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Toxicology Directorate

Reproductive and Developmental Toxicity of Per- and Polyfluoroalkyl Substances (PFAS) in Peromyscus spp., September 2018–October 2020

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14. ABSTRACT Concerns about per- and polyfluoroalkyl substances (PFAS) stem from their ubiquitous presence in the environment, bioaccumulation, resistance to degradation, and toxicity. In this study, the toxicity of four PFAS was evaluated in <i>Peromyscus spp.</i> , and data were used to derive Points of Departure (PODs) via Benchmark dose (BMD) modeling. Endpoints affected in <i>Peromyscus</i> are largely consistent with studies previously conducted. Specifically, prenatal exposure to perfluorooctane sulfonate (PFOS) caused neonatal mortality (benchmark dose low (BMDL) 0.12 mg/kg-day PFOS)). Additionally, prenatal exposure to tridecafluorohexane-1-sulfonic acid potassium salt (PFHxS) caused increased stillbirths (BMDL 5.72 mg/kg-day PFHxS) and reduced plaque forming cells (PFCs). At the doses tested, perfluorononanoic acid (PFNA) and 6:2 fluorotelomer sulfonate (FTS) did not markedly affect reproduction or development, but these experiments suggest they reduce PFCs in <i>Peromyscus</i> (BMDL 2.31 mg/kg-d PFNA; BMDL 2.26–2.63 mg/kg-day 6:2 FTS). Finally, <i>Peromyscus</i> exposed to perfluoroalkyl acids (PFAAs), but not 6:2 FTS, exhibited decreased serum thyroxine (T4) levels and increased liver weights.						
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Reproductive and Developmental Toxicity of Per- and Polyfluoroalkyl Substances (PFAS) in
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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations, 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 by the Laboratory Sciences (LS) Directorate prior to the study start in compliance with study protocol and modification requirements.

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TOXICOLOGICAL REPORT NO. S.0073458-18
REPRODUCTIVE AND DEVELOPMENTAL TOXICITY OF PER- AND POLYFLUOROALKYL
SUBSTANCES IN *PEROMYSCUS SPP.*,
SEPTEMBER 2018–OCTOBER 2020

1 SUMMARY

1.1 Purpose

Per- and polyfluoroalkyl substances (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. PFAS are also key ingredients in highly efficient fire suppressant agents known as aqueous film forming foams (AFFFs), which have been used by the Department of Defense (DOD) since approximately 1970 for fire training and emergency response activities.

Concerns about PFAS stem from their ubiquitous presence in the environment, bioaccumulation, resistance to degradation, and toxicity. In this study, PFAS were administered orally to white-footed mice (*Peromyscus leucopus*) or deer mice (*Peromyscus maniculatus*) for 28 consecutive days. Then, 1:1 mating pairs were initiated and reproduction and development were evaluated. With three chemicals (perfluorononanoic acid (PFNA), 6:2 fluorotelomer sulfonate (FTS), and tridecafluorohexane-1-sulfonic acid potassium salt (PFHxS)), immune response was evaluated. Data were used to derive Points of Departure (PODs) for *Peromyscus* via Benchmark dose (BMD) modeling.

1.2 Conclusions

Endpoints affected by four PFAS in *Peromyscus* are largely consistent with studies previously conducted in inbred and outbred rats and mice. Specifically, prenatal exposure to high doses of perfluorooctane sulfonate (PFOS) caused neonatal mortality (benchmark dose low (BMDL) 0.12 mg/kg-day PFOS)), though growth and development were unaffected by low doses. Additionally, prenatal exposure to PFHxS caused increased stillbirths (BMDL 5.72 mg/kg-day PFHxS) and reduced immune response. At the doses tested, PFNA and 6:2 FTS did not markedly affect reproduction or development, but these experiments suggest they reduce immune response in *Peromyscus* (BMDL 2.31 mg/kg-d PFNA; BMDL 2.26–2.63 mg/kg-day 6:2 FTS). Finally, *Peromyscus* exposed to perfluoroalkyl acids (PFAAs), but not 6:2 FTS, exhibited decreased serum thyroxine (T4) levels and increased liver weights.

2 REFERENCES

Appendix A contains a listing of references for this report.

