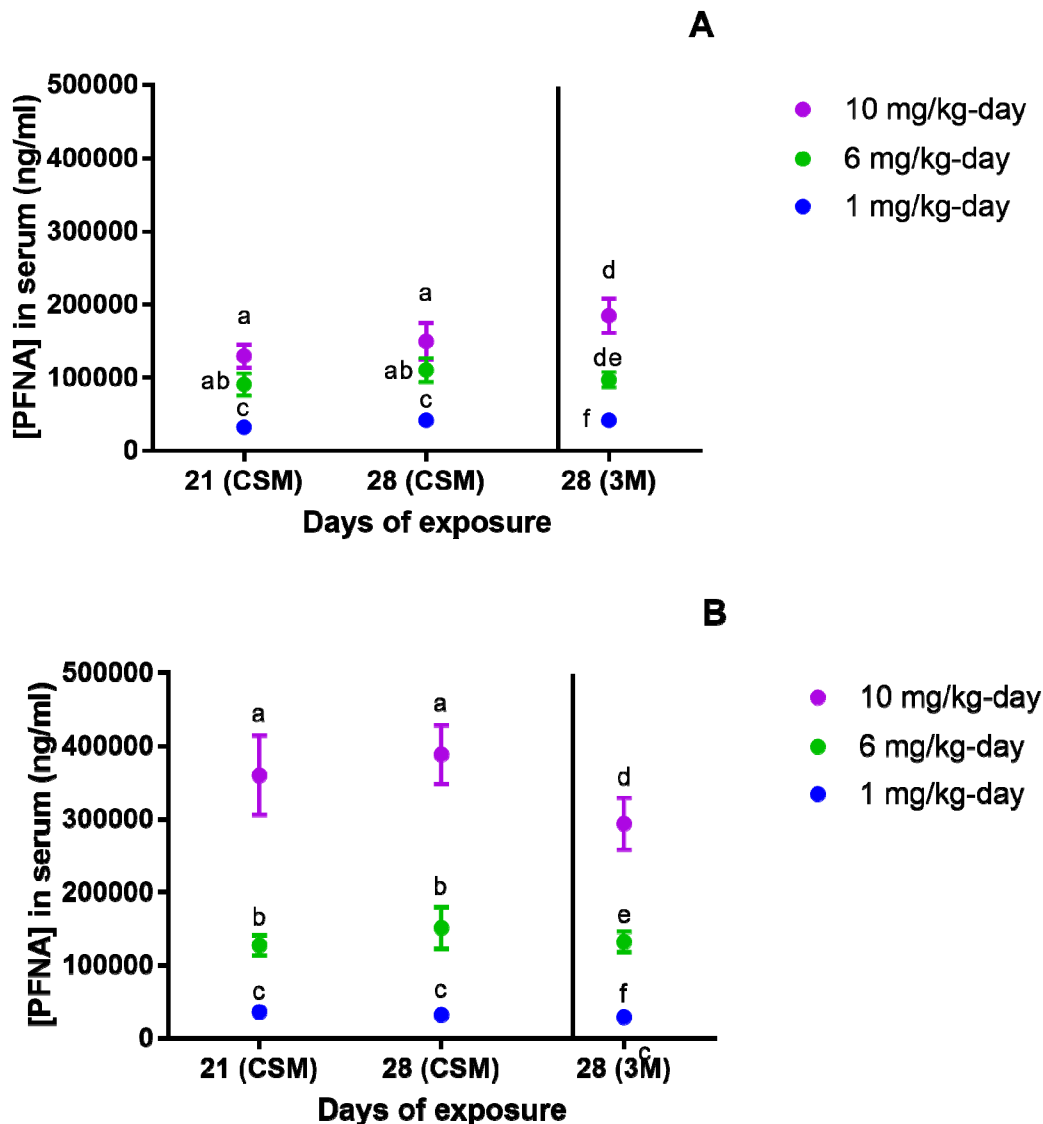
Mean ($\pm$ SEMs) [6:2 FTS] in serum of white-footed mice ($n=5-17/\text{dose}$) exposed to 6:2 FTS via oral gavage. Since there were no sex differences, data from male and female mice are presented together. Data from day 21 and 28 were generated at CSM and analyzed via repeated measures ANOVA to determine whether serum levels were similar at multiple time points. Data from day 28 were also generated at 3M, but were not compared to day 28 CSM data, due to differences in analytical methods. Means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$).

Table F-6. PFNA in Serum of White-Footed Mice Exposed to PFNA via Oral Gavage

Dose (mg/kg-day)	Sex	PFNA in serum (ng/mL)			
		Colorado School of Mines			3M
		Day 0	Day 21	Day 28	Day 28*
0	Male	<LOQ (n=3)	1138 (n=1) <LOQ (n=1) NS (n=1)	<LOQ (n=2) NS (n=1)	63 ± 16 (n=2)
1	Male	644 ± 61 (n=2) <LOQ (n=6) NS (n=1)	32448 ± 5916 ^c (n=8) NS (n=1)	41484 ± 7988 ^c (n=8) NS (n=1)	41409 ± 8605 ^f (n=8) NS (n=1)
6	Male	<LOQ (n=9)	90821 ± 15198 ^{ab} (n=9)	110122 ± 16516 ^{ab} (n=9)	97056 ± 10452 ^{de} (n=9)
10	Male	633 (n=1) <LOQ (n=8)	129196 ± 16040 ^a (n=9)	149380 ± 24836 ^a (n=9)	184389 ± 23262 ^d (n=9)
0	Female	<LOQ (n=3)	713 (n=1) <LOQ (n=1) NS (n=1)	<LOQ (n=2) NS (n=1)	141 (n=2) NS (n=1)
1	Female	1251 (n=1) <LOQ (n=8)	36024 ± 2874 ^c (n=8) NS (n=1)	31735 ± 4761 ^c (n=8) NS (n=1)	28788 ± 4292 ^f (n=8)
6	Female	2892 (n=1) <LOQ (n=8)	127310 ± 13733 ^b (n=9)	151012 ± 28476 ^b (n=9)	131772 ± 13813 ^e (n=9)
10	Female	782 (n=1) <LOQ (n=8)	359846 ± 54029 ^a (n=9)	387925 ± 40076 ^a (n=9)	293611 ± 35377 ^d (n=9)

Mean (± SEMs) PFNA in serum (ng/ml) of animals exposed to PFNA/sex were analyzed for PFOA. This experiment utilized a shared control group (N=9/sex) with 6:2 FTS and PFHxS; therefore, three control animals/sex were analyzed for PFNA. Data from day 0, 21, and 28 were generated at CSM and LOQ was 157.4 ng/mL. Data from day 28* were generated at 3M and LOQ was 1 ng/mL unless a sample was diluted, in which case the effective LOQ = working LOQ x dilution factor. Given the different methods, day 28 data from CSM and 3M are not comparable. Within each column, means that do not share a letter are different ($p < 0.05$) by Tukey HSD. Across timepoints (CSM day 21 and 28), means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$). NS = no sample.

Figure F-6. PFNA in Serum of White-Footed Mice Exposed to PFNA via Oral Gavage



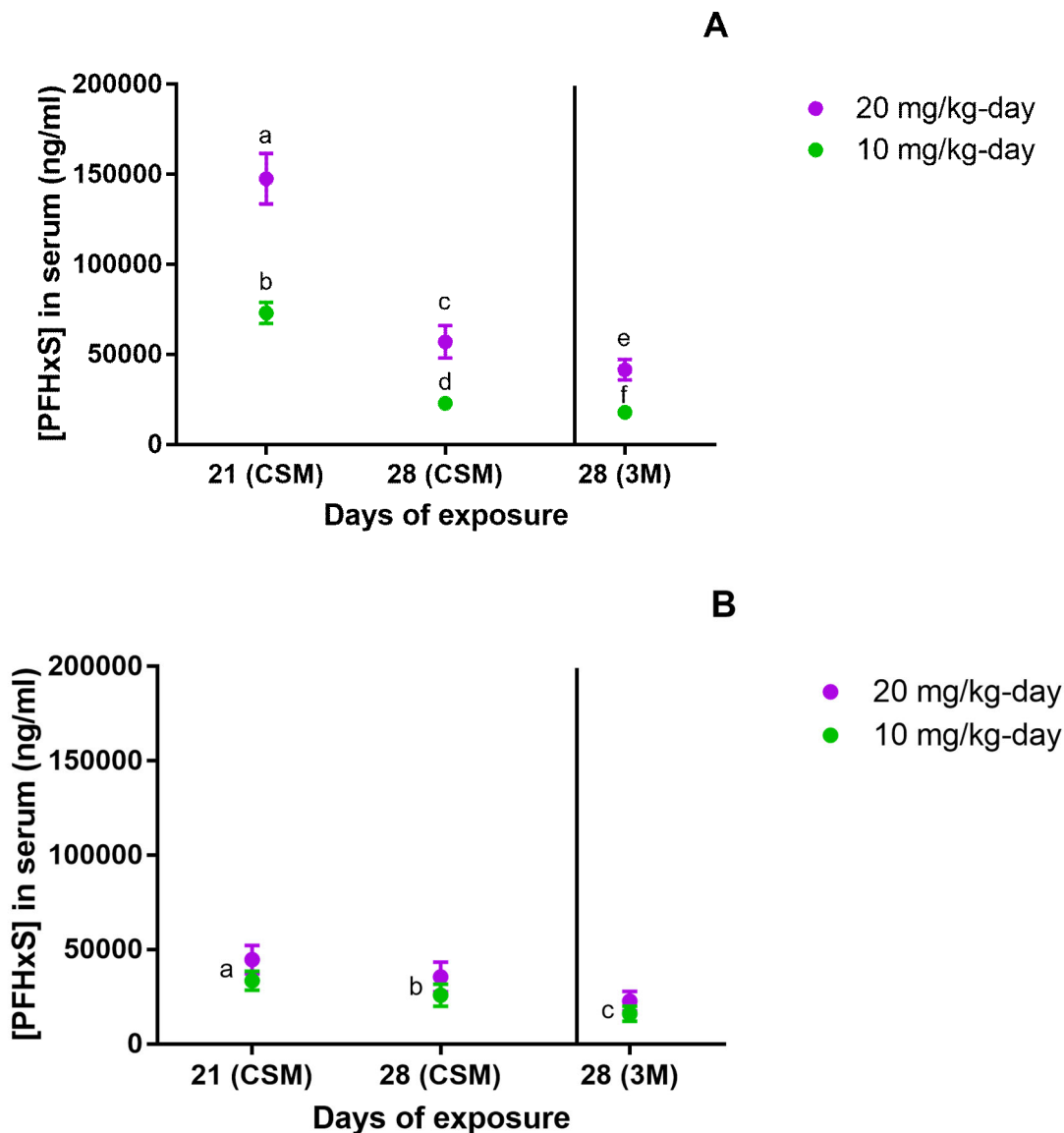
Mean ($\pm$ SEMs) [PFNA] in male (A, $n=3-9$) and female (B, $n=3-9$) white-footed mice exposed to PFNA via drinking water. Data from day 21 and 28 were generated at CSM and analyzed via repeated measures ANOVA to determine whether serum levels were similar at multiple time points. Data from day 28 were also generated at 3M, but were not compared to day 28 CSM data, due to differences in analytical methods. Means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$).

Table F-7. PFHxS in Serum of White-Footed Mice Exposed to PFHxS via Oral Gavage

Dose (mg/kg-day)	Sex	PFHxS in serum (ng/mL)			
		Colorado School of Mines			3M
		Day 0	Day 21	Day 28	Day 28*
0	Male	0 ± 0 (n=3)	0 ± 0 (n=3)	0 ± 0 (n=3)	7 ± 0 (n=2) <LOQ (n=1)
10	Male	0 ± 0 (n=6)	73020 ± 5887 ^b (n=6)	22957 ± 2975 ^d (n=6)	17925 ± 2486 ^f (n=6)
20	Male	0 ± 0 (n=7)	147559 ± 14014 ^a (n=7)	57057 ± 9100 ^c *** (n=7)	41629 ± 5656 ^e (n=7)
0	Female	0 ± 0 (n=3)	0 ± 0 (n=3)	0 (n=1) <LOQ (n=2)	25 ± 7 (n=3)
10	Female	0 ± 0 (n=6)	33546 ± 5053 ^a (n=6)	25977 ± 5953 ^b (n=6)	16157 ± 4103 ^c (n=6)
20	Female	143 ± 143 (n=6) <LOQ (n=1)	44793 ± 7473 ^a (n=7)	35589 ± 7899 ^b (n=7)	22798 ± 5248 ^c (n=6) NS (n=1)

Mean (± SEMs) PFHxS in serum (ng/ml) of animals exposed to PFHxS via oral gavage. This experiment utilized a shared control group (n=9/sex) with 6:2 FTS and PFNA; therefore, three control animals/sex were analyzed for PFHxS. Data from day 0, 21, and 28 were generated at CSM and LOQ was 157.4 ng/mL. Data from day 28* were generated at 3M and LOQ was 1 ng/mL unless a sample was diluted, in which case the effective LOQ = working LOQ x dilution factor. Given the different methods, day 28 data from CSM and 3M are not comparable. Within each column, means that do not share a letter are different ($p < 0.05$) by Tukey HSD. Across timepoints (CSM day 21 and 28), means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$). NS = no sample.

Figure F-7. PFHxS in Serum of White-Footed Mice Exposed to PFHxS via Oral Gavage

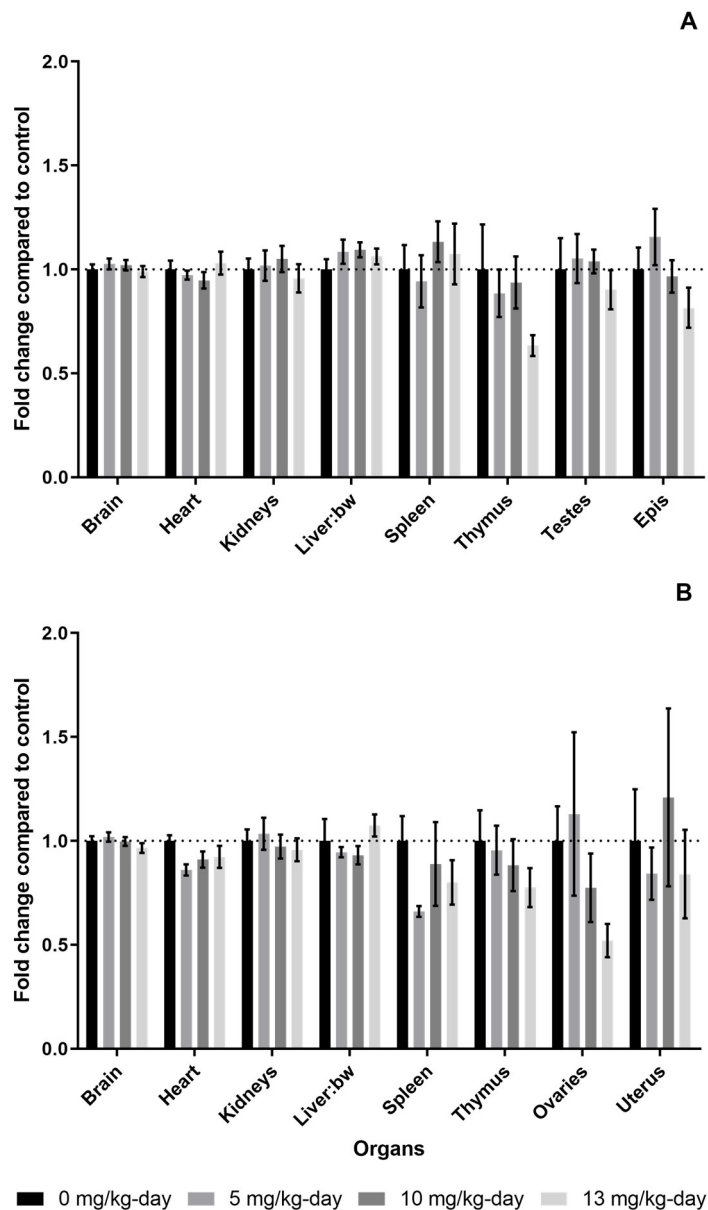


Mean ($\pm$ SEMs) [PFHxS] in serum of male (A, $n=3-9$ /sex/dose) and female (B, $n=3-9$ /sex/dose) white-footed mice exposed to PFHxS via oral gavage. Since there were sex differences, data from male and female mice are presented separately. Data from day 21 and 28 were generated at CSM and analyzed via repeated measures ANOVA to determine whether serum levels were similar at multiple time points. Data from day 28 were also generated at 3M, but were not compared to day 28 CSM data, due to differences in analytical methods. Means that do not share a letter are different ($p < 0.05$) by Tukey HSD. All other results were not statistically significant ($p > 0.05$).