3 AUTHORITY

This study was conducted with funding from the Strategic Environmental Research and Development Program (SERDP), Environmental Restoration Project No. ER-2625 (MIPR# W74RDV70043331). The study 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 reproductive and developmental effects of PFOS and perfluorooctanoic acid (PFOA) have been widely evaluated in inbred and outbred laboratory rodents. In contrast, there are relatively few studies on other PFAAs such as perfluorobutane sulfonate (PFBS), PFHxS, and PFNA. Prenatal exposure of rodents to PFAAs does not result in major malformations, and significant findings are often limited to higher exposure levels. Common effects resulting from prenatal exposures of rats and mice include increased mortality, decreased body weight, delayed eye opening, and liver hypertrophy. The present work was designed to identify the reproductive and developmental effects of three PFAAs – PFOS (8 carbon), PFNA (9 carbon), and PFHxS (6 carbon) – and one fluorotelomer – 6:2 FTS – in *Peromyscus spp.*

Most PFAA toxicity studies have been conducted in inbred (e.g., C67BL6, BALB/c) and outbred laboratory rodents (e.g., Sprague Dawley rats, Wistar rats, CD-1 mice). To date, no comprehensive toxicity study of PFAAs has been undertaken with a native mammalian species. Yet, some model species are less representative of native species due to domestication (Shorter et al., 2012). Additionally, genetic diversity in inbred strains is inherently limited and the genetic architecture of some model species are not useful for extrapolation to humans (Aitman et al., 2011; Yang et al., 2011). *Peromyscus* is useful as a mammalian epigenetic and a wildlife model (Shorter et al., 2012). Given that the serum elimination half-lives of various PFAAs can vary from hours to years based on the model species, sex, compound, and administered dose, extrapolation of results is difficult and often inaccurate. As such, the data generated in these experiments can be used to develop human- or wildlife-specific toxicity thresholds (e.g., reference doses (RfDs)).

The experiments conducted herein were designed to provide data that are conducive to modeling via the benchmark dose approach (Haber et al., 2018). Specifically, endpoints that can conceivably affect the sustainment of the population (e.g., mortality, morbidity, impaired reproduction or development, or changes in behavior related to feeding or predator avoidance) will be evaluated, as they are most valuable for inclusion in risk assessment (Mayfield and Skall, 2018; Williams et al., 2015). Conversely, endpoints of uncertain toxicological significance (e.g., altered liver enzymes or changes in clinical chemistry within normal ranges) are often not included in derivation of an RfD. Additionally, the length of time to which study subjects are exposed to the test chemical is important, with preference for inclusion given to studies with chronic exposures that are equal to or greater than 10% of the lifespan of the species. Finally, the experiments were conducted with robust sample sizes (i.e., 25–31 mating pairs per dose), which allows for more robust benchmark dose modeling (Haber et al., 2018).

Table 1 identifies the dates of critical study events.

Table 1. Critical Study Events

Critical Event	Date of Event
Animal Use Protocol Approved	08/13/2018
In-Life Initiation	09/03/2018
In-Life Completion	10/02/2020
Experimental Completion	10/22/2021
Study Completion	12/22/2021

5 MATERIALS AND METHODS

5.1 Test Substances

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

- Heptadecafluorooctanesulfonic acid potassium salt (PFOS),
- Perfluorononanoic acid (PFNA),
- Tridecafluorohexane-1-sulfonic acid potassium salt (PFHxS), and
- Tridecafluorooctane-1-sulphonic acid (6:2 FTS).

Three of the four test substances are PFAAs (PFOS, PFNA, and PFHxS). One test substance is a fluorotelomer (6:2 FTS).

Table 2. Test Substances

Experiment	Chemical	Abbreviation	Molecular weight	CAS-No.	Purity
1	Heptadecafluorooctanesulfonic acid potassium salt	PFOS	538.22 g/mol	2795-39-3	≥98%
2	Perfluorononanoic acid	PFNA	464.08 g/mol	375-95-1	97%
3	Tridecafluorohexane-1-sulfonic acid potassium salt	PFHxS	438.20 g/mol	3871-99-6	≥98%
4	Tridecafluorooctane-1-sulphonic acid	6:2 FTS	428.17 g/mol	27619-97-2	98%

Legend:

g/mol = molecular weight

5.2 Animals and Housing Conditions*

* The APHC Institutional Animal Care and Use Committee approved the Animal-use procedures. Animal care and use was conducted according to *The Guide for the Care and Use of Laboratory Animals* and all

* Animal-use procedures were approved by the APHC Institutional Animal Care and Use Committee.

For Experiment 1, adult male ($n = 124$; 22.45 ± 0.31 g) and female ($n = 124$; 19.80 ± 0.26 g) *P. leucopus* were obtained from the Peromyscus Genetic Stock Center (PGSC) at the University of South Carolina. Animals were acclimated to the facility at the U.S. Army Public Health Center (APHC) for at least 6 weeks before initiation of dosing. Animals were acclimated to handling for at least 5 weeks before initiation of dosing.

For Experiment 2, adult male ($n=125$; 21.23 ± 0.25 g) and female ($n=125$; 18.90 ± 0.23 g) *P. leucopus* were obtained from the PGSC. Animals were acclimated to the facility at the APHC for at least 4 weeks before initiation of dosing. Animals were acclimated to handling for at least 3 weeks before initiation of dosing.