Appendix G

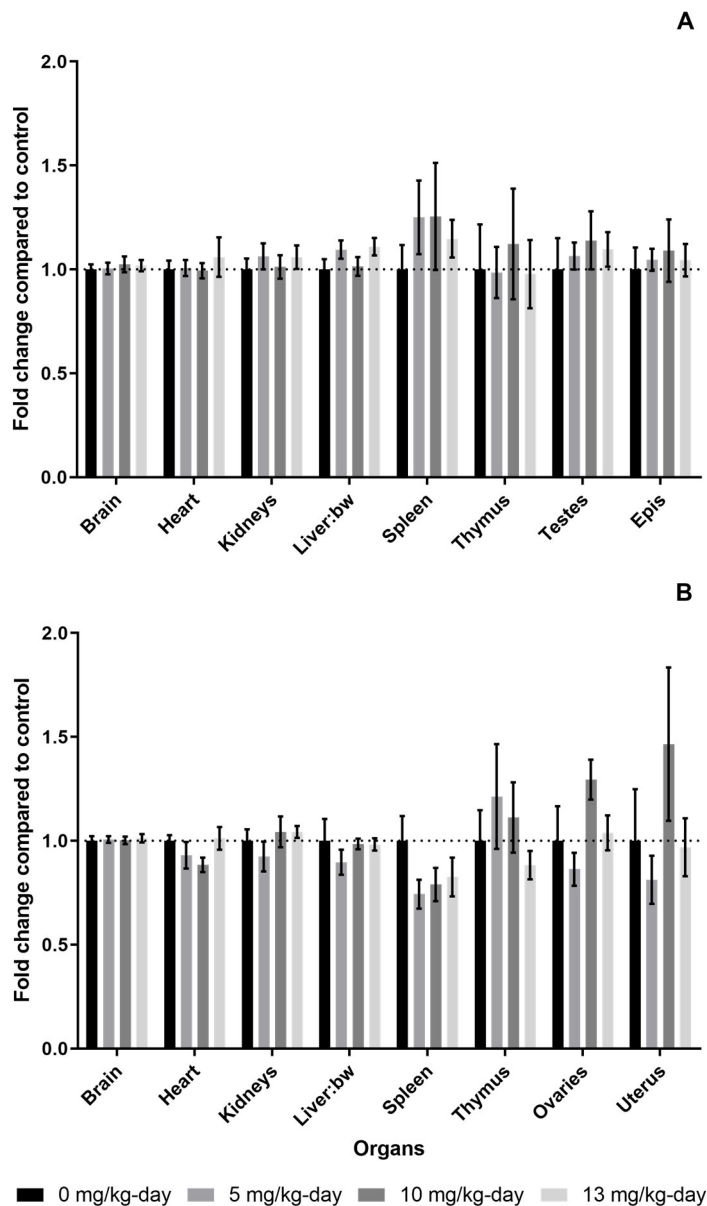
Organ Weights

Figure G-1. Organ Weights of White-Footed Mice Exposed to PFOA for 28 Days via Drinking Water



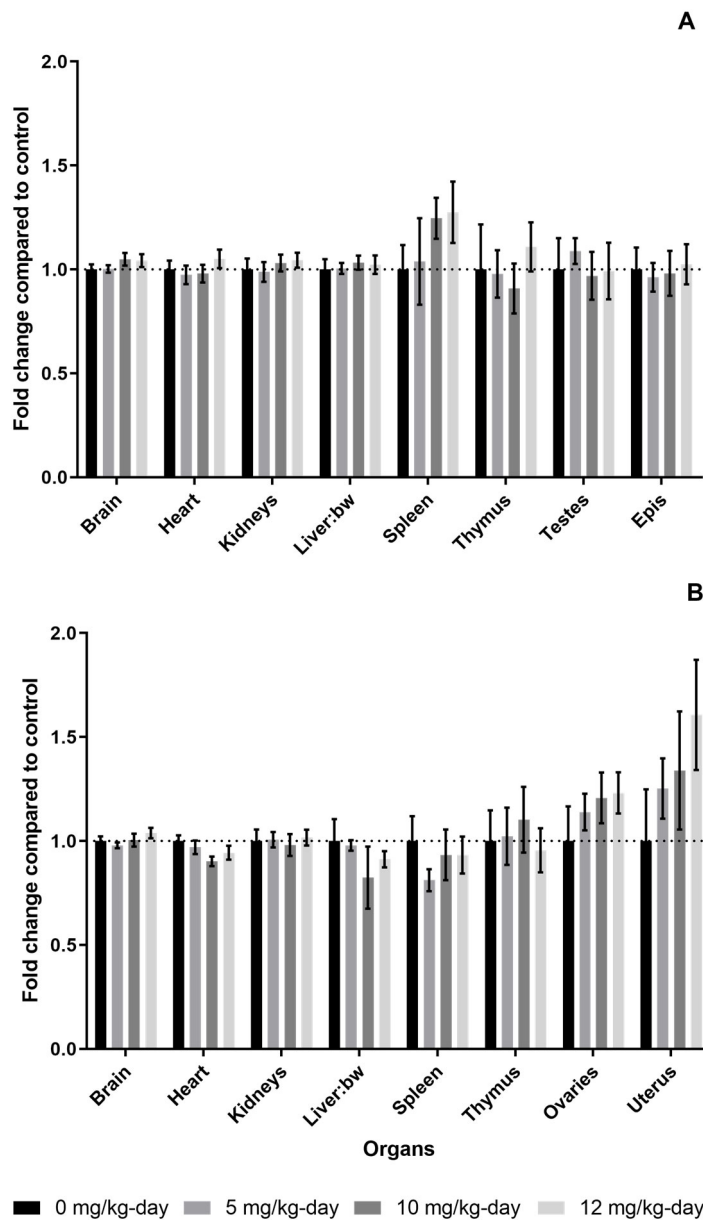
Graph and error bars represent mean and SEMs, respectively, of results obtained from male (A, n=7-8/sex/dose) and female (B, n=8-9/sex/dose) white-footed mice exposed to PFOA. Data are presented as fold change compared to control. Results were not statistically significant ($p > 0.05$).

Figure G-2. Organ Weights of White-Footed Mice Exposed to PFHxS for 28 Days via Drinking Water



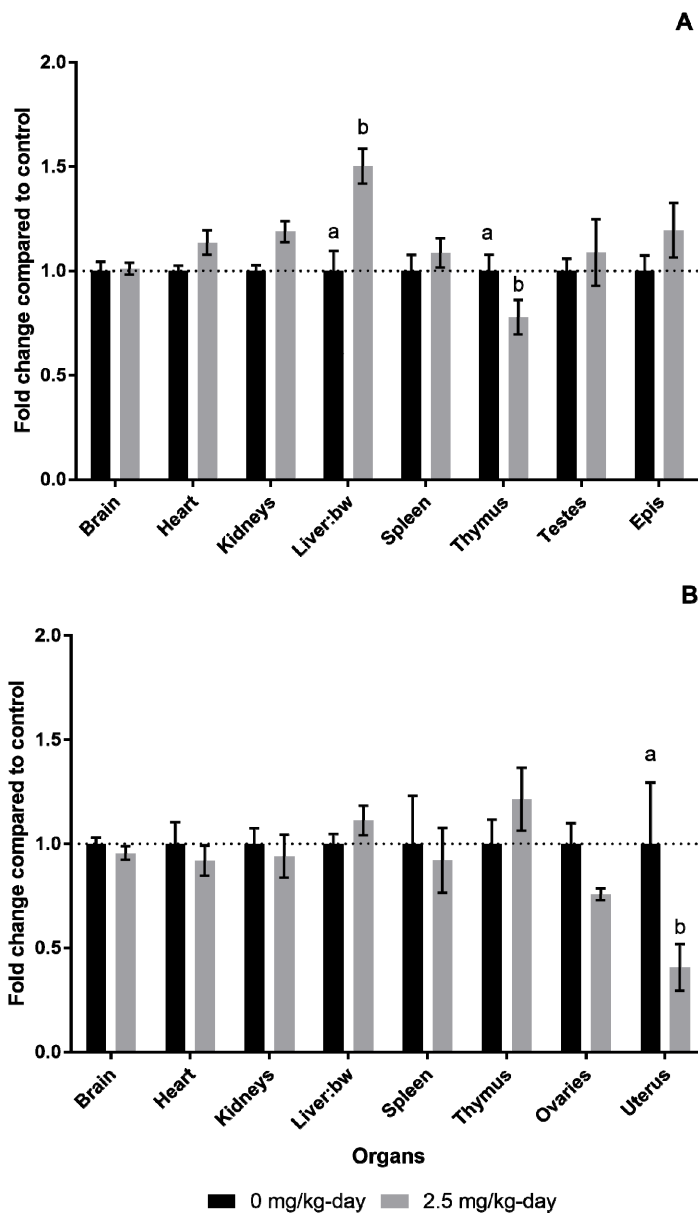
Graph and error bars represent mean and SEMs, respectively, of results obtained from male (A, n=7-9/sex/dose) and female (B, n=8-9/sex/dose) white-footed mice exposed to PFHxS. Data are presented as fold change compared to control. Statistical analyses revealed that, for each organ, results obtained at any exposure dose were not significantly from those obtained with no exposure ($p > 0.05$).

Figure G-3. Organ Weights of White-Footed Mice Exposed to PFBS for 28 Days via Drinking Water



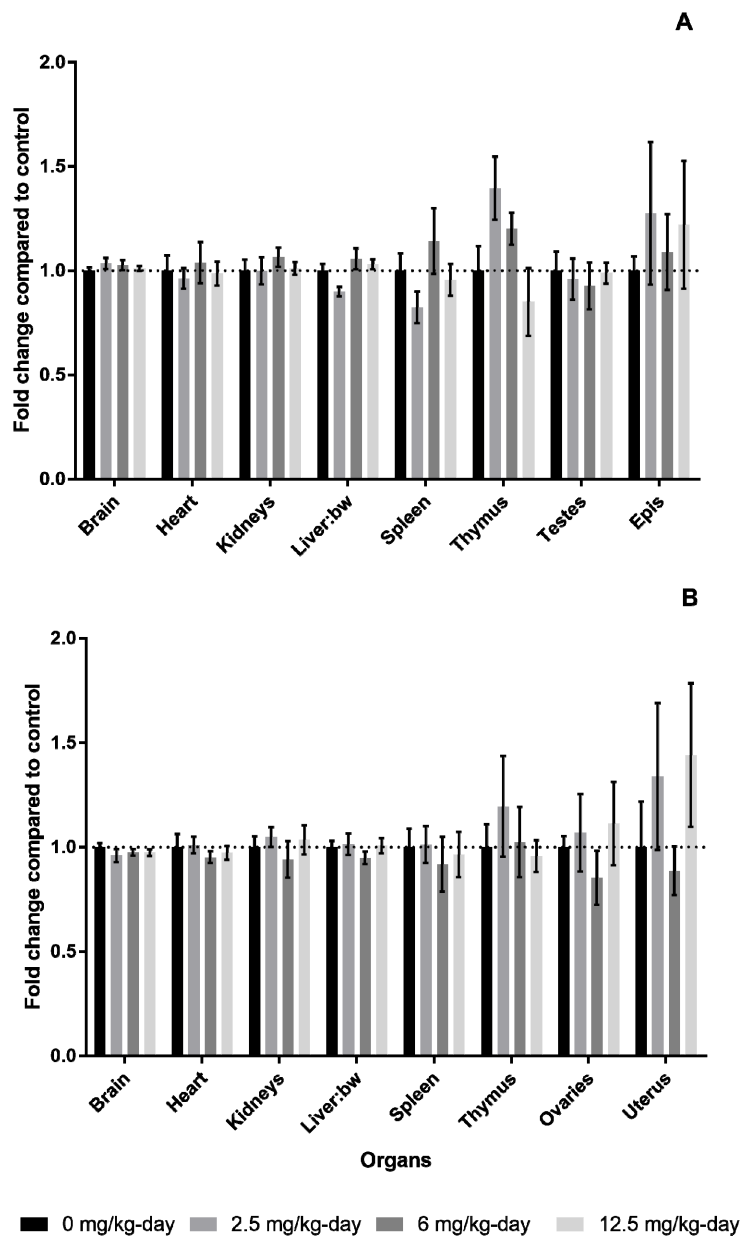
Graph and error bars represent mean and SEMs, respectively, of results obtained from male (n=7-9/sex/dose) and female (n=6-9/sex/dose) white-footed mice exposed to PFBS. Data are presented as fold change compared to control. Statistical analyses revealed that, for each organ, results obtained at any exposure dose were not significantly from those obtained at no exposure ($p > 0.05$).

Figure G-4. Organ Weights of White-Footed Mice Exposed to PFOS for 28 Days via Oral Gavage



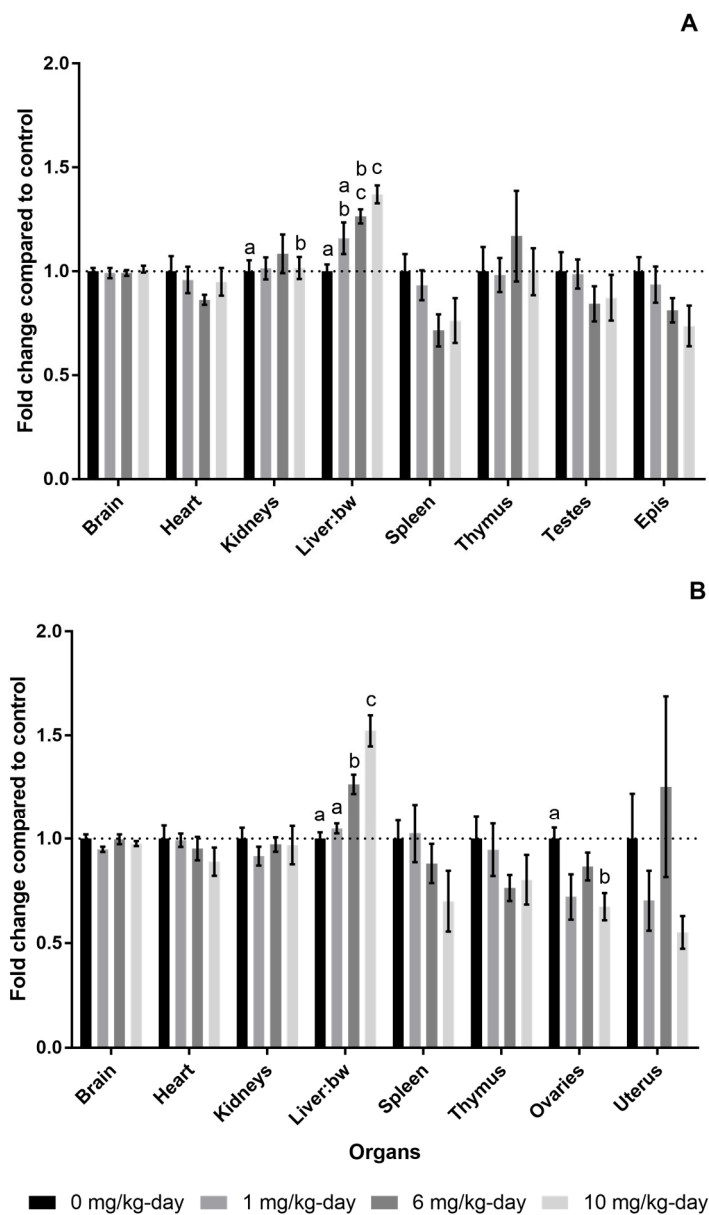
Graph and error bars represent mean and SEMs, respectively, of results obtained from male (A) (n=6/sex/dose) and female (B) (n=6-8/sex/dose) white-footed mice exposed to PFOS. Data are presented as fold change compared to control. Males exposed to PFOS had increased liver weights and decreased thymus weights. Females exposed to PFOS had decreased uterine weights. Statistical analyses revealed that, for all other organs, results from exposed animals were not significantly from control animals ($p > 0.05$). Means that do not share a letter are different ($p < 0.05$).

Figure G-5. Organ Weights of White-Footed Mice Exposed to 6:2 FTS for 28 Days via Oral Gavage



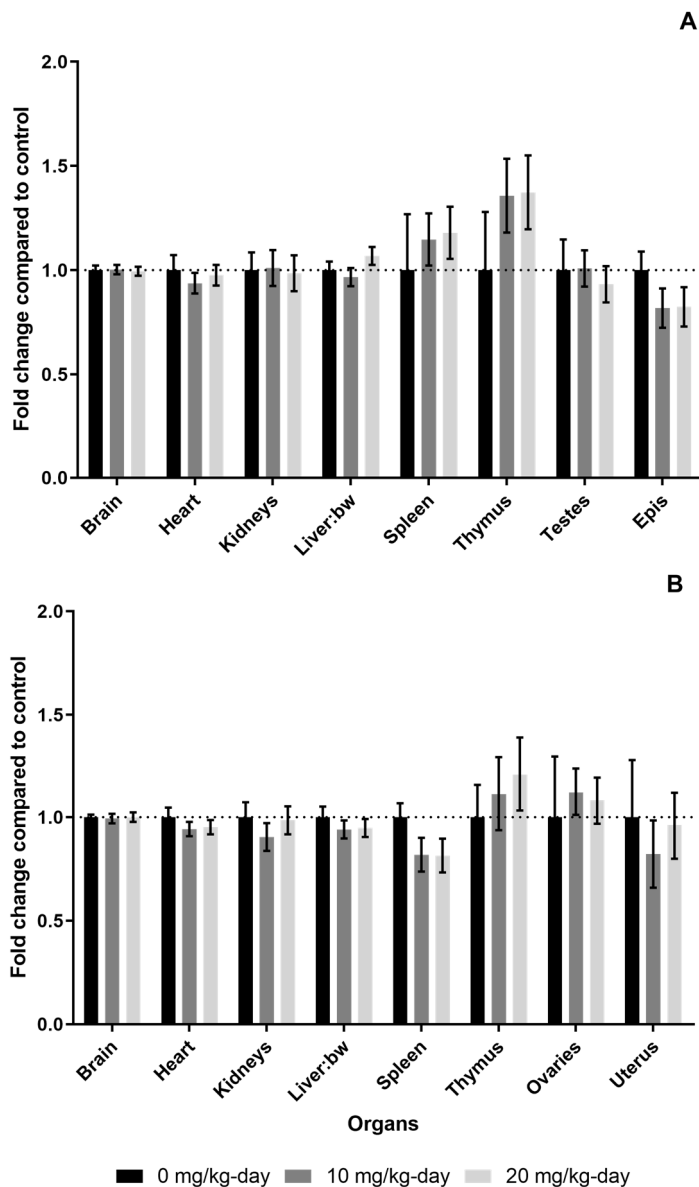
Graph and error bars represent mean and SEMs, respectively, of results obtained from male (A, n=8-9/sex/dose) and female (B, n=6-8/sex/dose) white-footed mice exposed to 6:2 FTS via oral gavage. Data are presented as fold change compared to control. For each organ, results from exposed animals were not statistically different from those obtained from non-exposed animals ($p > 0.05$).