For Experiment 3, adult male ($n=125$; 18.35 ± 0.13 g) and female ($n=125$; 16.33 ± 0.13 g) *P. maniculatus* were produced at the APHC from a colony obtained from Harvard University. Animals were acclimated to handling for at least 2 weeks before initiation of dosing.

For Experiment 4, adult male ($n=114$; 21.30 ± 0.24 g) and female ($n=121$; 19.10 ± 0.21 g) *P. leucopus* were obtained from the PGSC. Animals were acclimated to the facility at the APHC for at least 6 weeks before initiation of dosing. Animals were acclimated to handling for at least 5 weeks before initiation of dosing.

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

- Experiment 1 (PFOS): males were between 150 and 412 days; females were between 203 and 406 days.
- Experiment 2 (PFNA): males were between 118 and 349 days; females were between 117 and 357 days.
- Experiment 3 (PFHxS): males were between 80 and 199 days; females were between 77 and 196 days.
- Experiment 4 (6:2 FTS): males were between 149 and 259 days; females were between 196 and 307 days.

Several factors contributed to the large age range; 1) there is only one commercial vendor for the *Peromyscus*; 2) breeding is seasonal and sporadic; and 3) litter sizes are smaller (roughly 2.7 pups/litter) than those of traditional laboratory mice (roughly 8 pups/litter) (Schmidt and Hood, 2012).

In Experiment 1, animals were randomly assigned to treatment groups and stratified according to pre-study body weight, while taking age into consideration. In Experiment 1, mating pairs were randomly assigned within each dose group. In Experiments 2–4, animals were randomly assigned to treatment groups and stratified according to age while taking pre-study body weight into consideration. In Experiments 2–4, mating pairs were assigned according to age. Within

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.

each experiment, body weight did not differ among dose groups 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 20–26°C and 30–70% humidity. An automatically controlled 12/12-hour light/dark cycle was maintained for the first half of Experiment 1, with the dark period beginning at 1800 hours. For the second half of Experiment 1 and for all of Experiments 2–4, an automatically controlled 16/8-hour light/dark cycle was maintained, with the dark period beginning at 2000 hours. The appropriate light cycle for *Peromyscus* breeding is a 16/8-hour light/dark cycle.

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 Study Director 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 Das et al. (2015), Lau et al. (2003), and Narizzano et al. (2021). Doses were selected in order to evaluate male and female reproductive performance, conception, development of conceptus, parturition, litter survivability, and not to cause or detect overt toxicity in Parental (P) generation animals.

5.6 Exposure Route

Animals were exposed to PFAS via oral gavage, based on complications with drinking water and dietary exposures in previously conducted, 28-day studies (APHC, 2021, Narizzano et al., 2021).

5.7 Study Design

5.7.1 Experiment 1

Male and female white-footed mice were exposed to PFOS (31/sex/dose) for 28 consecutive days. Then, 1:1 mating pairs were established and reproduction and development were evaluated. Mating pairs were maintained until pups reached postnatal day (PND) 25 or 16 weeks had elapsed.

P generation animals were dosed daily for 11-27 total weeks at which point animals were euthanized and necropsied per 5.8.1. The first filial (F1) generation animals were exposed to PFOS *in utero* and via lactation, but not directly.

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 animal room drinking water using a graduated cylinder, and
- Adding the animal room drinking water to the polypropylene container.

Three dosing solutions, 0.02, 0.1 and 0.5 mg/mL were used throughout the experiment, which corresponded nominally to 0.2, 1 and 5 mg/kg-day. Solutions were prepared bi-weekly. Eurofins Eaton Analytical verified representative dosing solutions via liquid chromatography-tandem mass spectrometry (LC/MS/MS).

5.7.2 Experiment 2

Male and female white-footed mice were exposed to PFNA (25/sex/dose) for 28 consecutive days. Then, 1:1 mating pairs were established and reproduction and development were evaluated. Mating pairs were maintained until 12 weeks had elapsed or until generation of a litter. P generation animals were dosed daily for 16 total weeks regardless of whether a litter was produced. Five days prior to euthanasia, P generation animals were sensitized by intraperitoneal (IP) injection with 25% sheep red blood cells (SRBCs, Lampire Biological Labs, Pipersville, Pennsylvania) in phosphate buffered solution (PBS). P generation animals were euthanized and necropsied per 5.8.2. The F1 generation animals were exposed to PFNA *in utero* and via lactation, but not directly.

Dosing solutions and suspensions were prepared by—

- Weighing the required amount of neat PFNA,
- Adding methanol to make a stock solution (500 mg/mL),
- Adding the appropriate amount of stock solution to a volumetric flask,
- Adding roughly half the volume of animal room drinking water, and
- Bringing to volume with animal room water.