Figure G-6. Organ Weights of White-Footed Mice Exposed to PFNA for 28 Days via Oral Gavage



Graph and error bars represent mean and SEMs, respectively, of results obtained from male (A, n=8-9/sex/dose) and female (B, n=8-9/sex/dose) white-footed mice exposed to PFNA. Data are presented as fold change compared to control. Males exposed to PFNA had increased liver and kidney weights. Females exposed to PFNA had increased liver and decreased ovarian weights. Means that do not share a letter are statistically different ($p < 0.05$) by Tukey HSD or Sidak post-hoc. For all other organs, results obtained from exposed animals were not statistically different from those of non-exposed ($p > 0.05$).

Figure G-7. Organ Weights of White-Footed Mice Exposed to PFHxS for 28 Days via Oral Gavage



Graph and error bars represent mean and SEMs, respectively, of results obtained from male (A, n=6-8/sex/dose) and female (B, n=6-8/sex/dose) white-footed mice exposed to PFHxS via oral gavage. Data are presented as fold change compared to control. For all organs, results obtained from exposed and non-exposed animals were not significantly different ($p > 0.05$).

Appendix H
Sperm Analysis

Table H-1. Total Sperm Count (M) in White-Footed Mice Exposed to 6:2 FTS, PFNA, or PFHxS via Oral Gavage

	Sperm (M)			
PFAS	Control	Low	Medium	High
6:2 FTS	40.93 ± 9.49	38.73 ± 6.22	29.97 ± 8.46	27.34 ± 4.91
PFNA		29.58 ± 5.01	16.17 ± 3.26	18.82 ± 7.29
PFHxS		n/a	32.60 ± 8.01	29.19 ± 6.09

Appendix I

Protocol and Modifications

ANIMAL USE PROTOCOL
ARMY PUBLIC HEALTH CENTER
ABERDEEN PROVING GROUND MD 21010-5403

PROTOCOL TITLE: Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)

PROTOCOL NUMBER: 67-17-04-01

DATE OF APPROVAL: 20 April 2017

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ACRONYMS:

AFFF: Aqueous Film Forming Foam
ANCOVA: Analysis of Covariance
ANOVA: Analysis of Variance
APHC: Army Public Health Center
AV: Attending Veterinarian
CFR: Code of Federal Regulations

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CO₂: Carbon dioxide

DOD: Department of Defense

FEDRIP: Federal Research in Progress

GLP: Good Laboratory Practices

IACUC: Institutional Animal Care and Use Committee

IAW: In accordance with

IP: Intraperitoneal

PPE: personal protective equipment

PFASs: Perfluoroalkyl substance

PFBS: Perfluorobutanesulfonate

PFHxS: Perfluorohexanesulfonate

PFOA: Perfluorooctanoate

PFOS: Perfluorooctanesulfonate

SOP: Standing Operating Procedure

SRBC: Sheep red blood cell

TOX: Toxicology Directorate

TSCA: Toxic Substance Control Act

TRV: Toxicity Reference Value

USEPA: United States Environmental Protection Agency

I. NON-TECHNICAL SYNOPSIS:

PFASs are compounds manufactured for use in paints, cleaning agents, fire suppressants, non-stick cookware and food containers, and water-impermeable products in general. Concerns about PFASs stem from their ubiquitous presence in the environment, widespread reports of general toxicity, and the resistance of these compounds to degradation. The primary goal of this range finding study is to ensure bioaccumulation and sufficient body burden of the chemicals for follow-up reproductive/developmental toxicity studies; data from this study will help determine exposure levels and refine the design for upcoming reproductive/developmental toxicity studies. In turn, data from the reproductive/developmental toxicity studies will be used to derive TRVs. TRVs are critical components of environmental risk assessments that help determine if the risk of environmental exposure is acceptable.

In the current study, PFASs will be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days via drinking water. Blood samples will be collected every 7 days via facial/submandibular venipuncture and analyzed for the concentration of PFASs. Five days prior to euthanasia, animals will receive an IP injection of SRBC in sterile saline to evaluate immune function. Cardiac blood samples will be collected at termination from anesthetized animals and will be evaluated for immune parameters and PFASs concentration and subjected to clinical chemistry and hematology assessments (as available). Selected tissues will be weighed and processed for histopathology. Immunological organs (thymus and spleen) will also be

processed and evaluated for additional endpoints. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure via drinking water (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

II. BACKGROUND

II.1. Background:

Ecological and human health risk assessment of these chemicals are still in their infancy; however, PFOS and PFOA, the two most abundant PFASs in the environment and biota, have attracted increased regulatory scrutiny because of their resistance to degradation, ability to bioaccumulate, and growing evidence of toxicity in animals. In 2016, the USEPA published lifetime drinking water Health Advisory values of 0.2 and 0.4 µg/L for PFOS and PFOA, respectively. These recommended levels are several orders of magnitude lower than the concentration of PFOS and PFOA observed in groundwater at historical fire training sites.

Production of PFOS was phased out by the manufacturer 3M in 2002, and DuPont ceased production of PFOA by 2014. However, a new generation of shorter-chain PFASs have emerged in commerce, with little information available regarding their environmental fate and distribution as well as potential health effects.

The current understanding of adverse effects associated with PFAS exposure is based on laboratory animal models and includes hepatotoxicity, tumor induction, developmental toxicity, immunotoxicity, neurotoxicity, and endocrine disruption. General toxicological features of these chemicals include 1) major species and sex differences in pharmacokinetic disposition among PFASs with different carbon-chain lengths and functional groups and 2) differential potencies based on their primary mechanism of action. Furthermore, the serum elimination half-lives can vary greatly from hours to years based on species, sex, and dose, making extrapolation difficult and inaccurate.

Although many toxicity tests have been done with laboratory species, no comprehensive test for any PFASs has been undertaken with a wild mammalian species. Toxicity data from this series of studies will be used to determine a wild mammalian-specific TRV. Additionally, toxicity information gained in a new species may help to eliminate or reduce uncertainty factors used for human-based risk assessments.

The present study will compare the bioaccumulation of four PFASs most commonly found in AFFFs (highly efficient fire suppressant agents) and in environmental sites

Animal Use Protocol: Range finding for exposure to perfluoroalkyl acids via drinking water in the white-footed mouse (*Peromyscus leucopus*)

contaminated by AFFF use in the white-footed mouse (*Peromyscus leucopus*) exposed via drinking water.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: PubMed, RePORT, EmBase, FEDRIP

II.2.2. Date of Search: 26 January 2017 (PubMed), 3 February 2017 (EmBase), 6 February 2017 (FEDRIP), 7 February 2017 (RePORT)

II.2.3. Period of Search: 1960-2016 (PubMed), 1970-2017 (EmBase), 1992-2017 (FEDRIP), 1992-2017 (RePORT)

II.2.4. Key Words of Search:

PubMed: Search 1: (perfluorinated OR PFOS OR PFOA OR PFBS OR PFHxS) returned 4093; Search 2: ((peromyscus OR white footed mouse)) returned 2211; Search 3: (((perfluorinated OR PFOS OR PFOA OR PFBS OR PFHxS))) AND (((peromyscus OR white footed mouse))) returned 0; Search 4: (((perfluorinated OR PFOS OR PFOA OR PFBS OR PFHxS))) AND ((mus musculus OR lab mouse)) returned 276; Search 5: ((((((perfluorinated OR PFOS OR PFOA OR PFBS OR PFHxS))) AND ((mus musculus OR lab mouse)))))) AND toxicity returned 195; Search 6: (((((((perfluorinated OR PFOS OR PFOA OR PFBS OR PFHxS))) AND ((mus musculus OR lab mouse)))))) AND toxicity)) AND oral returned 41; Search 7: (((((((perfluorinated OR PFOS OR PFOA OR PFBS OR PFHxS))) AND ((mus musculus OR lab mouse)))))) AND toxicity)) AND drinking water returned 9.

EmBase: Search 1: pfoa AND tox* NOT ('human'/exp OR human) returned 464; Search 2: pfoa AND tox AND ('animal'/exp OR animal) returned 34; Search 3: pfoa AND tox AND ('animal'/exp OR animal) NOT ('human'/exp OR human) returned 17; Search 4: pfos AND tox* NOT ('human'/exp OR human) AND ('animal'/exp OR animal) returned 423; Search 5: pfos AND tox* AND ('mouse'/exp OR mouse) NOT ('human'/exp OR human) returned 78; Search 6: pfos:ti AND tox*:ti AND ('mouse'/exp OR mouse) NOT ('human'/exp OR human) returned 2; Search 7: perfluoro:ti AND tox*:ti AND ('rat'/exp OR rat) NOT ('human'/exp OR human) returned 4; Search 8: perfluoro:ti AND tox*:ti AND ('mouse'/exp OR mouse) NOT ('human'/exp OR human) returned 1; Search 9: perflu*:ti AND tox*:ti AND ('mouse'/exp OR mouse) NOT ('human'/exp OR human) returned 19; Search 10: perflu*:ti AND tox*:ti AND mouse:ti NOT ('human'/exp OR human) returned 3; Search 11: perflu*:ti AND tox*:ti AND rat:ti NOT ('human'/exp OR human) returned 12; Search 12: tox*:ti AND peromyscus:ti NOT ('human'/exp OR human) returned 7; Search 13: toxi* AND peromyscus:ti NOT ('human'/exp OR human) returned 51; Search 14: toxi*:ti AND peromyscus:ti NOT ('human'/exp OR human)

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returned 5; Search 15: toxi*:ti AND 'white footed':ab,ti NOT ('human'/exp OR human) returned 5; Search 16: oxi*:ab,ti AND 'white footed':ab,ti NOT ('human'/exp OR human) returned 10; Search 17: toxi*:ab,ti AND pfbs:ab,ti NOT ('human'/exp OR human) returned 21; Search 18: toxi*:ab,ti AND pfbs:ab,ti AND rat:ab,ti NOT ('human'/exp OR human) returned 7; Search 19: toxi*:ab,ti AND pfaa:ab,ti AND rat:ab,ti NOT ('human'/exp OR human) returned 1; Search 20: toxi*:ab,ti AND pfaa:ab,ti AND mouse:ab,ti NOT ('human'/exp OR human) returned 2; Search 21: toxi*:ab,ti AND pfh:ab,ti NOT ('human'/exp OR human) returned 6; Search 22: toxi*:ab,ti AND perfluoro:ab,ti NOT ('human'/exp OR human) returned 49; Search 23: toxi*:ab,ti AND perfluoro:ab,ti AND mouse:ab,ti NOT human:ab,ti returned 4; Search 24: toxi*:ab,ti AND perfluoro:ab,ti AND rat:ab,ti NOT human:ab,ti returned 12; Search 25: toxi*:ab,ti AND perfluoro:ab,ti AND guinea:ab,ti NOT human:ab,ti returned 1; Search 26: toxi*:ab,ti AND perfluoro:ab,ti AND hamster:ab,ti NOT human:ab,ti returned 2; Search 27: perfluoro:ab,ti AND rat:ab,ti NOT human:ab,ti returned 36; Search 28: perfluoro:ti AND rat:ab,ti NOT human:ab,ti returned 13; Search 29: perfluoro:ti AND mouse:ab,ti NOT human:ab,ti returned 8; Search 30: perfluoro:ti AND animal:ab,ti NOT human:ab,ti returned 6.

FEDRIP: Search 1: pfos OR pfoa returned 7.

RePORT: Search 1: "Perfluorooctanoic acid" and "toxicity" and "oral" returned 1, Search 2: "Perfluorooctanesulfonic acid" and "toxicity" returned 4.

II.2.5. Results of Search:

From the PubMed searches, 4093 publications provide data on PFASs. Of these, 276 provide data on PFASs in mice and 195 provide toxicity data for PFASs in mice. Of these, 41 publications provide toxicity data in mice exposed via the oral route to PFASs and nine publications provide toxicity data in mice exposed via drinking water to PFASs. Some of these publications provide data that serve as the basis for the potential utility of the work proposed here; however, none of the publications retrieved report data on the white-footed mouse (*Peromyscus leucopus*) exposed to PFOS, PFOA, PFBS, or PFHxS. Data for drinking water exposure to PFOA in C57BL/6N mice were reported by DeWitt (2009). DeWitt *et al.* did not see a difference in drinking water consumption between dose groups, suggesting that there is not a palatability issue with PFASs. Data for gestational and chronic low-dose PFOA exposure are reported by White (2011). White *et al.*, saw PFOA-induced developmental delays in CD-1 mice. Data for gestational exposure to PFOS was reported by Thibodeaux (2003). Thibodeaux *et al.*, saw serum PFOS levels increased with dosage, and liver levels approximately fourfold higher than serum. Furthermore, Thibodeaux saw a dose-dependent suppression in maternal weight gain. White *et al.* and Thibodeaux *et al.* are applicable to the current

Animal Use Protocol: Range finding for exposure to perfluoroalkyl acids via drinking water in the white-footed mouse (*Peromyscus leucopus*)

study in that CD-1 mice are sensitive to PFASs and therefore, will be used as a positive control.

The EmBase searches found hundreds of publications. Many of the publications were outside the scope of this project and/or had a more mechanistic focus. Some of the publications provide data on the toxicity of PFASs in rodents and were not captured by the PubMed search, but most were of inferior quality (e.g., poor study design, inappropriate interpretation of results) to publications retrieved from PubMed. Data on concurrent exposure to PFOS and stress in pregnant mice was reported by Fuentes (2006). Fuentes *et al.*, saw a significant general effect of restraint on body weight gain. Although restraint of this sort will not occur in the current study, it is worth considering the impact of stress from routine handling and procedures, given that the white-footed mouse (*Peromyscus leucopus*) is a wild-type prey species and thus, prone to stress. Sample sizes have been adjusted accordingly, in consultation with the AV and statistician. Data comparing radiation toxicities among *Mus musculus* and *Peromyscus leucopus* was reported by Liu (2013). Liu *et al.*, said although these species are roughly the same size, which allows for similar dosimetry, and virtually identical anatomically, the innate genetic differences are pronounced. Furthermore, he stated that while it may be interesting to consider the outbred white-footed mouse (*Peromyscus leucopus*) as an animal model more similar to the human population with its innate genetic diversity and outbred genetic status, there is probably as much danger of making a wrong assumption as there is when projecting expectations from mice onto humans. Importantly, none of the publications retrieved report data on the white-footed mouse (*Peromyscus leucopus*) exposed to PFOS, PFOA, PFBS, or PFHxS.

From the FEDRIP search, seven publications were retrieved. Of these, three publications pertained to persistent organic pollutants in general. The other four publications included two USEPA documents (Drinking Water Health Advisory for PFOS, 2016; Drinking Water Health Advisory for PFOA, 2016) and two textbooks pertaining to PFAS. Each USEPA document included hundreds of references. The USEPA documents (and references) and one textbook (DeWitt, 2015) were heavily consulted in the design of this study.

From the RePORT searches, five publications provide data on PFASs. White *et al.* was also captured in the PubMed search, and is described above. Of the remaining four publications, none reported relevant information; two provide data on zebrafish exposure and two pertain to how PFASs form bilayers.

III. OBJECTIVE/HYPOTHESIS:

Animal Use Protocol: Range finding for exposure to perfluoroalkyl acids via drinking water in the white-footed mouse (*Peromyscus leucopus*)

The main objective of the range finding study is to determine how (and to what extent) four PFASs accumulate in the white-footed mouse (*Peromyscus leucopus*). Data will also help determine exposure levels and refine the design for upcoming reproductive/developmental toxicity studies with PFASs.

IV. MILITARY RELEVANCE:

AFFFs have been used by the DOD since approximately 1970 for fire-training and emergency response activities. Environmental releases have been substantial; PFASs are distributed globally, even to remote locations such as the Arctic and the Antarctic, by atmospheric and/or oceanic transfers. Furthermore, a plethora of studies have documented the prevalence of these chemicals in a variety of wildlife species and humans. Therefore, an improved understanding of the ecotoxicity of compounds associated with the release of AFFFs is needed.

Data from this study will help determine exposure levels and refine the design for upcoming reproductive/developmental toxicity studies. Data from the reproductive/developmental toxicity studies will, in turn, be used to derive TRVs for a wild mammalian species. TRVs are critical components of environmental risk assessments that help determine if the risk of exposure is acceptable. These TRVs, and increased understanding of these unique chemicals in general, will assist in the development of appropriate site-specific risk assessment and decisions related to mitigation of exposures and/or future cleanup.

V. MATERIALS AND METHODS

Test Article: This study will be conducted with PFOS, PFOA, PFBS, and PFHxS. Each PFAS will be mixed with deionized or PFAS-free water. The vehicle 0.5% Tween-20 may be used. Concentration verification and stability analysis will be conducted for each chemical IAW appropriate methods. Neat test material will be stored at room temperature (e.g., 20±5°C).

V.1. Experimental Design and General Procedures:

This study includes four experiments with four compounds. PFOS, PFOA, PFBS, and PFHxS will be administered orally via drinking water to male and female white-footed mice (*Peromyscus leucopus*) for 28 consecutive days. PFASs will be administered at constant dietary concentrations and will be provided *ad libitum* via water bottles. Animals will be assigned to dose groups within sex using a stratified random procedure based on pre-study body weight and age, as possible. At the commencement of the study, there will be no significant differences in the mean body weights (and age, if possible) between groups within each sex, or groups will be re-assigned. Cages will be

arranged in racks randomly to minimize possible effects due to cage placement. All animals will be monitored throughout the study for body weight changes and clinical signs of toxicity. Blood samples will be collected on days 0, 7, 14, 21 (as described in section V.4.4.3.1) and analyzed for the concentration of PFAS. Five days prior to euthanasia, animals will receive an IP injection of SRBC in sterile saline as described in section V.4.4.1 to evaluate immune function. At termination (following 28 consecutive days of exposure), blood samples will be collected from anesthetized animals (as described in section V.4.4.3.2) and will be evaluated for immune parameters and PFAS concentration and subjected to clinical chemistry and hematology assessments (as available). Selected tissues will be weighed and processed for histopathology. Immunological organs (thymus and spleen) will also be processed and evaluated for additional endpoints.