Four dosing solutions, 0.004, 0.02, 0.1, and 0.5 mg/mL were used throughout the experiment, which corresponded nominally to 0.04, 0.2, 1, and 5 mg/kg-day. Solutions were prepared bi-weekly. Eurofins Eaton Analytical verified representative dosing solutions via LC/MS/MS. Given that the volume of methanol added to each dosing solution accounted for approximately 0.1% total volume, methanol was not expected to affect the study outcome, based on its median lethal dose (LD50) of 7,300–10,000 mg/kg in mice (Smith and Taylor, 1982).

5.7.3 Experiment 3

This experiment was conducted using animals produced from an in-house colony. In order to accomplish this, the experiment was conducted in two iterations to ensure a tighter age range.

In the first iteration of this experiment, male and female deer mice were exposed to PFHxS (10/sex/dose) for 10 consecutive days, at 0.04, 0.2, 1, and 5 mg/kg-day. Then, doses were increased to 2.5, 5, 10, and 20 mg/kg-day and animals were maintained on these doses for approximately 15 weeks. After 5 weeks of dosing, 1:1 mating pairs were established and reproduction and development were evaluated. Mating pairs were maintained until 11 weeks had elapsed or until generation of a litter.

In the second iteration of this experiment, male and female deer mice were exposed to PFHxS (15/sex/dose) for 28 consecutive days at 2.5, 5, 10, and 20 mg/kg-day. Then, 1:1 mating pairs were established and reproduction and development were evaluated. Mating pairs were maintained until 12 weeks had elapsed or until generation of a litter.

In both iterations of this experiment, P generation animals were dosed daily for 16 total weeks regardless of whether a litter was produced. Five days prior to euthanasia, P generation animals were sensitized by IP injection with 25% SRBC (Lampire Biological Labs, Pipersville, Pennsylvania) in PBS. P generation animals were euthanized and necropsied per 5.8.2. The F1 generation animals were exposed to PFHxS *in utero* and via lactation, but not directly.

Dosing solutions and suspensions were prepared by—

- Weighing the required amount of neat PFHxS and transferring to a volumetric flask,
- Adding roughly half the volume of animal room drinking water,
- Stirring using a magnetic stir bar and stir plate until dissolved, and
- Bringing to volume with animal room drinking water.

Four dosing solutions, 0.25, 0.5, and 1, and 2 mg/mL, were used throughout the experiment, which corresponded nominally to 2.5, 5, 10, and 20 mg/kg-day. Solutions were prepared bi-weekly. Eurofins Eaton Analytical verified representative dosing solutions via LC/MS/MS.

5.7.4 Experiment 4

Male and female white-footed mice were exposed to 6:2 FTS (25/sex/dose) for 28 consecutive days. Then, 1:1 mating pairs were established and reproduction and development were evaluated. Mating pairs were maintained until 12 weeks had elapsed or until generation of a litter. P generation animals were dosed daily for 16 total weeks regardless of whether a litter was produced. Five days prior to euthanasia, P generation animals were sensitized by IP injection with 25% SRBC (Lampire Biological Labs, Pipersville, Pennsylvania) in PBS. P generation animals were euthanized and necropsied per 5.8.2. The F1 generation animals were exposed to 6:2 FTS *in utero* and via lactation, but not directly.

Dosing solutions were prepared by—

- Weighing the required amount of neat 6:2 FTS,
- Adding methanol to make a stock solution (500 mg/mL),
- Adding the full volume of stock solution to a volumetric flask,
- Adding roughly half of animal room drinking water, and
- Bringing to volume with animal room drinking water.

Four dosing solutions, 0.02, 0.1, and 0.5, and 2.5 mg/mL, were prepared for 6:2 FTS, which corresponded nominally to 0.2, 1, 5, and 25 mg/kg-day. Solutions were prepared bi-weekly. Representative 6:2 FTS dosing solutions were verified via LC/MS/MS by 3M. Given that the volume of methanol added to each dosing solution accounted for approximately 0.5% total volume, methanol was not expected to affect the study outcome, based on its LD50 of 7300-10000 mg/kg in mice (Smith and Taylor, 1982).

To determine occurrence of contaminants in the 6:2 FTS dosing solution, suspect screening analysis was performed via LC-QTOF-MS, as previously described (APHC, 2021, Narizzano et al., 2021).

5.8 Clinical Observations and Body Weight

5.8.1 Experiment 1

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

F1 generation animals were observed at least once daily prior to weaning in conjunction with dosing of P generation animals. F1 generation animals were weighed on PND0, 4, 7, 10, 14, 17, 20, 25 and then at least weekly post-weaning.

5.8.2 Experiments 2-4

P generation animals were removed from their home cages and observed daily by study personnel in conjunction with dosing for signs of toxicity, morbidity, and mortality. Body weights of P generation males were recorded at the start of test substance administration, at least weekly thereafter, and at termination. Body weights of P generation females were recorded at the start of test substance administration, at least weekly thereafter prior to establishing mating pairs, daily while in mating pairs and through PND30, and at termination.

F1 generation animals were observed at least once daily prior in conjunction with dosing of P generation animals. F1 generation animals were weighed on PND0, 1, 4, 7, and 10.

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

5.9.1 Experiment 1

P generation animals were euthanized in blocked batches when their litters were approximately PND25 or after 20 total weeks of dosing. F1 generation animals were euthanized and necropsied at PND57-63 in blocked batches.

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. The brain, heart, kidneys, liver, ovaries, uterus, spleen, thymus, testes, and epididymides were removed, trimmed, and weighed. Testes and ovaries were weighed as pairs.

5.9.2 Experiments 2-4

P generation animals were euthanized and necropsied after 16 total weeks of dosing. F1 generation animals were euthanized at PND10.

Surviving animals were rendered unconscious with 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 P generation animals following euthanasia. The brain, heart, kidneys, liver,

ovaries, uterus, spleen, thymus, testes, and epididymides were removed, trimmed, and weighed. Testes and ovaries were weighed as pairs.

5.10 Sperm Analysis

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 Immune Assay

In Experiments 2-4, immune function was evaluated via a modified Jerne plaque forming assay (Cunningham, 1976). Briefly, spleens were collected aseptically at necropsy and dissociated into single cells through a cell strainer into PBS. The cell count of each spleen was determined using an automated cell counter (Bio-Rad® TC20), and samples were diluted to approximately 2-E6 cell/mL in cold RPMI-1640 without Fetal Bovine Serum. The cell suspensions were combined with RPMI-1640 at 1:5, 2:5, and 5:5 for control/low, medium, and high dose groups, respectively. SRBCs (25%, 100 µL) and guinea pig complement (Cedarlane, Burlington, North Carolina 50 µL) were added to the cell suspension/RPMI-1640. The mixture was vortexed, pipetted onto parafilm and loaded into two chambers of pre-made counting slides via capillary action. Slides were sealed with paraffin and incubated at 37°C for 2 hours. Plaques were counted under low power (10X) using a simple light microscope. Responses were determined as plaque forming cells (PFCs) per million cells.

5.12 Hormone Analysis

In Experiments 1, 2 and 4, estradiol, progesterone, testosterone, total triiodothyronine (T3), and total T4 were determined using the MAGPIX® analyzer and the Milliplex® Multi-Species Hormone Magnetic Bead Panel. The assay was conducted according to the manufacturer's instructions (Millipore, 2013), and quality control standards were within the target reference ranges.

In Experiment 3, total T3 and total T4 were determined using the MAGPIX® analyzer and the Milliplex® Multi-Species Hormone Magnetic Bead Panel. The assay was conducted according to the manufacturer's instructions (Millipore, 2013), and quality control standards were within the target reference ranges.

5.13 Histopathology

Preserved tissues were processed using standard techniques, sectioned 4–5 µm thick, and stained with hematoxylin and eosin using a routine automatic stainer. Testis and epididymis were additionally stained with Periodic-acid-Schiff (PAS). A board certified veterinary pathologist performed detailed microscopic examination of tissues. In Experiment 1, histopathologic

findings were subjectively graded across a 6-point scale, where Grade 0 (essentially normal) referred to tissues with changes observed in <1% of the sampled tissue. Grade 1 (minimum) referred to a change, which affected <5% of the presented tissue area, and Grade 2 (mild) referred to a change which affected 6–20% of the tissue area. Grade 3 (moderate) was scaled to refer to a change which affected 21–40% of the tissue area, Grade 4 (marked) was scaled for lesions affecting 41–80% of the tissue area, and Grade 5 (severe) indicated that >80% of the tissue was affected. In Experiments 2-3, histopathologic findings were categorized as present/absent and livers were evaluated. In experiment 4, histopathologic findings were categorized as present/absent, and livers, spleens, and thymuses were evaluated.

5.14 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. Analyses were conducted for male and female mice separately, with the exception of F1 organ weight data in Experiment 1, F1 body weight data in Experiments 1-4, and immune data in Experiment 2.

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.

Continuous data were analyzed using analysis of variance (ANOVA) with dose group as a fixed factor. Absolute organ weight was analyzed by analysis of covariance (ANCOVA) for brain, heart, kidneys, epididymides, spleen, testes, thymus, and uterus because they demonstrate a nonlinear 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. Weights of liver 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). Weights of 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 repeated measures ANOVA to determine dependence of effects. If the interaction between the within subjects factor and the between-subjects factor was significant, the effect of dose group on the parameter was determined within each sampling week using a one-way ANOVA. Thyroid and sex hormone levels were analyzed in separate multivariate analysis of variance (MANOVAs) followed by ANOVA if results were significant and appropriate post hoc tests. 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)).