Group	No. males	No. females	Pain category
PFOS Dose 1	9	9	18D
PFOS Dose 2	9	9	18D
PFOS Dose 3	9	9	18D
PFOA Dose 1	9	9	18D
PFOA Dose 2	9	9	18D
PFOA Dose 3	9	9	18D
PFBS Dose 1	9	9	18D
PFBS Dose 2	9	9	18D
PFBS Dose 3	9	9	18D
PFHxS Dose 1	9	9	18D
PFHxS Dose 2	9	9	18D
PFHxS Dose 3	9	9	18D
Control	9	9	18D
	TOTAL = 117	TOTAL = 117	TOTAL = 234D

V.1.1. Dose Selection:

Dose levels will be based on the literature and will vary for individual chemicals. The primary goal of this range finding study is to ensure bioaccumulation and sufficient body burden of the chemicals for follow-up reproductive/developmental toxicity studies. It is generally recommended that the highest dose level be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death. Three drinking water dose solutions will be used throughout the study. The control group will be an untreated group allowed *ad libitum* access to drinking water.

V.1.2. Administration of Test Substance:

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PFOS, PFOA, PFBS, and PFHxS will be administered via drinking water for 28 consecutive days. The drinking water exposure route was selected because it is environmentally relevant and will reduce animal handling procedures, and thus stress. Animals will be provided the PFAS-treated drinking water *ad libitum* via water bottles. Water bottles and sipper tube will be color coded according to treatment group.

V.1.3. Serum Analysis:

Serum analysis will be performed IAW appropriate methods.

V.1.4. Clinical Chemistry and Hematology:

Fasted blood samples will be collected from all animals at termination (as described in V.4.4.3.2) and may be subjected to hematology and clinical chemistry analyses.

V.1.5. Gross Necropsy, Organ Weight, and Tissue Preparation:

At the time of termination or premature death, all animals will be necropsied and examined macroscopically for any structural abnormalities or pathological changes. Wet weights of the organs listed below from all animals will be determined as soon as possible after dissection to avoid drying. This tissue list may be altered at the discretion of the pathologist/PI based on observed toxicity and gross pathology findings.

- Brain
- Liver
- Kidneys
- Heart
- Spleen
- Thymus
- Testes
- Epididymides
- Ovaries
- Uterus

Additional tissues that will be collected at necropsy, but not weighed include the lymph node and bone marrow. Testes and epididymides from each animal will be placed in Davidson's fixative overnight (no longer than 24 hours) or 10% buffered formalin for at least 24 hours; however, fixing in Davidson's solution for less than 24 hours is preferred. The fixative used will be documented in the study records. All other organs will be placed in 10% buffered formalin for at least 24 hours for fixation. All organ weights and gross pathological changes will be recorded on TOX DOC 4. All training of necropsy

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personnel will be verified prior to any necropsy procedures. Personnel and the procedures they perform will be documented at the time of necropsy.

V.1.6. Histopathology:

Full histopathology of the organs listed in section V.1.5. will be performed for all high-dose and control animals at the discretion of the pathologist and based on observed toxicity and gross pathology findings. Organs demonstrating treatment-related changes may also be examined in animals in the lower dose groups. Gross lesions will be subjected to histopathological analysis at the discretion of the pathologist.

V.1.7. Study Time Frame

The proposed experimental start date is June 2017 and anticipated study end date is March 2018.

V.2. Sample Size Evaluation, Data Analysis Plan, and Archiving of Data:

The sample size is slightly larger than what is typical for a range finding, at the recommendation of the AV and statistician. Given that the white-footed mouse (*Peromyscus leucopus*) is a wild-type prey species and prone to stress, extra animals have been built into each group (i.e., N=9 rather than N=6) to better ensure adequate statistical power in the event of stress-related deaths.

For variables that are measured only at the end of the study, the dose groups will be compared using a one-factor ANOVA. Organ to brain and organ to body weight ratios will be calculated and analyzed similarly to other parameters measured at the end of the study. If the dose group effect is significant, post hoc tests will be used to compare pairs of dose groups and dose groups to the control group; a Tukey's multiple comparison test if the variance of the groups is similar and a Dunnett's T3 test if the variances are unequal. Data will be tested for normality and log transformed and retested if significantly different from normal. Variance equality will be determined by a Levene's test.

For absolute organ weights, comparison of the dose groups will be made using an ANCOVA, with body weight at the end of the study being the covariate used. Even though the dose groups will be assigned prior to initiating exposure, the weights can change during the study dependent on dose group. The ANCOVA will adjust for any differences in body weights among the dose groups at the end of the study because heavier animals will tend to have heavier organs. If the dose group effect is significant, an appropriate post hoc test will be used to compare pairs of dose groups and dose groups to the control group.

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Dose groups will also be compared with respect to absolute body weights, as well as weekly changes in body weight and net weight changed using a repeated measures ANOVA. Dose groups will also be compared with respect to net food consumption for the study using a one-factor ANOVA. If the ANOVA is significant, the post hoc tests will be used to compare pairs of dose groups; a Tukey's multiple comparison test if the variance of the groups are similar, and a Dunnett's T3 test if the variance are unequal. Variance equality will be determined by a Levene's test.

Other observational data including gross necropsy observations and histopathology data may be converted to categorical data and analyzed using a Chi-square or Fisher's exact test.

An appropriate statistical software package, such as SPSS[®], SAS[®], or MiniTab[®] will be used to perform all analyses and statistical significance will be defined as $p < 0.05$ for all tests.

This study will be conducted in a manner consistent with the principles of 40 CFR Part 792 TSCA GLP Regulation (CFR, 1989). The investigators and technicians will adhere to the Guide for the Care and Use of Laboratory Animals (NRC, 2011).

Records will be kept in standard APHC laboratory notebooks and/or three ring binders. Daily records will be kept on survival and clinical signs collected on animals during the study. Procedures for preparation of any drug administration, animal blood collection, observation logs, morbidity/mortality logs, etc., will be stored with the study records. All post mortem procedures not listed in this protocol will be documented in the study records and kept with the study raw data. These records will be made available to oversight organizations such as the USEPA, Laboratory and Toxicology Accreditation and Compliance Office, and the IACUC. The protocol, protocol amendments, raw data, statistical analysis, tabular calculations, and graphic analysis of the data will be saved with the study records. Additionally, memoranda on the study file, study logs, signature logs, final reports, and final report amendments will be archived at APHC. Some ancillary records such as maintenance and calibration logs, environmental monitoring logs, animal room husbandry and health rounds sheets, training files, etc. may be stored in the archives but not stored with the study files.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered:

The main objective of the study is to determine how four PFASs accumulate in the white-footed mouse (*Peromyscus leucopus*). There are no appropriate animal substitutes (e.g., computer models, tissue/cell cultures) for the data that will be

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produced in this study. No non-animal alternative would provide the necessary toxicological information provided by this study. Therefore, it is necessary to perform this study in an animal model.

V.3.2. Animal Model and Species Justification:

Indigenous species have great utility when evaluating the effectiveness of site remediation efforts (Tice *et al.*, 1987). The white-footed mouse was selected because it is a terrestrial species and has been shown to be an excellent small mammal model (Rattner and Hoffman, 1984; Reddy *et al.*, 1997; Johnson *et al.*, 2000). Additionally, this wild mammalian species has been historically used for toxicity studies by APHC TOX. The genus *Peromyscus* is the most widespread and geographically variable of the North American rodents (Godin, 1977).

V.3.3. Laboratory Animals

V.3.3.1. Genus species: *Peromyscus leucopus*

V.3.3.2. Strain / Stock / Breed: N/A

V.3.3.3. Source / Vendor: Peromyscus Genetic Stock Center, University of South Carolina, Columbia, SC 29208; USDA license number 56-R-0003. Animals on Animal Use Protocol Number 15-04-01 may be transferred to this protocol.

V.3.3.4. Age: Adults, as available from the vendor

V.3.3.5. Weight: Appropriate for age

V.3.3.6. Sex: Both

V.3.3.7. Special Considerations: N/A

V.3.4. Number of Animals Required (by Species): 234

V.3.5. Refinement, Reduction, Replacement (3 Rs):

V.3.5.1. Refinement:

All animals will be socially housed and will be provided with a certified plastic-free form of enrichment (e.g., Nestlet) in their cage/pen. Animals will be exposed via drinking water partially because it will reduce animal handling procedures, and thus reduce stress. Animals will be considered for early removal from this study as described in section V.4.5.

V.3.5.2. Reduction:

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Tissue sharing may be allowed. Tissues will only be shared after all samples for the study have been collected and only if doing so will not affect the outcome of the study. Animals on Animal Use Protocol Number 15-04-01 may be transferred to this protocol. The minimum number of animals for valid statistical analysis is being used.

V.3.5.3. Replacement:

No non-animal alternatives are known to exist that will provide the required data. At the same time, there are no non-animal alternatives that can fully replicate the complex processes that occur within an intact mammalian organism.

V.4. Technical Methods:

V.4.1. Pain / Distress Assessment:

V.4.1.1. APHIS Form 7023 Information:

V.4.1.1.1. Number of Animals

V.4.1.1.1.1. Column B: 0

V.4.1.1.1.2. Column C: 0

V.4.1.1.1.3. Column D: 234

V.4.1.1.1.4. Column E: 0

V.4.1.2. Pain Relief / Prevention

V.4.1.2.1. Anesthesia / Analgesia / Tranquilization:

Animals will be anesthetized with CO₂ prior to blood collection via cardiac puncture. Animals will be brought to the necropsy room in a home cage or transport cage. The stainless steel lid will be placed on the cage. If using a cage with a grommet for automatic water, the grommet will be covered with tape, magnet, or stopper. CO₂ will displace 10-30% of the chamber volume per minute IAW QSARC 800 (APHC, 2017a). Animals will remain in the cage until they are recumbent and breathing is slow and shallow. Once recumbent, a toe or space between the toes will be pinched to assess appropriate depth of anesthesia. If there is no response to toe pinch, the animal will be removed and blood collected (as described in section V.4.4.3.2). Upon completion of blood collection the animal will be returned to the cage and euthanized as described in section V.4.6.

V.4.1.2.2. Pre- and Post-procedural Provisions:

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A thorough physical examination of each animal will be performed by study personnel at a similar time at least once per week. Twice daily during the week and once daily on weekends, an in-cage, general clinical observation of each animal will be performed by study personnel and/or animal care staff. All animals will be observed for signs of toxicity, morbidity, and mortality. All data related to the observation of animals will be documented in the study records by study personnel. Details related to the observations and/or physical examination of animals are described in V.4.4.8.2.

V.4.1.2.3. Paralytics: N/A

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Source(s) Searched: PubMed, ALTBIB, AnimAlt-ZEBET

V.4.1.3.2. Date of Search: 17 March 2017

V.4.1.3.3. Period of Search: 1900-2017 (PubMed), 1980-2000 (ALTBIB), 2002-2017 (AnimAlt-ZEBET)

V.4.1.3.4. Key Words of Search:

PubMed: Search 1: (mouse or mice AND heart OR cardiac AND blood draw AND alternatives) returned 0.

ALTBIB: Search 1: (mouse AND pain AND alternatives AND cardiac blood draw) returned 0; Search 2: (mouse AND distress AND alternatives AND cardiac blood draw) returned 0;

AnimAlt-ZEBET: Search 1: (mouse or mice AND heart OR cardiac AND draw AND alternatives) returned 0.

V.4.1.3.5. Results of Search:

The literature search did not identify any references pertaining to alternatives to painful procedures.

V.4.1.4. Unalleviated Painful or Distressful Procedure Justification:

The nature of this study precludes the use of totally painless procedures. An attempt to alleviate pain or distress by the administration of anesthetics, analgesics, or drugs may alter the manifestation of the toxic response. Typical pain relievers such as opiates and non-steroidal anti-inflammatories as well as anesthetics have the ability to mask certain toxic signs that may be observed due to the administration of the test compound, especially those signs resulting from pain or distress. In addition, certain side effects

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such as alterations in blood chemistry and hematology may arise from the use of these drugs and could be misinterpreted by the investigator as clinical signs caused by the test material. Animals determined to be moribund with no possibility for recovery will be euthanized as described in section V.4.6.

V.4.2. Prolonged Restraint and Restraint Methods: N/A

V.4.3. Surgery

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Survival Operative Procedures

V.4.3.6.1. Procedures: N/A

V.4.3.6.2 Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections:

Animals will be administered 0.1 mL of a SRBC suspension in sterile saline via IP injection, as described by Harper *et al.*, 1993. Briefly, animals will be held, angled head-down and ventral side superior (Trendelenburg position) to facilitate settling the abdominal organs away from the injection site, thereby reducing the likelihood the needle will perforate the intestines. The needle (21-28 gauge, 5/8-1 inch) will be inserted bevel side up at approximately a 30 degree angle (relative to the abdomen), in the lower left quadrant (avoiding the midline) at a depth suitable to ensure penetration of peritoneal cavity. The plunger will be drawn back to verify the needle is not in the intestine (aspirate luminal contents), blood vessels (aspirate blood), or bladder (aspirate urine). If no aspirate is observed, the solution will be injected into the peritoneal cavity rapidly.

V.4.4.2. Use of Non-pharmaceutical-grade chemicals:

The compounds being tested are not available in pharmaceutical grade composition. They are under investigation as described in the objectives section (III) of this protocol.

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V.4.4.3. Biosamples:

V.4.4.3.1. Facial/Submandibular venipuncture:

Animals will be physically restrained (just long enough to complete the procedure) in such a manner to maximize viewing of the face. Fur may be clipped and skin may be cleansed with alcohol or other cleansing solutions. Petroleum jelly or a similar lubricant may be placed over the submandibular area. Using a 22-25 gauge or smaller needle or a 5 mm or smaller lancet, the submandibular area will be entered by advancing the needle or lancet perpendicular to the animal's face. The volume of blood taken will be less than 10% (~150 uL) of the animal's total blood volume, given the one week recovery period between procedures.

V.4.4.3.2. Cardiac blood collection:

Animals will be anesthetized as described in V.4.1.2.1. Using an appropriately-sized needle (e.g., 22-25 gauge, 1 inch) and syringe (e.g., 1-3 cc), the needle will enter the chest cavity either through the diaphragm or through the intercostal muscles. When the needle has entered the heart, the plunger will be pulled back and blood will be collected. The animal will be euthanized immediately after blood collection as described in V.4.6.

V.4.4.4. Adjuvants: N/A

V.4.4.5. Monoclonal Antibody (MAb) Production: N/A

V.4.4.6. Animal Identification:

Animals will be identified by cage cards according to QSARC 804 (APHC, 2017b). The animals will be received from the vendor with a hole punch or ear tag identification number which will also ensure individual identification of animals. This number will be written on the animal's cage card. An identification number (e.g., the last 3 digits of the animal number or the number from the vendor) may be marked on the tail of each animal with a water-insoluble marker.

V.4.4.7. Behavioral Studies: N/A

V.4.4.8. Other Procedures:

V.4.4.8.1. Body Weight and Food/Water Consumption

Animals will be weighed at the start of test compound administration, at least weekly thereafter, and at termination. Food and water consumption will be monitored at least weekly for all animals by weighing the food hopper/water bottle or measuring the amount of water remaining. Food and water consumption will be measured per cage.

V.4.4.8.2 Observations:

A thorough physical examination of each animal will be performed by study personnel at a similar time at least once a week. The examination process may take place in conjunction with collection of biosamples. The examination process will consist of each animal being removed from its home cage, individually handled, and carefully observed. Observations will include, but not be limited to, evaluation of the skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or abnormal behavior (e.g., self-mutilation, walking backward). Twice daily during the week and once daily on weekends, an in-cage, general clinical observation of each animal will be performed by study personnel and/or animal care staff. All animals will be observed for signs of toxicity, morbidity and mortality.

V.4.4.9. Tissue Sharing:

Tissues from animals euthanized on this study may be made available to other personnel with an approved protocol if coordinated through the PI/SD. Tissue sharing will be allowed only if doing so does not affect the quality and validity of the study, or changes the euthanasia methods.

V.4.5. Study Endpoint:

The study endpoint for study animals is intervention euthanasia of moribund animals or euthanasia following the final day of test substance administration.

Intervention euthanasia will be conducted on moribund animals, but animals are not expected to become ill on this study, based on the literature. Furthermore, the primary goal of this range finding study is to ensure bioaccumulation and sufficient body burden of the chemicals for follow-up reproductive/developmental toxicity studies. However, given that the white-footed mouse is a wild-type prey species and prone to stress, it is not unexpected for animals to die during handling/procedures. Animals will be assessed for morbidity based on a weight of evidence of the following signs: impaired ambulation which prevents animals from reaching food/water; excessive weight loss or emaciation (20% body weight loss compared to controls); lack of physical or mental alertness; prolonged labored breathing (e.g., lasting longer than 8 hours and accompanied by extreme lethargy); unabated seizure activity; inability to urinate or defecate for greater than 24 hours; or a prolonged inability to remain upright (e.g., lasting more than 2 hours). The AV may be consulted to evaluate potentially moribund

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animals, unless the PI/SD plans to immediately euthanize the animal. Intervention euthanasia will be conducted on animals determined to be moribund.

V.4.6. Euthanasia:

Euthanasia will be accomplished by asphyxiation from CO₂ exposure IAW QSARC 800 (APHC, 2017a). Death of all animals euthanized by CO₂ will be ensured by thoracotomy or by opening the abdomen and puncturing the diaphragm. Thoracotomy will be accomplished by inserting a sharp blade into the chest cavity behind a rib and moving the blade the length of a rib.

V.5. Husbandry & Veterinary Care:

V.5.1. Husbandry Considerations:

Animal rooms will be maintained IAW QSARC 707 (APHC, 2017c) except as described herein. Animals will be provided *ad libitum* rodent chow that is certified free of contaminants. Analytical information on the nutrient and dietary contaminant levels of the rodent chow will be provided at the beginning of the study and when there is a change in the batch used, and will be included in the study records. Water will be provided *ad libitum* by water bottles. The automated watering system or reservoirs that feed into the racks may be used in emergency situations only. Analytical information on the contaminant levels (e.g., pesticides, metals, organic contaminants) in the drinking water used in the study will be provided prior to the study. Light cycle will be 12 hours light, 12 hours dark. Room temperature will be set at 68-79°F and humidity at 30-70%. Cage sanitation will be confirmed to be acceptable at least once daily by animal care staff. The animals will be housed in conventional plastic, solid-bottom shoebox cages with Diamond Dry bedding. All animals will undergo a 5-day acclimation period. Body weight and observational data may also be collected for animals by study personnel during the acclimation period in an attempt to more accurately monitor the health status of the animals in preparation for their use on study. However, animals will not be weighed or handled by study personnel within the first 24 hours after their arrival to the facility.