Fisher's exact test or Chi-squared test was used to determine significant differences between treated and control groups for nominal or count data (e.g., mating, fertility, histology, etc.). Histopathology severity categories were consolidated to present/absent for statistical analyses. If multiple Chi-squared tests were performed, a Bonferroni-Holm Correction was performed.

5.15 Determination of BMD

Dose response modeling was conducted using United States Environmental Protection Agency (USEPA) Benchmark Dose Software (BMDS) 3.2. Dichotomous endpoints (e.g., total litter loss), continuous endpoints (e.g., organ weights), and nested endpoints (e.g., stillbirths per litter) were fit with corresponding models. All available models present in BMDS 3.2 (including e.g., Bayesian and unrestricted) were fit for each endpoint. Largely, default settings were relied upon for model fit and automated model selection. This implies that model Akaike Information Criterion (AIC) value, goodness of fit p -value, and scaled residuals at control and dose near BMD are all considered in best fitting model. Adaptations were made to constant and nonconstant variance models based on BMDS 3.2 output of constant variance tests but also professional interpretation of deviation data. In cases of ties or nonsensical model fits (e.g., extreme slopes in Hill models fitted to gently sloping data, BMDL below lowest study dose) professional interpretation and peer consensus was used to choose model fit that was compromise between model simplicity and reasonable BMDL estimate. All models selected as final selection fit “viable” category in BMDS automated model selection.

6 RESULTS

6.1 Experiment 1: PFOS

6.1.1 Analytical Verification of Dosing Solutions

Comparisons of the measured concentrations of PFOS in solution to the nominal concentrations of the solutions revealed ~80–99% accuracy for the solutions containing ≤ 0.1 mg/mL PFOS at days 14 and 28 (Appendix D). All results were within the pre-established acceptance of 70–130% accuracy level. The analytically verified concentrations of PFOS revealed that PFOS levels in the more concentrated solution (0.5 mg/mL) at day 14 corresponded to ~68% of the nominal concentration (Appendix D). As such, the doses administered were reported as nominal: 0.2, 1.0, and 5.0 mg/kg-day PFOS.

6.1.2 Clinical Observations and Body Weight

Clinical observations in the P generation were limited to wounds, malocclusions, and hair loss. Body weight of P generation males was unaffected by PFOS ($F_{3, 86} = 1.056$, $p = 0.372$, Appendix E). Body weight of P generation females was unaffected by PFOS ($F_{3, 119} = 0.444$, $p = 0.722$). Finally, maternal weight gain was affected by PFOS ($F_{3, 44} = 3.047$, $p = 0.038$). Post-hoc examination revealed a difference between the 0.2 and 5.0 mg/kg-day PFOS groups, not PFOS-exposed and control.

Clinical observations in the F1 generation were limited to wounds and hair loss. Litter weights on PND0, 4, 7, 10, 14, 17, and 20 were unaffected by PFOS ($F_{2, 23} = 2.423$, $p = 0.111$, Appendix E). Developmental landmarks (i.e., pinna unfolding and eye opening) were unaffected by PFOS (pinna unfolding: $F_{2, 29} = 1.406$, $p = 0.261$; eye opening: $F_{2, 29} = 1.187$, $p = 0.320$).

6.1.3 Reproduction and Fertility

Fertility (i.e., the number of pregnant animals divided by the number of mating pairs) was affected by PFOS (Pearson χ^2 (3, n = 121) = 8.651, p = 0.034, Appendix F). Fertility in PFOS-treated animals did not differ from controls on post hoc test examination (p > 0.05).

Pregnancy (i.e., the number of females that delivered a live litter divided by the number of animals identified as pregnant) was affected by PFOS (Pearson χ^2 (3, n = 48) = 21.548, p = 0.001). Specifically, pregnancy was decreased in P generation animals exposed to 5.0 mg/kg-d PFOS (Pearson χ^2 (1, n = 19) = 14.702, p = 0.001) and 1.0 mg/kg-d PFOS (Pearson χ^2 (1, n = 30) = 4.588, p = 0.032) compared to controls (Appendix F).

Live litter size was decreased in animals exposed to PFOS compared to controls ($F_{3,44} = 4.081$, p = 0.012); however, total litter size was unaffected ($F_{3,44} = 2.162$, p = 0.106). Stillbirths were increased in animals exposed to PFOS compared to controls (number: $F_{3,44} = 5.963$, p = 0.002, percent per litter: $F_{3,44} = 7.229$, p < 0.001, Appendix F).

Total litter loss by PND1 was affected by PFOS (Pearson χ^2 (3, n = 48) = 23.632, p < 0.001). Specifically, total litter loss was increased in animals exposed to 5.0 mg/kg-day PFOS (Pearson χ^2 (1, n = 19) = 19.000, p < 0.001) and 1.0 mg/kg-day PFOS (Pearson χ^2 (1, n = 30) = 6.982, p = 0.008) compared to controls. (Appendix F). Notably, total litter loss occurred in 100% of 5.0 mg/kg-d PFOS litters and 41% of 1.0 mg/kg-d PFOS litters.