V.5.1.1. Study Room:

Studies will be conducted at the APHC animal facility, Bldg E-2100 or Bldg E-2101, housing room as assigned. All live animal work (dosing, weighing, bleeding) will occur in the assigned animal room.

V.5.1.2. Special Husbandry Provisions

Compound delivery will occur via water bottles and dose calculations will be determined based on consumptions of PFAS solutions over time. Water bottles and sipper tubes will be color coded according to treatment group. Water bottles and sipper tubes will be sanitized weekly. Water bottles must be made of polypropylene, given the compounds under investigation. PFAS dosing solutions may be stored in the animal room and will be labeled with concentration, preparation date, expiration date, and initials of the person responsible for mixing the solution. Food consumption for all study animals will be monitored based on weight of the food hopper. Therefore, water and food for all study animals will be provided by study staff in exception of QSARC SOP 707 (APHC, 2017c). Food and water levels will be monitored daily to ensure continuous supply. However, to reduce production of hazardous waste and fouling of solutions with repetitive refilling, water bottles may not necessarily be refilled when $\frac{1}{2}$ full as indicated in QSARC SOP 707 (APHC, 2017c). Care should be taken not to cause excessive spillage from the water bottles and the SD/PI should be notified when leaks or spills occur so they may be accounted for and cleaned up as necessary. Food enrichment may not be used due to food consumption monitoring.

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care:

Animals will routinely be observed no less than once daily by assigned veterinary medical personnel for husbandry conditions, humane care, and general health status. IAW current IACUC Policy, in the event an animal becomes ill or injured, veterinary or toxicology personnel will contact the AV or his/her designated backup who will consult with the SD/PI and determine the appropriate course of action. Animals will be observed by study personnel as described in 4.4.8.2. Animals appearing ill or displaying toxic signs will be assessed for moribundity and early removal from the study as described in section V.4.5.

V.5.2.2. Emergency Veterinary Medical Care:

In the event an animal requires after-hours emergency veterinary care, a veterinarian is available 24 hours a day, 7 days a week. In the case of an emergency health problem, if the PI or Primary Co-Investigator is unavailable or the investigator staff and veterinary staff cannot reach consensus on treatment of a study animal, the veterinarian has the authority to treat the animal, remove it from the experiment, institute appropriate measures to relieve severe pain or distress, or perform euthanasia if necessary. However, all decisions involving the treatment of a study animal in which a consensus cannot be reached will only be made after the veterinarian or designated backup

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veterinarian has actually observed and examined the animal in question. To facilitate communication, the animal care staff will maintain an emergency contact roster. In an emergency, the animal care staff will phone the numbers (office, home, and mobile) listed for the PI and Primary Co-Investigator. If the PI or Primary Co-Investigator cannot be reached by phone within 1 hour, then they are considered unavailable, and animal care staff has decision-making authority.

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy:

All animals will be socially housed and will be provided with certified, plastic-free form of enrichment (e.g., Nestlet) in their cage/pen.

V.5.3.2. Enrichment Restrictions:

Enrichment devices made from plastic or nylon (e.g., Nylabones, rodent retreats, rodent tubes) are unacceptable, given the compounds under investigation. Food enrichment may not be used due to food consumption monitoring.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

Personnel on Protocol	Activities to be performed on this protocol	Formal Training with lab mice or <i>Peromyscus</i>	Qualifications & Experience
Allison Jackovitz	Handling & observations	6/4/09; 2/18/15; 3/12/15; 12/8/16; 12/20/16; 12/22/16; 1/24/17	B.S., Biology 7+ Yrs animal research experience
	Facial/Submandibular venipuncture	6/19/12; 12/20/16; 12/22/16; 12/30/16	
	IP injections	2/18/15; 3/12/15; 12/20/16	
	CO ₂ anesthesia/euthanasia	3/12/15	
James Cox	Handling & observations	12/20/16	M.S., Biology 6+ Yrs animal research experience
	Facial/Submandibular venipuncture	12/20/16	
	IP injections	12/20/16	
Lee Crouse	Handling & observations	12/8/16; 12/20/16	M.S., Environmental Sciences 18+ Yrs animal research experience
	Facial/Submandibular venipuncture	12/20/16	
	IP injections	12/20/16	

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Adam Deck	Handling & observations	12/12/16; 12/20/16	B.S., Biology 15+ Yrs animal research experience
	Facial/Submandibular venipuncture	12/20/16	
	IP injections	12/20/16	
	CO ₂ anesthesia/euthanasia	8/25/11	
Theresa Hanna	Handling & observations	6/30/11; 2/12/15; 2/18/15; 2/25/15; 3/12/15; 12/12/16; 12/20/16	ALAT 15+ Yrs animal research experience
	Facial/Submandibular venipuncture	6/30/11; 6/19/12; 12/20/16	
	IP injections	6/30/11; 2/12/15; 2/18/15; 2/25/15; 3/12/15; 12/20/16	
	CO ₂ anesthesia/euthanasia	2/12/15; 3/12/15	
Adolph Januskiewicz	Handling & observations	12/8/16; 12/20/16	Ph.D., Pharmacology & Toxicology, M.S., Pharmacology & Toxicology, B.S., Biology & Chemistry 40+ Yrs animal research experience
	Facial/Submandibular venipuncture	12/20/16	
	IP injections	12/20/16	
	CO ₂ anesthesia/euthanasia	8/25/11	
Emily Lent	Handling & observations	10/12/07; 12/11/07; 1/23/08; 10/14/08; 2/12/15; 2/18/15; 12/12/16; 12/20/16	Ph.D., Natural Resources and Environmental Studies, M.S., Wildlife Biology 18+ Yrs animal research experience
	Facial/Submandibular venipuncture	10/14/08; 12/20/16	
	IP injections	10/14/08; 2/12/15; 2/18/15; 12/20/16	
	CO ₂ anesthesia/euthanasia	10/12/07; 12/11/07; 1/23/08	
Caroline Procell	Handling & observations	12/20/16; 12/22/16; 1/24/17	B.S., Biology 2+ Yrs animal experience
	Facial/Submandibular venipuncture	12/20/16; 12/22/16	
	IP injections	12/20/16	
Michael Quinn	Handling & observations	6/21/05; 10/14/08; 6/30/11; 2/25/15; 3/12/15; 12/20/16; 12/22/16; 1/24/17	Ph.D., Animal Science 18+ Yrs animal

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	Facial/Submandibular venipuncture	6/30/11; 5/1/12; 12/20/16; 12/22/16	research experience
	IP injections	6/30/11; 2/25/15; 3/12/15; 12/20/16	
Mark Way	Handling & observations	5/17/07; 6/24/09; 2/12/15; 2/18/15; 2/25/15; 3/12/15; 12/8/16; 12/20/16; 1/24/17	B.S., Biology 17+ Yrs animal research experience
	Facial/Submandibular venipuncture	12/20/16; 12/22/16	
	IP injections	2/12/15; 2/18/15; 2/25/15; 3/12/15; 12/20/16	
	CO ₂ anesthesia/euthanasia	6/24/09; 2/12/15; 3/12/15;	

VII. BIOHAZARD/SAFETY: Risks associated with this protocol include bites/scratches/needle sticks, transmission of zoonotic diseases, the development of animal allergies, and exposure to PFASs. To minimize risk, appropriate handling techniques will be used and appropriate PPE will be worn for all animal handling work. This includes (but may not be limited to) facemask, gloves, and disposable lab coat. Personnel will take precautions when filling water bottles to avoid splashes of test article treated water. Personnel will wash their hands upon completion of animal work. Applicable current TOX SOPs and PHC regulations (TOX SOP 046, TOX SOP 044, and USACHPPM 385-5) will be followed. These documents specify hazardous waste disposal, bite/scratch procedures, and zoonotic disease prevention. A sharps container will be present at all times when using sharps, and needles will not be recapped after entering animal tissue. Drinking water with the test article cannot be disposed of down the drain.

VIII. Enclosures

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IX. ASSURANCES:

IX.1. As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard/Safety: I have taken into consideration and made the proper coordination regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedures: (*Applicable if the research being conducted has the potential to cause more than momentary or slight pain or distress even if an anesthetic or analgesic is used to relieve the pain and/or distress.*)

I am conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress WILL / WILL NOT (circle one or both, if applicable) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

I. Unexpected Adverse Events: I acknowledge the responsibility for reporting unexpected adverse events IAW the most current version of IACUC Local Guidance Document 008 "Reportable Events".

Allison M. Jackovitz
Principal Investigator


(Signature)

4/17/17
(Date)

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IX.2. As the Primary Co-Investigator on this protocol, I provide the following assurances:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Authority: I understand that, as the Primary Co-Investigator, I am authorized and responsible for performing all procedures and manipulations as assigned to the SD/PI in the SD/PI's absence. This includes euthanasia of distressed animals.

C. Training: I verify that I am technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

D. Responsibility: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that I will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible, and conducting humane and lawful research.

E. Painful Procedures: I am conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress WILL or WILL NOT (circle one or both, if applicable) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

F. Unexpected Adverse Events: I acknowledge the responsibility for reporting unexpected adverse events IAW the most current version of IACUC Local Guidance Document 008 "Reportable Events".

Michael S. Quinn

(PRINT) First name, MI, Last name of Primary Co-Investigator

Michael S. Quinn

(Signature)

4/19/17

(Date)

Animal Use Protocol: Range finding for exposure to perfluoroalkyl acids via drinking water in the white-footed mouse (*Peromyscus leucopus*)

IX.2. As a Co-Investigator on this protocol, I provide the following assurances:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Authority: I understand that, as a Co-Investigator, I am authorized, responsible for, and willing to perform all procedures and manipulations as assigned to me by the SD/PI.

C. Training: I verify that I am technically competent and have been or will be properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the assigned procedures/manipulations performed by me.

D. Responsibility: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that I will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to participate in this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible, and conducting humane and lawful research.

E. Painful Procedures: I am participating in biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. I will follow the direction of the SD/PI relative to potential pain and/or distress and relief by use of anesthetics, analgesics, and/or tranquilizers.

F. Unexpected Adverse Events: I acknowledge the responsibility for reporting unexpected adverse events IAW the most current version of IACUC Local Guidance Document 008 "Reportable Events".

<u>Adam T. Deck</u>	<u></u>	<u>4/18/2017</u>
(PRINT)	(SIGNATURE)	(DATE)
First name, MI, Last name of Co-Investigator		

_____	_____	_____
(PRINT)	(SIGNATURE)	(DATE)
First name, MI, Last name of Co-Investigator		

_____	_____	_____
(PRINT)	(SIGNATURE)	(DATE)
First name, MI, Last name of Co-Investigator		

_____	_____	_____
(PRINT)	(SIGNATURE)	(DATE)
First name, MI, Last name of Co-Investigator		

APPENDIX A

REFERENCES

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Animal Use Protocol: Range finding for exposure to perfluoroalkyl acids via drinking water in the white-footed mouse (*Peromyscus leucopus*)

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PROTOCOL REVIEW, SUPPORT, APPROVAL SHEET				
PROTOCOL NUMBER: -67-17-04-01		TITLE: Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (Peromyscus leucopus)		
SUB-JONO TEST TYPE IACUC NUMBER				
1. SCIENTIFIC MERIT (PEER REVIEW)				
1a. Printed Name (First, MI, Last) Matthew A Bazar		1b. Title Biologist	1c. Signature BAZAR.MATTHEW.A.1241429322	1d. Date (yyyy/mm/dd) 20170321
2. DIRECTOR				
2a. Printed Name (First, MI, Last)		2b. Title	2c. Signature	2d. Date (yyyy/mm/dd)
3. PROGRAM MANAGER				
3a. Printed Name (First, MI, Last)		3b. Title	3c. Signature	3d. Date (yyyy/mm/dd)
4. ATTENDING VETERINARIAN				
4a. Printed Name (First, MI, Last) Michael R Bonhage		4b. Title Attending Veterinarian	4c. Signature BONHAGE.MICHAEL.R.1014029881	4d. Date (yyyy/mm/dd) 20170321
5. ANALYTICAL CHEMISTRY (If Applicable)				
5a. Printed Name (First, MI, Last)		5b. Title	5c. Signature	5d. Date (yyyy/mm/dd)
6. SAFETY MANAGER				
6a. Printed Name (First, MI, Last)		6b. Title	6c. Signature	6d. Date (yyyy/mm/dd)
7. STATISTICIAN (If Applicable)				
7a. Printed Name (First, MI, Last) Angela M Mound		7b. Title Statistician	7c. Signature MOUND.ANGELA.M.1243525361	7d. Date (yyyy/mm/dd)

PROTOCOL NUMBER: - 67 - 17-04-01 SUB-JONO TEST TYPE IACUC NUMBER		TITLE: Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (Peromyscus leucopus)	
8. SIO-QAT (GLP COMPLIANCE AND QA SUPPORT)			
8a. Printed Name (First, MI, Last)	8b. Title	8c. Signature	8d. Date (yyyy/mm/dd)
9. CHAIRMAN, IACUC			
9a. Printed Name (First, MI, Last) Kristin T. Newkirk	9b. Title Animal Care and Use Specialist	9c. Signature NEWKIRK, KRISTIN T. ORELL.1014786895	9d. Date (yyyy/mm/dd) 2017/04/20
10. INSTITUTIONAL OFFICIAL			
10a. Printed Name (First, MI, Last) John J. Resta	10b. Title Director, Army Public Health Center	10c. Signature RESTA, JOHN J. 229129305	10d. Date (yyyy/mm/dd)
11. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR			
11a. Printed Name (First, MI, Last) Allison M. Jackovitz	11b. Title Biologist	11c. Signature JACKOVITZ, ALISON M. ON.M.1367161236	11d. Date (yyyy/mm/dd) 20170424
12. OTHER ORGANIZATION(S) PROVIDING SUPPORT (AS NEEDED):			
12a. Printed Name (First, MI, Last)	12b. Title	12c. Signature	12d. Date (yyyy/mm/dd)
13. STUDY SPONSOR:			
13a. Printed Name (First, MI, Last)	13b. Title	13c. Signature	13d. Date (yyyy/mm/dd)

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01

Protocol Approval Date: 20 APRIL 2017

PROTOCOL
TITLE:

Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 1

Submission Date: Aug 30, 2017

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

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DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USA,
cn=JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2017.08.30 18:38:18 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: Sep 5, 2017

AURO receipt:

NEWKIRK.KRISTIN.TORELL.1014786895

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ou=USA, cn=NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2017.09.05 09:55:17 -04'00'

IACUC Designated Member Reviewer(s) (sign when review is completed):

NEWKIRK.KRISTIN.TORELL.1014786895

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DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USA, cn=NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2017.09.18 16:29:13 -04'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☒ YES ☐ NO ☐

Sep 18, 2017

Date

MOCCIA.KRINON.DODEKA.1167935563

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cn=MOCCIA.KRINON.DODEKA.1167935563
Date: 2017.09.18 16:01:07 -04'00'

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Sep 18, 2017

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USA,
cn=NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2017.09.18 16:30:01 -04'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Sep 18, 2017

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: **1**

Submission Date: **18 September 2017**

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

In the current study, four different perfluoroalkyl substances (PFASs) will be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days via drinking water. Blood samples will be collected every 7 days and analyzed for the concentration of PFASs. Blood samples will also be collected at termination and will be evaluated for PFASs concentration. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure via drinking water (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

The Attending Veterinarian and Study Staff discussed a concern that although the blood volume collected during training has been successful rather consistently, there may be a few animals where the volume collected from each facial/submandibular venipuncture (V.4.4.3.1.) may not always be sufficient for chemical analysis. To provide an additional option for blood collection in case this unlikely event occurs, we are requesting to add the modified tail clip method to our protocol as a potential method. This procedure has been vetted and approved by the APHC IACUC previously for a field study with *peromyscus* that required blood collection for lead analysis.

Similarly during training, the Attending Veterinarian and Study Staff concluded that the blood volume required at termination for analysis could not be collected via the originally proposed method (i.e., cardiac puncture). Therefore, animals will be deeply anesthetized with CO₂ as described in the protocol, euthanized via decapitation, and trunk blood will be collected via exsanguination.

Other modifications include: one typographical error, the addition of 6 control animals, the addition of one qualified technician, and a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
--	----------------	---

ADMINISTRATIVE - AURO/QAU/SEMO REVIEW	Administrative Modifications (e.g., <i>correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance</i>)	X
	Addition or deletion of a qualified technician, co-investigator, or study staff	X
MINOR Modifications (examples)	Change in animal usage [e.g., <i>vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)</i>]	X
	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: <u>Decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%. Addition of modified tail clip as a method for blood collection.</u>	X
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: <u>Change in blood collection method at termination and euthanasia method.</u>	X

3. PREVIOUSLY APPROVED MODIFICATIONS: (Hit 'Enter' after description of each modification to list all individually.)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
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4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 234 B: 0 C: 0 D: 234 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

Blood samples collected at termination will not be evaluated for immune parameters; it was misstated in the protocol.

New reference: APHC (2017). QSARC 801. Use and Maintenance of Decapitation Equipment (Aberdeen Proving Ground, MD).

New reference: Parasuraman S, Raneendran R, and Kesavan R 2010. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother; 1(2): 87-93.

Due to the need for additional assistance with weighing, holding, and bleeding animals, Thomas Sussan (HEF) will be added to the protocol as a qualified technician.

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

N/A

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). *(Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).*

This study includes four experiments with four compounds: PFOS, PFOA, PFHxS, and PFBS. Animals in each experiment should be physically and visually separate. Additionally, controls should be evenly spaced among the separate experiments for data integrity. For example, the first and second columns of a rack could be dedicated to PFOS and control animals, the third row could be empty, and the fourth and fifth columns could be dedicated to PFOA and control animals. Alternatively, separate racks could be used for each chemical, with control animals

evenly between the racks. Therefore, 3 additional males and 3 additional females are needed, making for a total of 12 control males and 12 control females, of which 3 per sex will be housed with each experiment/compound.

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.)*

***** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

For weekly blood draws, the volume taken will be decreased from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5% (V.4.4.3.1.). Weekly body weight measurements will be taken immediately prior to each weekly blood draw.

The Attending Veterinarian and Study Staff discussed a concern that although the blood volume collected during training has been successful rather consistently, there may be a few animals where the volume collected from each facial/submandibular venipuncture (V.4.4.3.1.) may not always be sufficient for chemical analysis. Analysis of circulating PFAS concentrations is a key objective of this study and including the modified tail clip method as an option will better ensure that the appropriate blood volume is collected. To perform this technique, each mouse will be placed on a stainless steel surface and the handler will hold the base of the tail; the handler merely limits movement if the mouse tries to leave the working surface. Another technician will prepare the tip of the tail with an appropriate antiseptic. Topical anesthesia may be applied to the tail at the discretion of the AV. If topical anesthesia is applied, adequate time (e.g., 30 minutes) will elapse prior to performing the tail clip in accordance with Parasuraman et al., 2010. Using a new, sterile razor or a new, sterile scalpel blade, the second technician will clip a maximum of 2 mm of the distal end of the tail with one short, quick movement from the top of the tail straight downward. A clotting agent such as a styptic pencil may be used if necessary to stop blood flow. To increase blood flow, the tail may be warmed prior to the cut being made and the tail may be stroked toward the site of the cut or "milked" immediately after the cut is made. Although some literature suggests that milking the tail can increase white blood cell counts and decrease the quality of the sample for clinical chemistry and hematology parameters, neither of these will be evaluated. Furthermore, serum does not contain white blood cells, so chemical analysis of serum will not be compromised. Blood may be collected in a capillary tube, vial, or other appropriate device. This method was selected by former Attending Veterinarian LTC Fitzhugh and study staff and approved by the APHC IACUC after rigorous efforts were made to assess the best survival blood collection techniques for peromyscus. LTC Fitzhugh noted that peromyscus did not appear to be adversely effected upon immediate release back into their enclosure. Again, it is unlikely that this technique will be used often in the study; however, the chemical analysis is vitally important for the success of the project and we feel it is important to have this secondary option available. If repeat sampling via modified tail-clip is necessary, the clot (if still there) will be removed. If the clot is no longer there or blood does not flow from where the clot was

removed, the AV will be consulted and will make a decision as to whether the entire procedure may be repeated.

The Attending Veterinarian and Study Staff discussed a concern that the blood volume required at termination for analysis could not be collected via the originally proposed method (i.e., cardiac puncture.) Therefore, rather than performing cardiac blood collection (V.4.4.3.2.) following CO2 anesthesia (V.4.1.2.1.), animals will be deeply anesthetized with CO2 as described, euthanized via decapitation, and trunk blood will be collected via exsanguination. As such, exsanguination will be the secondary method of euthanasia. Decapitation will be accomplished with sharp scissors or shears, IAW QSARC 801. Briefly, scissors or shears of the appropriate size for the species will be inspected for rust, cleanliness, and lubrication. The animal will be removed from its home cage and anesthetized as currently described in V.4.1.2.1. Once the animal has taken its last breathe and is unresponsive to a strong toe pinch, the shears/scissors will be placed around the neck of the animal as close to the head as possible and the head will be cut off. This will ensure rapid and quick severance in the correct position. Next, the animal will be inverted and exsanguinated and trunk blood will be collected in an appropriately-sized tube or container.

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.).

(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)

N/A

G. Changes to the personnel conducting this protocol (Section VI.). *(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)*

New procedures: Modified tail clip

Qualified personnel: Allison Jackovitz, Michael Quinn, Mark Way, Thomas Sussan

Training dates: 6, 13 September 2017

New procedures: Decapitation and exsanguination

Qualified personnel: Allison Jackovitz, Michael Quinn, Mark Way, Thomas Sussan

Training dates: 6 July, 30 August, 6, 13 September 2017

New qualified personnel: Thomas Sussan

Activities to be performed on this protocol: Handling & observations, facial/submandibular venipuncture, modified tail clip, decapitation and exsanguination

Formal Training with lab mice or Peromyscus: Handling & observations, facial/submandibular venipuncture, modified tail clip (6, 13 September 2017), decapitation and exsanguination (13 September 2017).

Qualifications & Experience:

Ph.D. Biochemistry, Cellular, and Molecular Biology

17+ Yrs animal research experience, specifically with a variety of mice

H. Changes in Biohazard/ Safety (Section VII).

N/A

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01 **Protocol Approval Date:** 20 APRIL 2017

PROTOCOL TITLE: Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (Peromyscus leucopus)

Modification Request #: 2 **Submission Date:** Sep 19, 2017

Study Director/ Principal Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USA,
cn=JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2017.09.19 15:45:26 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: Sep 26, 2017

AURO receipt:

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
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cn=NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2017.09.26 11:22:44 -04'00'

IACUC Designated Member Reviewer(s) (sign when review is completed):

Attending/ Alternate Veterinarian Review Signature: Approved via VVC: N/A ☒ YES ☐ NO ☐

Sep 26, 2017

Date

BONHAGE.MICHAEL.ROBERT.1014029881

Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
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cn=BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2017.09.26 13:44:05 -04'00'

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Sep 26, 2017

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
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cn=NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2017.09.26 14:49:25 -04'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Sep 26, 2017

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: 2

Submission Date: 19 September 2017

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

In the current study, four different perfluoroalkyl substances (PFASs) will be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days via drinking water. Blood samples will be collected every 7 days and analyzed for the concentration of PFASs. Blood samples will also be collected at termination and will be evaluated for PFASs concentration. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure via drinking water (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

The proposed modification is the option to separate animals in the event of incompatibility between cage-mates. Additionally, the proposed modification includes the clarification that the 6 animals added to the protocol in the previous modification belong to pain category D.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
ADMINISTRATIVE - AURO/QAU/SEMO REVIEW	Administrative Modifications (e.g., correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance)	
	Addition or deletion of a qualified technician, co-investigator, or study staff	
MINOR Modifications (examples)	Change in animal usage [e.g., vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)]	
	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	X

	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: <u>Clarification: 6 animals added in previous modification belong to pain category D.</u>	X
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: _____	

3. PREVIOUSLY APPROVED MODIFICATIONS: (*Hit 'Enter' after description of each modification to list all individually.*)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood collection, and a change in blood collection method at termination and euthanasia method.	6	18 September 2017

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 240 **B:** 0 **C:** 0 **D:** 240 **E:** 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

N/A

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

N/A

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3.) *(Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).*

N/A

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.).*
****** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

In the previous modification, 6 animals were added to the protocol, but pain category was not specified. All 6 animals belong to pain category D.

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.). *(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)*

At the recommendation of Animal Health Technician Rebecca Kilby, we would like the option to separate animals in the event of incompatibility between cage-mates. Ms. Kilby has maintained training peromyscus at APHC for many months and may have to singly house some peromyscus on this protocol for their safety.

Section "V.5.1.3. Exceptions" of the protocol is changed from "N/A" to "Every attempt will be made to group house all animals. In instances of incompatibility and aggression between cage mates that cannot be remedied, animals may be separated and housed individually."

Add the following statement to Section "V.5.3.1. Enrichment Strategy": "Animals that have been singly housed due to unresolved cage aggression may be offered additional enrichment (e.g., Nestlet) and will be within sight and smell of conspecifics on the cage rack."

G. Changes to the personnel conducting this protocol (Section VI.). *(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)*

N/A

H. Changes in Biohazard/ Safety (Section VII).

N/A

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01

Protocol Approval Date: 20 APRIL 2017

**PROTOCOL
TITLE:**

Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 3

Submission Date: Oct 6, 2017

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2017.10.06 11:59:16 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 6 Oct 17

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by
SEYFERT.ALLISON.MARIE.1473772210
Date: 2017.10.06 13:29:33 -04'00'

IACUC Designated Member Reviewer(s) (*sign when review is completed*):

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USA, cn=NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2017.10.17 10:28:52 -04'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☒ YES ☐ NO ☐

Oct 17, 2017

Date

BONHAGE.MICHAEL.ROBERT.1014029881

Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2017.10.17 10:53:52 -04'00'

Safety Review Signature (*if applicable*):

Date

LTACO/GLP Review Signature (*if applicable*):

Date

IACUC Chair Review Signature:

Oct 17, 2017

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USA, cn=NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2017.10.17 11:13:00 -04'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Oct 17, 2017

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: **3**

Submission Date: **6 October 2017**

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

In the current study, four different perfluoroalkyl substances (PFASs) will be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days via drinking water. Blood samples will be collected every 7 days and analyzed for the concentration of PFASs. Blood samples will also be collected at termination and will be evaluated for PFASs concentration. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure via drinking water (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

Peromyscus leucopus is a prey species and thus, prone to stress. Post-procedure, some animals appear stunned, dull, or non-responsive because of stress. Although some animals recover, others become moribund. The proposed modification adds the option for trained personnel to perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber). Study personnel have trained on cervical dislocation using CD-1 mouse cadavers in conjunction with CO2 anesthesia and decapitation training. Species-specific training has yet to occur for all study personnel. All training records will be submitted to the IACUC prior to any procedures being done on the study itself by personnel without training documented at the time of submission of this Modification #3 request.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
ADMINISTRATIVE - AURO/QAU/SEMO REVIEW	Administrative Modifications (e.g., <i>correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance</i>)	X
	Addition or deletion of a qualified technician, co-investigator, or study staff	
R Modifi cation s (exam	Change in animal usage [e.g., <i>vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)</i>]	

	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: <u>Trained personnel may perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber).</u>	X
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: _____	

3. PREVIOUSLY APPROVED MODIFICATIONS: (*Hit 'Enter' after description of each modification to list all individually.*)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood	6	18 September 2017

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
	collection, and a change in blood collection method at termination and euthanasia method.		
2	Single housing in the event of cagemate incompatibility and extra enrichment for those separated. Clarification about the 6 animals added in modification 1 belonging to Category D.		26 September 2017

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 240 B: 0 C: 0 D: 240 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

New reference: American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition.

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

N/A

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). (*Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]*).

N/A

E. Changes to technical methods (Section V.4.). (*Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.*).
***** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH**

FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.

V.4.6. The modification is to add cervical dislocation as an optional euthanasia method for clearly moribund animals. These animals would be likely to expire prior to being transported to the procedure room and placed into the CO2 chamber for euthanasia, therefore cervical dislocation is a more immediate and humane method of euthanasia. These animals may be exhibiting agonal breathing, appear non-responsive, and not likely to recover from the stress associated with handling during the bleeding procedure. Cervical dislocation is an American Veterinary Medical Association-approved method of euthanasia for small birds, poultry, mice, immature rats (< 200 g), and rabbits. The procedure for mice and rats will be done in accordance with QSARC 800: the animal will be positioned face down in a prone position, the thumb and index finger are placed on either side of the neck at the base of the skull; with the other hand, the base of the tail or the hind limbs are held and quickly pulled up and back until a distinct separation is felt (the separation of the cervical vertebra from the skull). Only trained personnel will perform the procedure.

Cervical dislocation, if performed, will take place in the animal room, as the intent of adding this procedure is to be able to immediately alleviate pain or distress in a moribund animal. An attempt to move the animal to the necropsy room to cervically dislocate it there will cause a delay in the alleviation of the distress, and potentially cause more distress. Every attempt will be made to perform the procedure out of the line of sight of other animals in the room. Death will be confirmed by bilateral thoracotomy.

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.).

(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)

N/A

G. Changes to the personnel conducting this protocol (Section VI.).

(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)

New procedure: Cervical dislocation

Qualified personnel: Training & dates:

Allison Jackovitz	06/2009- Small Animal Handling Workshop (MRICD); 09/2017- CD-1 Mus CO2 anesthesia and decapitation (cervical dislocation was performed on the euthanized mice prior to decapitation with sharp scissors) (APHC)
Adam Deck	08/2011- Euthanasia of anesthetized Peromyscus via cervical dislocation (USACHPPM)

H. Changes in Biohazard/ Safety (Section VII).

N/A

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01

Protocol Approval Date: 20 APRIL 2017

**PROTOCOL
TITLE:**

Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 4

Submission Date: Jan 12, 2018

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.01.12 12:19:54 -05'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 12 January 2018

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.01.19 09:50:32 -05'00'

IACUC Designated Member Reviewer(s) (*sign when review is completed*):

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.01.25 11:26:39 -05'00'

BONHAGE.MICHAEL.ROBERT.1014029881

Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2018.01.25 11:40:15 -05'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☒ YES ☐ NO ☐

Jan 25, 2018

Date

BONHAGE.MICHAEL.ROBERT.1014029881

Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2018.01.25 11:40:44 -05'00'

Safety Review Signature (*if applicable*):

Date

LTACO/GLP Review Signature (*if applicable*):

Date

IACUC Chair Review Signature:

Jan 25, 2018

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.01.25 12:08:13 -05'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Jan 25, 2018

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: 4

Submission Date: 12 January 2018

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

The original protocol included four experiments with four chemicals and utilized shared control animals. Briefly, four perfluoroalkyl substances (PFASs) will to be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days. Blood samples will be collected every 7 days and analyzed for the concentration of PFASs. Blood samples will also be collected at termination and will be evaluated for PFASs concentration. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

In the first portion of this range finding, three chemicals (PFOA, PFHxS, and PFBS) were administered to the white-footed mouse for 28 consecutive days via drinking water. This modification proposes to refine the exposure route for testing with the fourth chemical (PFOS), due to unforeseen solubility issues. Briefly, PFOS will be dissolved in an appropriate solvent and applied to food treats. Once a day for 28 days, animals will be placed in individual enclosures and given a food treat. Then, animals will be given an appropriate period of time (e.g., several hours) undisturbed to consume treats, in an effort to reduce stress. Later, animals will be returned to their home cages. As such, animals will still be housed socially, with the exception of behavioral incompatibility, as described in a previous modification.

Since we are proposing a refined exposure route for dosing with PFOS and the original protocol was designed to utilize shared controls, additional animals are needed to serve as controls for the second portion of the study and the alternative exposure route. As such, we are requesting that 12 animals from the training protocol be transferred to this protocol to serve as control animals.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
TRATIVE - AURO/Q AU/SEM O	Administrative Modifications (e.g., correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance)	X

	Addition or deletion of a qualified technician, co-investigator, or study staff	
MINOR Modifications (examples)	Change in animal usage [e.g., vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)]	X
	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	X
	Changes in dosing procedures (e.g., dose, volume, or timing)	X
	Other: _____	
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: _____	

3. PREVIOUSLY APPROVED MODIFICATIONS: (Hit 'Enter' after description of each modification to list all individually.)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
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Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood collection, and a change in blood collection method at termination and euthanasia method.	6	18 September 2017
2	Single housing in the event of cagemate incompatibility and extra enrichment for those separated. Clarification about the 6 animals added in modification 1 belonging to Category D.		26 September 2017
3	Option for trained personnel to perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber).		17 October 2017

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 240 B: 0 C: 0 D: 240 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

New references:

Hays, L. E., C. D. Carpenter, and S. L. Petersen. 2002. Evidence that GABAergic neurons in the preoptic area of the rat brain are targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin during development. Environ Health Perspect 110 Suppl 3:369-76.

McCaffrey, K. A., B. Jones, N. Mabrey, B. Weiss, S. H. Swan, and H. B. Patisaul. 2013. Sex specific impact of perinatal bisphenol A (BPA) exposure over a range of orally administered doses on rat hypothalamic sexual differentiation. Neurotoxicology 36:55-62.

Patisaul, H. B., K. T. Burke, R. E. Hinkle, H. B. Adewale, and D. Shea. 2009. Systemic administration of diarylpropionitrile (DPN) or phytoestrogens does not affect anxiety-related behaviors in gonadally intact male rats. Horm Behav 55 (2):319-28.

Patisaul, H. B., S. C. Roberts, N. Mabrey, K. A. McCaffrey, R. B. Gear, J. Braun, S. M. Belcher, and H. M. Stapleton. 2013. Accumulation and endocrine disrupting effects of the flame retardant mixture Firemaster(R) 550 in rats: an exploratory assessment. J Biochem Mol Toxicol 27 (2):124-36.

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

V.1.2. Administration of Test Substance. As a refinement to the previously approved exposure route, PFOS will be dissolved in an appropriate solvent (e.g., methanol) and applied to food treats via pipette. At least 24 hours will be given for the methanol to evaporate off, leaving only the test compound on the treat. "Dosing" will consist of giving animals a food treat containing the compound of interest. After administering the compound (e.g., dropping each treat into each enclosure) study staff will leave the room. Animals will be given an appropriate period of time (e.g., several hours) undisturbed to consume treats, in an effort to reduce stress. In the event that an animal does not consume the treat during undisturbed period, the treat will be removed, an alternative food treat will be provided, and more time will be given. Food treats are a well-documented oral route of exposure for toxicity tests with rodents (Hays, Carpenter, and Petersen 2002; McCaffrey et al. 2013; Patisaul et al. 2009; Patisaul et al. 2013). The concentration of the test compound in methanol will be verified by appropriate analytical methods.

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). *(Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).*

In the first portion of this range finding, three chemicals (PFOA, PFHxS, and PFBS) were administered to the white-footed mouse for 28 consecutive days via drinking water. This modification proposes to refine the exposure route for testing with the fourth chemical (PFOS), due to unforeseen solubility issues. Since we are proposing a refined exposure route for dosing with PFOS and the original protocol was designed to utilize shared controls, additional animals are needed to serve as controls for the second portion of the study. As such, we are requesting that 12 animals from the training protocol be transferred to this protocol.

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.).*
***** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

V.4.4.6. Animal Identification. Individual cages used during "dosing" will be labeled with the cage card corresponding to each animal inhabitant, according to QSARC 804 (APHC, 2017). As such, a second set of cage cards may be printed and maintained on the individual enclosures used for "dosing."