6.1.4 Necropsy and Organ Weights

Liver and kidney weights were increased in male P generation animals exposed to PFOS compared to controls (liver: $F_{3,102} = 9.40$, p < 0.001; kidney $F_{3,101} = 3.15$, p = 0.028; Appendix G). Liver weights were increased and thymus weights were decreased in female P generation animals exposed to PFOS compared to controls (liver: $F_{3,110} = 16.20$, p < 0.001; thymus $F_{3,108} = 3.36$, p = 0.02; Appendix G). All other results in both sexes of P generation animals were not significant (p > 0.05).

Liver weights were increased in F1 generation animals exposed to PFOS compared to controls ($F_{2,45} = 5.278$, p = 0.009). All other results in F1 generation animals were not significant (p > 0.05).

6.1.5 Sperm Analysis

Total sperm count of P generation males was unaffected by PFOS ($H_3 = 1.60$, p = 0.658, Appendix H).

6.1.6 Hormone Analysis

Serum levels of sex hormones (i.e., estradiol, progesterone, and testosterone) were unaffected by PFOS exposure in P generation males (Wilks' Lambda = 0.812, $F_{9,114.536} = 1.138$, p = 0.342) and females (Wilks' Lambda = 0.810, $F_{9,121.837} = 1.224$, p = 0.287).

Serum levels of thyroid hormones were affected by PFOS exposure in P generation males (Wilks' Lambda = 0.448, $F_{6, 116} = 9.546$, $p < 0.001$) and females (Wilks' Lambda = 0.461, $F_{6, 128} = 10.086$, $p < 0.001$). This multivariate effect was due to an effect on T4 in both males ($F_{3, 59} = 21.825$, $p < 0.001$) and females ($F_{3, 65} = 15.973$, $p < 0.001$). T4 levels were decreased in males exposed to 0.2, 1.0, and 5.0 mg/kg-day PFOS and females exposed to 1.0 and 5.0 mg/kg-day compared to controls (Appendix I).

6.1.7 Histopathology

Livers in males (Pearson χ^2 (3, $n = 111$) = 27.386, $p < 0.001$) and females (Pearson χ^2 (3, $n = 112$) = 27.172, $p < 0.001$) animals were affected by PFOS (Appendix J). Specifically, hepatic vacuolation increased in males exposed to 5.0 mg/kg-day PFOS (Pearson χ^2 (1, $n = 53$) = 20.485, $p < 0.001$) and 1.0 mg/kg-day PFOS (Pearson χ^2 (1, $n = 61$) = 5.160, $p = 0.023$) compared to controls. Hepatic vacuolation also increased in females exposed to 5.0 mg/kg-day PFOS (Pearson χ^2 (1, $n = 51$) = 25.697, $p < 0.001$) compared to controls. All other tissues examined (i.e., spleen, kidney, bone marrow, thymus, and lymph nodes) were unaffected by PFOS (Appendix J). Specific liver pathologies in animals exposed to 5.0 mg/kg-day PFOS are described in Appendix J.

6.1.8 Determination of BMD

Table 3. Summary of Effects in P Generation *P. leucopus* Exposed to PFOS

Critical effect	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	BMD (mg/kg-d)	BMDL (mg/kg-d)	BMR	Model
Increased total litter loss by PND1	0.2	1.0	0.26	0.12	10%	Frequentist restricted Multistage Degree 2
Increased stillbirths per litter	0.2	1.0	0.68	0.19	10%	Frequentist restricted nested Logistic (lsc+ilc+)
Increased liver: body weight (males)	0.2	1.0	1.33	0.51	1 St. dev.	Frequentist restricted Hill
Increased hepatocyte cytoplasmic vacuolization (males)	0.2	1.0	0.50	0.33	10%	Frequentist unrestricted Quantal Linear
Decreased serum T4 (males)	n/a	0.2	0.31	0.19	1 St. dev.	Frequentist restricted Exponential 4
Decreased serum T4 (females)	0.2	1.0	0.30	0.18	1 St. dev.	Frequentist restricted Exponential 4

6.2 Experiment 2: PFNA

6.2.1 Analytical Verification of Dosing Solutions

Comparisons of the measured concentrations of PFNA in solution to the nominal concentrations of the solutions revealed ~50-70% accuracy for the solutions containing 0.004, 0.02, and 0.5 mg/mL PFNA at days 14 and 28 (Appendix D). Results were outside the pre-established acceptance of 70-130% accuracy level. The analytically verified concentrations of PFNA revealed that 0.1 mg/mL PFNA corresponded to ~70% of the nominal concentration and was reported as nominal (Appendix D). As such, the doses administered were reported as 0.03, 0.14, 1.0, and 3.0 mg/kg-day rather than the targeted doses of 0.04, 0.2, 1.0 and 5.0 mg/kg-day.