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.).

(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)

V.5.1.2. Special Husbandry Provisions. Compound delivery will no longer occur via water bottles; therefore, animals will receive water ad libitum via the automatic watering system (Lixits). Animals have been successfully drinking from the automatic watering system for several months; therefore a transition period is not needed prior to initiating the experiment. In lieu of receiving compound in drinking water via water bottles, once a day for 28 days, animals will be removed from their home cages and placed in individual enclosures to allow consumption of the test compound, as described in 5.B. (V.1.2.). Then, animals will be returned to their home cages. As such, animals will still be housed socially, with the exception of behavioral incompatibility, as described in a previous modification. Animals will be acclimated to removal from the home cage and receiving treats prior to initiation of the study.

G. Changes to the personnel conducting this protocol (Section VI.). *(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)*

N/A

H. Changes in Biohazard/ Safety (Section VII).

N/A

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01

Protocol Approval Date: 20 APRIL 2017

PROTOCOL
TITLE:

Rane finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 5

Submission Date: Feb 1, 2018

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.02.01 14:48:28 -05'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☐

VVC ☐

Received Date: Feb 1, 2018

AURO receipt:

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by
NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.02.01 15:15:44 -05'00'

IACUC Designated Member Reviewer(s) (sign when review is completed):

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.02.01 15:16:04 -05'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☐ YES ☐ NO ☐

Feb 2, 2018

Date

MOCCIA.KRINON.DODEKA.1167935563

Digitally signed by MOCCIA.KRINON.DODEKA.1167935563
Date: 2018.02.02 12:37:05 -05'00'

Safety Review Signature (if applicable):

Date

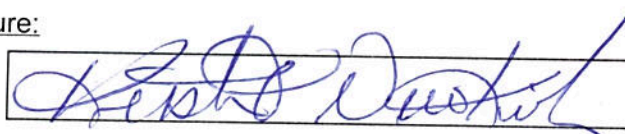
LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

2 Feb 2018

Date



Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: 2 Feb 2018

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: 5

Submission Date: 1 February 2018

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

The original protocol included four experiments with four chemicals and utilized shared control animals. Briefly, four perfluoroalkyl substances (PFASs) will to be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days. Blood samples will be collected every 7 days and analyzed for the concentration of PFASs. Blood samples will also be collected at termination and will be evaluated for PFASs concentration. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

In the first portion of this range finding, three chemicals (PFOA, PFHxS, and PFBS) were administered to the white-footed mouse for 28 consecutive days via drinking water. The previous modification was to refine the exposure route for testing with the fourth chemical (PFOS), due to unforeseen solubility issues. This modification includes clarification on the acclimation and treat-feeding processes. Briefly, PFOS will be dissolved in an appropriate solvent and applied to food treats.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
ADMINISTRATIVE - AURO/QAU/SEMO REVIEW	Administrative Modifications (e.g., correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance)	☒
	Addition or deletion of a qualified technician, co-investigator, or study staff	☐
MINOR Modifications (examples)	Change in animal usage [e.g., vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)]	☐
	Need to repeat an experiment without the addition of animals	☐
	Addition/ change of sample collection times	☐

	Additional noninvasive sampling	
	Changes in acclimation or recovery period	X
	Special housing request or change in husbandry procedures	X
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: _____	
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: _____	

3. PREVIOUSLY APPROVED MODIFICATIONS: (Hit 'Enter' after description of each modification to list all individually.)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood collection, and a change in blood collection method at termination and euthanasia method. Single housing in the event of cagemate	6	18 September 2017

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
2	incompatability and extra enrichment for those separated. Clarification about the 6 animals added in modification 1 belonging to Category D.		26 September 2017
3	Option for trained personnel to perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber).		17 October 2017
4	Refine exposure route for testing with the fourth chemical. Addition of 12 animals.	12	25 January 2018

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 252 B: 0 C: 0 D: 252 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

N/A

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

N/A

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). (Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).

N/A

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.).*
***** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

N/A

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.). *(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)*

V.5.1.2. Special Husbandry Provisions. As discussed in the previous modification, compound delivery will no longer occur via water bottles. In lieu of receiving compound in drinking water via water bottles, once a day for 28 days, animals will be removed from their home cages and placed in individual enclosures to allow consumption of the test compound, as described in 5.B. (V.1.2.). Although animals will receive water ad libitum via the automatic watering system (Lixits) during dosing, animals will not have ad libitum access to rodent chow during the compound delivery period (i.e., when separated and offered the compound-treated food 'treat').

Animals will be acclimated to removal from the home cage and receiving food treats prior to initiation of the study. A variety of food treats may be used. Methanol may be applied via pipette to the food treats and given at least 24 hours to evaporate off, during the acclimation period to test acceptance and any aversion to the food treat options.

G. Changes to the personnel conducting this protocol (Section VI.). *(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)*

N/A

H. Changes in Biohazard/ Safety (Section VII).

N/A

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01

Protocol Approval Date: 20 APRIL 2017

**PROTOCOL
TITLE:**

Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 6

Submission Date: Mar 2, 2018

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.03.02 11:04:20 -05'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 2 Mar 18

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by
SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.03.02 11:50:14 -05'00'

IACUC Designated Member Reviewer(s) (*sign when review is completed*):

BONHAGE.MICHAEL.ROBERT.1014029881

Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2018.03.13 15:38:45 -04'00'

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.03.14 14:15:19 -04'00'

Attending/ Alternate Veterinarian Review Signature: Approved via VVC: N/A ☒ YES ☐ NO ☐

Mar 13, 2018

Date

BONHAGE.MICHAEL.ROBERT.1014029881

Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2018.03.13 15:38:59 -04'00'

Safety Review Signature (*if applicable*):

Date

LTACO/GLP Review Signature (*if applicable*):

Date

IACUC Chair Review Signature:

Mar 14, 2018

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.03.14 14:16:02 -04'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Mar 14, 2018

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: 6

Submission Date: 2 March 2018

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

The original protocol included four experiments with four chemicals and utilized shared control animals. Briefly, four perfluoroalkyl substances (PFASs) will be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days. Blood samples will be collected every 7 days and analyzed for the concentration of PFASs. Blood samples will also be collected at termination and will be evaluated for PFASs concentration. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

Given the sensitivity of the species involved, the Study Director/Principal Investigator attempted to reduce handling and invasive procedures while still meeting the study objectives. As such, in the first portion of this range finding, three chemicals (PFOA, PFHxS, and PFBS) were administered to the white-footed mouse for 28 consecutive days via drinking water. Due to unforeseen solubility issues with PFASs (e.g., the chemical partitioning at the air-water interface) and the fact that PFASs are more soluble in methanol than water, the exposure route was refined in Modification 4. Per Modification 4, PFOS was dissolved in an appropriate solvent (methanol), applied to food treats via pipette, and methanol was given time to evaporate off, leaving only the test compound on the treat. Per Modification 5, all animals were acclimated to food treats (with methanol applied to them and with at least 24 hours for the solvent to evaporate off) prior to initiating the study. During the acclimation period, all animals consumed their food treats. However, within three days of testing with PFOS, medium- and high-dose animals developed an aversion to consuming various treats and even un-dosed treats. Therefore, the current modification proposes to expose animals to PFOS via oral gavage.

Oral gavage is the only other physiologically relevant exposure route. Since PFASs are environmental contaminants, the test compound must be administered via a route that undergoes first pass metabolism through the liver. Therefore, an alternate exposure route (such as injection) is not physiologically relevant and not an option. Although the proposed change in exposure route is more invasive than previously approved methods, it will not change the overall Pain Category, as all animals are already classified as Category C, based on facial/submandibular venipuncture. Given that the animals have been acclimating at the Army Public Health Center for many months and based on the lessons learned thus far, the

SD/PI anticipates that with additional training, oral gavage can be successfully performed on the animals.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
ADMINISTRATIVE - AURO/QAU/SEMO REVIEW	Administrative Modifications (e.g., correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance)	X
	Addition or deletion of a qualified technician, co-investigator, or study staff	X
MINOR Modifications (examples)	Change in animal usage [e.g., vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)]	
	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	X
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: _____	
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	

	Other: <u>change in exposure route</u>	X
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3. PREVIOUSLY APPROVED MODIFICATIONS: (*Hit 'Enter' after description of each modification to list all individually.*)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood collection, and a change in blood collection method at termination and euthanasia method.	6	18 September 2017
2	Single housing in the event of cagemate incompatibility and extra enrichment for those separated. Clarification about the 6 animals added in modification 1 belonging to Category D.		26 September 2017
3	Option for trained personnel to perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber).		17 October 2017
4	Refine exposure route for testing with the fourth chemical. Addition of 12 animals.	12	25 January 2018
5	Clarification about acclimation and treat-feeding procedures.		02 February 2018

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 252 B: 0 C: 0 D: 252 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

New reference:

American Association for Laboratory Animal Science. 2002. Laboratory Animal Data: Quick Reference Guide, 2nd edition.

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

V.1.2. Administration of Test Substance. The test substance will be dissolved or suspended in distilled water. Dosing solutions will be prepared shortly before dosing. Dosing solutions will be constantly mixed during dosing, to prevent the test substance from partitioning at the water-air interface (a property which precludes dosing via water bottles). Constant concentration dosing via oral gavage will be used based on maximum dose volume restrictions and minimum feasible delivery options. The maximum dose volume given in a single dose will not exceed 10 mL/kg of body weight, per Laboratory Animal Data: Quick Reference Guide (2002). Doses will be calculated according to the body weight measured on the day of dosing.

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). *(Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).*

N/A

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.).*
****** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

V.4.1.2.2. Pre- and Post-Procedural Provisions. Animals will be observed post-gavage in the manner used post-bleeding. Specifically, Study Staff will scan through cages in numerical order (also the order in which procedures are conducted) repeatedly. No one cage will be observed for an extended period of time, as it induces stress in this species. Study Staff will use a similar procedure to observe animals 1-2 hours after the last animal is dosed for signs of distress (e.g., labored breathing, wheezing, gasping, dullness, changes in gait, etc.) on weekdays. On weekends and holidays, post-procedural observations will be conducted by Animal Health Technicians.

V.4.4.8 Other Procedures. Once daily for 28 consecutive days, each peromyscus will be gently restrained or "scruffed" by pinching the skin at the base of the neck, behind the ears, between the thumb and fingers. Animals will be positioned upright in a vertical position. Oral gavage will be performed with an appropriately sized oral gavage needle or feeding tube for the species (e.g., 18-24 gauge needle, 1-2 inches), per Laboratory Animal Data: Quick Reference Guide (2002). The gavage needle will be gently slid down the animal's esophagus until the hub of the gavage needle is at the opening of the animal's mouth. If any resistance is felt during the gavage procedure, the gavage needle will be removed and the animal is briefly released before the procedure is attempted again. Once the material has been dispensed and upon return to the home cage, the animal will be observed for any signs of aspiration and/or test material around the outside of the mouth. Based on previous studies at APHC and discussions with several Subject Matter Experts (SMEs), flexible gavage tubes will not be used, given the lack of control with the material.

V.4.5. Study Endpoint. The study endpoint for study animals is still euthanasia following the final day of test substance administration or intervention euthanasia for moribund animals. For gavage incidents, such as aspiration of test material, animals will be observed frequently throughout the remainder of the day and a decision about euthanasia will be made by the end of the day. If a punctured esophagus is suspected, animals will be euthanatized as soon as they shows signs (e.g., changes in gait and/or significant, unexpected weight loss).

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.).

(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)

V.5.1.2. Special Husbandry Provisions. Since compound delivery will no longer occur via food treats, animals will no longer be placed in individual enclosures to allow consumption of the test compound. As such, animals will always be socially housed, with the exception of behavioral incompatibility, as described in a previous modification. Additionally, since compound delivery will no longer occur via food treats, animals will have ad libitum access to water and rodent chow.

G. Changes to the personnel conducting this protocol (Section VI.).

(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)

New procedures: Oral gavage

Qualified personnel: Allison Jackovitz, Michael Quinn, Mark Way

Training dates: 5, 6, 7 March 2018; December 2016

Qualified personnel: Thomas Sussan

Training dates: 5, 6 March 2018

New qualified personnel: Jarod Hanson

Activities to be performed on this protocol: Handling & observations, oral gavage

Formal training with peromyscus: 5, 6, 7 March 2018

Qualifications & Experience: DVM, Ph.D., Infectious Diseases/Virology, 24+ Yrs animal research experience

Training records will be submitted to the AURO office as completed.

H. Changes in Biohazard/ Safety (Section VII).

N/A

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01 **Protocol Approval Date:** 20 APRIL 2017

PROTOCOL TITLE: Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (Peromyscus leucopus)

Modification Request #: 7 **Submission Date:** Mar 23, 2018

Study Director/ Principal Investigator Signature: JACKOVITZ.ALLISON.MARIE.1367161236 Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.03.23 15:01:51 -04'00'

For review use only: Review Process: ADMIN ☒ FCR ☐ DMR ☐ VVC ☐

Received Date: 23 March 18 AURO receipt: SEYFERT.ALLISON.MARIE.1473772210 Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.03.23 15:04:19 -04'00'

IACUC Designated Member Reviewer(s) (sign when review is completed):

Attending/ Alternate Veterinarian Review Signature: Approved via VVC: N/A ☐ YES ☐ NO ☐

Date

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Mar 23, 2018

Date

KEFAUVER.MICHAEL.P.1229209678 Digitally signed by KEFAUVER.MICHAEL.P.1229209678
Date: 2018.03.23 15:15:21 -04'00'

IACUC Chair Review Signature:

Mar 23, 2018

Date

NEWKIRK.KRISTIN.TORELL.1014786895 Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.03.23 15:45:33 -04'00'

Modification IACUC APPROVED ☐ GLP REVIEWED ☒ Date: 23 March 18

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: 7

Submission Date: 23 March 2018

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

The original protocol included four experiments with four chemicals and utilized shared control animals. Briefly, four perfluoroalkyl substances (PFASs) will be administered to the white-footed mouse (*Peromyscus leucopus*) for 28 consecutive days. Blood samples will be collected every 7 days and analyzed for the concentration of PFASs. Blood samples will also be collected at termination and will be evaluated for PFASs concentration. This study will provide information on how various PFASs accumulate in the body with repeated, daily exposure (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs.

Given the sensitivity of the species involved, the Study Director/Principal Investigator attempted to reduce handling and invasive procedures while still meeting the study objectives. As such, in the first portion of this range finding, three chemicals (PFOA, PFHxS, and PFBS) were administered to the white-footed mouse for 28 consecutive days via drinking water. Due to unforeseen solubility issues with PFASs (e.g., the chemical partitioning at the air-water interface) and the fact that PFASs are more soluble in methanol than water, the exposure route was refined in Modification 4. Per Modification 4, PFOS was dissolved in an appropriate solvent (methanol), applied to food treats via pipette, and methanol was given time to evaporate off, leaving only the test compound on the treat. Per Modification 5, all animals were acclimated to food treats (with methanol applied to them and with at least 24 hours for the solvent to evaporate off) prior to initiating the study. During the acclimation period, all animals consumed their food treats. However, within three days of testing with PFOS, medium- and high-dose animals developed an aversion to consuming various treats and even un-dosed treats. Therefore, the previous modification proposed to expose animals to PFOS via oral gavage.

The current administrative modification proposes to use corn oil as the vehicle (and vehicle control) rather than distilled water.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
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ADMINISTRATIVE - AURO/QAU/SEMO REVIEW	Administrative Modifications (e.g., <i>correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance</i>)	X
	Addition or deletion of a qualified technician, co-investigator, or study staff	
MINOR Modifications (examples)	Change in animal usage [e.g., <i>vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)</i>]	
	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: _____	
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: _____	

3. PREVIOUSLY APPROVED MODIFICATIONS: (*Hit 'Enter' after description of each modification to list all individually.*)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood collection, and a change in blood collection method at termination and euthanasia method.	6	18 September 2017
2	Single housing in the event of cagemate incompatibility and extra enrichment for those separated. Clarification about the 6 animals added in modification 1 belonging to Category D.		26 September 2017
3	Option for trained personnel to perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber).		17 October 2017
4	Refine exposure route for testing with the fourth chemical. Addition of 12 animals.	12	25 January 2018
5	Clarification about acclimation and treat-feeding procedures.		02 February 2018
6	Change in exposure route to oral gavage.		14 March 2018

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 252 B: 0 C: 0 D: 252 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

Because of its unique chemical properties, when PFOS is mixed with water, it partitions at the air-water interface. Specifically, the hydro-oleophobic perfluorocarbon chain stretches into the air bubbles and only the polar head (e.g., sulfonate) is in the water. Therefore, when samples

are taken from the solution beneath the bubbles, the concentration of PFOS is much lower in (and in an inconsistent manner) than intended.

When PFOS is mixed with corn oil and rocked, it forms a uniform suspension, which is superior for delivering the intended dosage accurately and consistently.

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

N/A

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). *(Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).*

N/A

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.).*
***** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

N/A

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.). *(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)*

N/A

G. Changes to the personnel conducting this protocol (Section VI.). *(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)*

N/A

H. Changes in Biohazard/ Safety (Section VII).

N/A

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01 **Protocol Approval Date:** 20 APRIL 2017

PROTOCOL TITLE: Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (Peromyscus leucopys)

Modification Request #: 8 **Submission Date:** May 8, 2018

Study Director/ Principal Investigator Signature: JACKOVITZ.ALLISON.MARIE.1367161236 Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.05.08 08:57:28 -04'00'

For review use only: Review Process: ADMIN ☐ FCR ☐ DMR ☒ VVC ☐

Received Date: 8 May 18 AURO receipt: SEYFERT.ALLISON.MARIE.1473772210 Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.05.08 10:04:07 -04'00'

IACUC Designated Member Reviewer(s) (sign when review is completed):

BONHAGE.MICHAEL.ROBERT.1014029881 Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2018.05.16 15:44:03 -04'00'

NEWKIRK.KRISTIN.TORELL.1014786895 Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.05.21 09:52:51 -04'00'

Attending/ Alternate Veterinarian Review Signature: Approved via VVC: N/A ☒ YES ☐ NO ☐

May 16, 2018

Date

BONHAGE.MICHAEL.ROBERT.1014029881 Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2018.05.16 15:44:50 -04'00'

Safety Review Signature (if applicable):

May 16, 2018

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

May 21, 2018

Date

NEWKIRK.KRISTIN.TORELL.1014786895 Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.05.21 09:53:38 -04'00'

Modification IACUC APPROVED ☒ GLP REVIEWED ☐ Date: May 21, 2018

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: 8

Submission Date: 8 May 2018

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

The original protocol included four experiments with four chemicals where the white-footed mouse (*Peromyscus leucopus*) was exposed to one of four per- and polyfluoroalkyl substances (PFAS) for 28 consecutive days.

Given the sensitivity of the species involved, the Study Director/Principal Investigator attempted to reduce handling and invasive procedures while still meeting the study objectives: to provide information on how various PFASs accumulate in the body with repeated, daily exposure (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs. As such, in the first portion of this range finding, three chemicals (PFOA, PFHxS, and PFBS) were administered to the white-footed mouse for 28 consecutive days via drinking water.

Due to unforeseen solubility issues with PFAS (e.g., the chemical partitioning at the air-water interface) and the fact that PFAS are more soluble in methanol than water, the exposure route was refined in Modification 4 for testing with the fourth chemical. Per Modification 4, PFOS was dissolved in an appropriate solvent (methanol), applied to food treats via pipette, and methanol was given time to evaporate off, leaving only the test compound on the treat. Per Modification 5, all animals were acclimated to food treats (with methanol applied to them and with at least 24 hours for the solvent to evaporate off) prior to initiating the study. During the acclimation period, all animals consumed their food treats. However, within three days of testing with PFOS, medium- and high-dose animals developed an aversion to consuming various treats and even un-dosed treats. Therefore, per Modification 6, animals were exposed to PFOS via oral gavage. Per Modification 7, the vehicle (and vehicle control) was adjusted from water to corn oil.

Modification 8 seeks to add 152 animals to the protocol for testing with two additional chemicals, perfluorononanoic acid (PFNA) and tridecafluorooctane-1-sulphonic acid (6:2 FTS), at the request of the sponsor. PFNA and 6:2 FTS are found in drinking water and human serum throughout the United States, oftentimes near military bases and training sites, and both are gaining increased attention by regulators.

As an additional part of Modification 8, we would like to repeat a smaller, modified experiment with PFHxS. Within three days of exposing the white-footed mouse to PFOS suspended in oil

and delivered via oral gavage, we began seeing toxicity. However, in 28 days of testing with PFOA, PFHxS, and PFBS via drinking water, no toxicity was observed, and tissue weights and gross pathology were unaffected. By conducting a smaller, modified experiment where PFHxS is suspended in oil and delivered via oral gavage (as was done successfully with PFOS), we will compare the results of the two exposure routes, and extrapolate the results to the other two chemicals administered via drinking water (PFOA and PFBS) for follow-up studies. Through a smaller, modified experiment we hope to gain data to best design reproductive studies while minimizing the number of animals involved and without entirely repeating experiments. As an additional measure to minimize animal use, testing with PFHxS will only be conducted at two doses and shared controls will be utilized for testing with PFNA, 6:2 FTS, and PFHxS.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
ADMINISTRATIVE – AURO/QAU/SEMO REVIEW	Administrative Modifications (e.g., <i>correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance</i>)	
	Addition or deletion of a qualified technician, co-investigator, or study staff	X
MINOR Modifications (examples)	Change in animal usage [e.g., <i>vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)</i>]	
	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: _____	
MAJOR Modifications (examples)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	X
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	X

	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	
	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: <u>Conduct a smaller, modified experiment with PFHxS using additional animals</u>	X

3. PREVIOUSLY APPROVED MODIFICATIONS: (*Hit 'Enter' after description of each modification to list all individually.*)

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood collection, and a change in blood collection method at termination and euthanasia method.	6	08 Sep 17
2	Single housing in the event of cagemate incompatibility and extra enrichment for those separated. Clarification about the 6 animals added in modification 1 belonging to Category D.		26 Sep 17
3	Option for trained personnel to perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber).		17 Oct 17
4	Refine exposure route for testing with the fourth chemical. Addition of 12 animals.	12	25 Jan 18
5	Clarification about acclimation and treat-feeding procedures.		02 Feb 18
6	Change in exposure route to oral gavage.		14 Mar 18

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
7	Change in vehicle (and vehicle control).		23 Mar 18

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 252 B: 0 C: 0 D: 252 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

Removal of Michael Quinn as a co-investigator.

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

V.1. Experimental Design and General Procedures. This Modification seeks to add 152 animals to the protocol for testing with two additional chemicals, perfluorononanoic acid (PFNA) and tridecafluorooctane-1-sulphonic acid (6:2 FTS), at the request of the sponsor. PFNA and 6:2 FTS are found in drinking water and human serum throughout the United States, oftentimes near military bases and training sites, and both are gaining increased attention by regulators.

As an additional part of Modification 8, we would like to repeat a smaller, modified experiment with PFHxS. Within three days of exposing the white-footed mouse to PFOS suspended in oil and delivered via oral gavage, we began seeing toxicity. However, in 28 days of testing with PFOA, PFHxS, and PFBS via drinking water, no toxicity was observed, and tissue weights and gross pathology were unaffected. By conducting a smaller, modified experiment where PFHxS is suspended in oil and delivered via oral gavage (as was done successfully with PFOS), we will compare the results of the two exposure routes, and extrapolate the results to the other two chemicals administered via drinking water (PFOA and PFBS) for follow-up studies. Through a smaller, modified experiment we hope to gain data to best design reproductive studies while minimizing the number of animals involved and without entirely repeating experiments. As an additional measure to reduce the use of animals, testing with PFHxS will only be conducted at two doses and shared controls will be utilized for testing with PFNA, 6:2 FTS, and PFHxS.

A table is attached and the end of Modification 8 detailing how the 152 additional animals will be utilized. As with previous rounds of this range finding, PFNA and 6:2 FTS will be administered at three doses to male and female peromyscus (N=9 per group). As an additional measure to minimize animal use, testing with PFHxS will be limited to two doses (N=6-7 per group) and shared controls will be utilized for testing with all three chemicals (N=9 per sex).

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). *(Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).*

V.3.5.2. Reduction. Modification 8 includes a shared control group as a reduction method. Additionally, the experiment with PFHxS will be limited to two dose groups.

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.).*

****** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

The additional animals used for the new compounds to be tested, and the repeated testing of PFHxS revise the animal usage section (total protocol usage) to: V.4.1.1.1.3. Column D: 404

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.). *(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)*

N/A

G. Changes to the personnel conducting this protocol (Section VI.). *(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)*

N/A

H. Changes in Biohazard/ Safety (Section VII).

N/A

Group	No. males	No. females	TOTAL	Pain category
controls	9	9	18	D
6:2 ETS dose 1	9	9	18	D
6:2 ETS dose 2	9	9	18	D
6:2 ETS dose 3	9	9	18	D
PFNA dose 1	9	9	18	D
PFNA dose 2	9	9	18	D
PFNA dose 3	9	9	18	D
PEHxS dose 2	6	6	12	D
PEHxS dose 3	7	7	14	D
TOTAL	76	76	152	D

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: ALLISON JACKOVITZ

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

PROTOCOL #: 67-17-04-01 **Protocol Approval Date:** 20 APRIL 2017

PROTOCOL TITLE: Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (Peromyscus leucopys)

Modification Request #: 9 **Submission Date:** Jun 12, 2018

Study Director/ Principal Investigator Signature: JACKOVITZ.ALLISON.MARIE.1367161236 Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.06.12 08:07:34 -04'00'

For review use only: Review Process: ADMIN ☒ FCR ☐ DMR ☐ VVC ☐

Received Date: 12 June AURO receipt: SEYFERT.ALLISON.MARIE.1473772210 Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.06.12 08:16:51 -04'00'

IACUC Designated Member Reviewer(s) (sign when review is completed):

Attending/ Alternate Veterinarian Review Signature: Approved via VVC: N/A ☐ YES ☐ NO ☐

Jun 14, 2018

Date

BONHAGE.MICHAEL.ROBERT.1014029881 Digitally signed by BONHAGE.MICHAEL.ROBERT.1014029881
Date: 2018.06.14 14:55:38 -04'00'

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Jun 12, 2018

Date

KEFAUVER.MICHAEL.P.1229209678 Digitally signed by KEFAUVER.MICHAEL.P.1229209678
Date: 2018.06.12 13:25:20 -04'00'

IACUC Chair Review Signature:

Jun 12, 2018

Date

DECK.ADAM.T.1267159900 Digitally signed by DECK.ADAM.T.1267159900
Date: 2018.06.12 13:48:35 -04'00'

Modification IACUC APPROVED ☒ GLP REVIEWED ☒ Date: 12 Jun 18

APHC IACUC Animal Use Protocol Modification

Study Director/ Principal Investigator: **Allison Jackovitz**

PROTOCOL #: **67-17-04-01**

Protocol Approval Date: 20 April 2017

PROTOCOL TITLE: **Range finding for exposure to perfluoroalkyl substances via drinking water in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: allison.m.jackovitz.civ@mail.mil

Modification Request #: 9

Submission Date: 12 June 2018

1. Brief non-technical synopsis of existing protocol or background information pertaining to the modification request.

The original protocol included four experiments with four chemicals where the white-footed mouse (*Peromyscus leucopus*) was exposed to one of four per- and polyfluoroalkyl substances (PFAS) for 28 consecutive days.

Given the sensitivity of the species involved, the Study Director/Principal Investigator attempted to reduce handling and invasive procedures while still meeting the study objectives: to provide information on how various PFASs accumulate in the body with repeated, daily exposure (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs. As such, in the first portion of this range finding, three chemicals (PFOA, PFHxS, and PFBS) were administered to the white-footed mouse for 28 consecutive days via drinking water.

Due to unforeseen solubility issues with PFAS (e.g., the chemical partitioning at the air-water interface) and the fact that PFAS are more soluble in methanol than water, the exposure route was refined in Modification 4 for testing with the fourth chemical. Per Modification 4, PFOS was dissolved in an appropriate solvent (methanol), applied to food treats via pipette, and methanol was given time to evaporate off, leaving only the test compound on the treat. Per Modification 5, all animals were acclimated to food treats (with methanol applied to them and with at least 24 hours for the solvent to evaporate off) prior to initiating the study. During the acclimation period, all animals consumed their food treats. However, within three days of testing with PFOS, medium- and high-dose animals developed an aversion to consuming various treats and even un-dosed treats. Therefore, per Modification 6, animals were exposed to PFOS via oral gavage. Per Modification 7, the vehicle (and vehicle control) was adjusted from water to corn oil.

In Modification 8, 152 animals were added to the protocol for testing with two additional chemicals, perfluorononanoic acid (PFNA) and tridecafluorooctane-1-sulphonic acid (6:2 FTS), and a smaller, modified experiment with PFHxS. PFHxS was previously tested via drinking water and by a smaller, modified experiment where PFHxS is suspended in oil and delivered via oral gavage (as was done successfully with PFOS), we will compare the results of the two exposure routes, and extrapolate the results to the other two chemicals administered via drinking water (PFOA and PFBS) for follow-up studies.

Modification 9 seeks to dissolve the test article in an appropriate solvent to create a stock solution, and then to dissolve or suspend the stock solution in an appropriate vehicle to create a solution or suspension, respectively. Forming a uniform solution or suspension is superior for delivering the intended dosage accurately and consistently. Dissolving the test article in an appropriate solvent was previously approved in Modification 4.

2. Type(s) of Modification Requested: (check all that apply)

	Type Of Change	X
ADMINISTRATIVE – AURO/QAU/SEMO REVIEW	Administrative Modifications (<i>e.g., correct typographical errors/grammar, contact information update, change in study start/completion dates, change in references, protocol title change, change that requires review and approval for GLP compliance</i>)	X
	Addition or deletion of a qualified technician, co-investigator, or study staff	
MINOR Modifications (<i>examples</i>)	Change in animal usage [<i>e.g., vendor, sex, age, weight, strain, small increase in # of animals used (less than 10% of overall number of approved animals)</i>]	
	Need to repeat an experiment without the addition of animals	
	Addition/ change of sample collection times	
	Additional noninvasive sampling	
	Changes in acclimation or recovery period	
	Special housing request or change in husbandry procedures	
	Changes in dosing procedures (<i>e.g., dose, volume, or timing</i>)	
	Other: _____	
MAJOR Modifications (<i>examples</i>)	Change in SD/PI	
	Change in objectives of the study	
	Addition of a test article to be evaluated	
	Change that results in greater pain, distress, or degree of invasiveness (changes to pain categories D or E from C require a literature search for alternatives to be completed)	
	Addition of animals to pain Category E (literature search for alternatives required)	
	Addition of or change in Species	
	Addition of more than 10% of the total # of animals originally approved	
	Addition of blood sampling	
	Change in frequency of observations for morbidity	
	Change in study endpoint	
	Changes in housing or use of animals in a location that is not part of the animal program overseen by the IACUC	
	Changes from nonsurvival to survival surgery	

	Change that impacts personnel safety	
	Other change that relates to the specific experimental design and aims of the original protocol	
	Other: _____	

3. PREVIOUSLY APPROVED MODIFICATIONS: *(Hit 'Enter' after description of each modification to list all individually.)*

Mod #	Short Description of the Amendment(s) (Include pain category breakdown of animal usage if changes were made from the original protocol)	No. & Species of Animal Requested	Approval Date
1	One typographical error, the addition of 6 control animals, the addition of one qualified technician, a decrease in the blood volume to be taken weekly from less than 10% (~150 uL) of the animal's total blood volume to approximately 7.5%, the addition of modified tail clip as a method for blood collection, and a change in blood collection method at termination and euthanasia method.	6	08 Sep 17
2	Single housing in the event of cagemate incompatibility and extra enrichment for those separated. Clarification about the 6 animals added in modification 1 belonging to Category D.		26 Sep 17
3	Option for trained personnel to perform cervical dislocation as a method of euthanasia on clearly moribund animals (e.g., those that would expire before making it to the CO2 chamber).		17 Oct 17
4	Refine exposure route for testing with the fourth chemical. Addition of 12 animals.	12	25 Jan 18
5	Clarification about acclimation and treat-feeding procedures.		02 Feb 18
6	Change in exposure route to oral gavage.		14 Mar 18
7	Change in vehicle (and vehicle control).		23 Mar 18
8	Addition of 152 animals for testing with two new chemicals and a smaller, modified experiment with one of the chemicals previously tested via the drinking water exposure route.	152	21 May 18

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: 404 B: 0 C: 0 D: 404 E: 0

5. FOR ITEMS A-H BELOW: Describe the requested changes to be implemented and the justification(s) for each.

A. Administrative change: Editorial (typographical, grammar, contact info update, etc), GLP compliance changes, or change in personnel (not change of study director/principal investigator):

New reference: Bartsch W., Sponer G., Dietmann K., Fuchs G. 1976. Acute toxicity of various solvents in the mouse and rat. LD50 of ethanol, diethylacetamide, dimethylformamide, dimethylsulfoxide, glycerine, N-methylpyrrolidone, polyethylene glycol, 400, 1,2-propanediol and Tween. Arzneimittelforschung; 26(8): 1581-3.

Because of the unique chemical properties of PFAS, PFNA, 6:2 FTS, and PHFxS do not form a uniform solution when added to water. Additionally, grinding PFNA, 6:2 FTS, and PFHxS and adding corn oil was not enough to create a uniform suspension. Complications included 6:2 FTS forming a wax ball and all three chemicals "clumping" when corn oil was added.

Instead, the test article will be dissolved in an appropriate solvent (e.g, Tween 20, methanol, ethanol, etc.) to create a stock solution. The stock solution will then be dissolved or suspended in an appropriate vehicle (e.g., water, corn oil, etc.) to create a solution or suspension, respectively. The solvent and vehicle used for the three test articles may be different, but each will be prepared consistently throughout the study. The solvent and vehicle used for each chemical will be recorded in the Study Records.

Solvents such as Tween 20, methanol, and ethanol are frequently used in toxicological work to improve the solubility of otherwise poorly soluble compounds (Bartsch et al., 1976). Forming a uniform solution or suspension is superior for delivering the intended dosage accurately and consistently. Dissolving the test article in an appropriate solvent was previously approved in Modification 4. Using water and corn oil as appropriate solvents were previously approved in Modifications 6 and 7, respectively.

B. Specific changes or additions to the experimental design of the protocol (Section V.1. Experimental Design).

N/A

C. Change in sample size evaluation, data analysis plan, archiving of data, (Section V.2.).

N/A

D. Change in the animals used, (Section V.3). (Include any changes in amount used, sex, age/weight, vendor/source, refinement, reduction, and replacement [3Rs]).

N/A

E. Changes to technical methods (Section V.4.). *(Include changes in USDA pain category classifications [section V.4.1.1.1.1 thru V.4.1.1.1.4.], changes in anesthesia, analgesia, restraint, injections, identification, behavior studies, 'other procedures', study endpoint, euthanasia, etc. Include reference source for basis of dose/treatment change.)*

****** If the modification requested will be adding animals to COLUMN D or E and the protocol did not previously have animals in those categories, a LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL OR DISTRESSFUL PROCEDURES must be completed and submitted with this modification request.**

N/A

F. Changes to the Husbandry and Veterinary Care procedures (Section V.5.). *(Changes in husbandry considerations, special provisions, exceptions to the Guide, AWAR, or IACUC Policy that have an impact on animal care and use; any changes to the veterinary medical care or environmental enrichment.)*

N/A

G. Changes to the personnel conducting this protocol (Section VI.). *(Include record of completed training for any animal handling or use procedures assigned to new personnel. Signed assurance page(s) must be included with the modification if changing SD/PI, Primary Co-investigator, or adding a co-investigator.)*

N/A

H. Changes in Biohazard/ Safety (Section VII).

N/A