6.2.2 Clinical Observations and Body Weight

Clinical observations in the P generation were limited to wounds, malocclusions, and hair loss. Body weight of P generation males was unaffected by PFNA ($F_{4, 108} = 0.825$, $p = 0.512$, (Appendix E). Body weight of P generation females was unaffected by PFNA ($F_{4, 119} = 0.269$, $p = 0.898$). Finally, maternal weight gain was unaffected by PFNA ($F_{4, 89} = 0.863$, $p = 0.490$).

Clinical observations in the F1 generation were limited to wounds. Litter weights on PND0, 1, 4, 7, and 10 did not differ between PFNA-treated and control animals ($F_{4, 75} = 0.444$, $p = 0.777$, (Appendix E). The developmental landmark of pinna unfolding was affected by PFNA ($F_{4, 75} = 4.077$, $p = 0.005$, (Appendix E). Post-hoc examination revealed a difference between various PFNA- groups, not PFNA-treated and control.

6.2.3 Reproduction and Fertility

Fertility (Pearson χ^2 (4, $n = 120$) = 2.488, $p = 0.647$) and pregnancy (Pearson χ^2 (4, $n = 98$) = 8.830, $p = 0.065$) were unaffected by PFNA (Appendix F).

Total litter size ($F_{4, 93} = 1.026$, $p = 0.398$), live litter size ($F_{4, 93} = 1.112$, $p = 0.355$), and stillbirths (number: $F_{4, 93} = 1.874$, $p = 0.121$, percent per litter: $F_{4, 93} = 1.696$, $p = 0.157$, Appendix F) were unaffected by PFNA.

Additionally, total litter loss by PND1 was unaffected by PFNA (Pearson χ^2 (4, $n = 98$) = 5.155, $p = 0.276$, (Appendix F). Notably, pup survivability to PND10 was unaffected by PFNA ($F_{4, 93} = 1.298$, $p = 0.276$).

6.2.4 Necropsy and Organ Weights

Liver and kidney weights were increased in male P generation animals exposed to PFNA compared to controls (liver: $F_{4, 108} = 28.87$, $p < 0.001$; kidney $F_{4, 109} = 19.11$, $p < 0.001$, (Appendix G). Liver weights were increased in female P generation animals exposed to PFNA compared to controls (liver: $F_{4, 107} = 4.41$, $p = 0.002$, Appendix G). All other results in both sexes of P generation animals were not significant ($p > 0.05$).

6.2.5 Sperm Analysis

Total sperm count was unaffected by PFNA ($H_4 = 0.97$, $p = 0.915$. Appendix H).

6.2.6 Hormone Analysis

Serum levels of sex hormones (i.e., estradiol, progesterone, and testosterone) were unaffected by PFNA exposure in P generation males (Wilks' Lambda = 0.872, $F_{12, 225.180} = 1.000$, $p = 0.450$) and females (Wilks' Lambda = 0.832, $F_{12, 209.306} = 1.256$, $p = 0.247$).

Serum levels of thyroid hormones were affected by PFNA exposure in P generation males (Wilks' Lambda = 0.524, $F_{8, 196} = 9.348$, $p < 0.001$) and females (Wilks' Lambda = 0.820, $F_{8, 200} = 2.604$, $p = 0.010$). In males, this multivariate effect was due to an effect on T3 ($F_{4, 99} = 2.790$, $p = 0.030$) and T4 ($F_{4, 99} = 16.478$, $p < 0.001$) in males exposed to PFNA. Serum T3 levels in PFNA-treated males did not differ from controls on post hoc test examination ($p > 0.05$). T4 levels decreased in males exposed to 1.0 and 3.0 mg/kg-day PFNA compared to controls. In females, this multivariate effect was due to an effect on T4 ($F_{4, 101} = 4.879$, $p = 0.001$) in females exposed to PFNA. T4 levels decreased in females exposed to 3.0 mg/kg-day PFNA compared to controls (Appendix I).

6.2.7 Histopathology

Livers in males were unaffected by 3.0 mg/kg-d PFNA; however, incidence of hepatic vacuolation were increased in controls (Pearson χ^2 (1, $n = 45$) = 4.590, $p = 0.032$).

Livers in females were unaffected by 3.0 mg/kg-day PFNA (Pearson χ^2 (1, $n = 47$) = 2.374, $p = 0.123$) compared to controls.

6.2.8 Immune Assay

Formation of PFCs was decreased in animals exposed to 3.0 mg/kg-day PFNA compared to controls and all other treatment groups ($F_{4, 160} = 7.681$, $p < 0.001$, Appendix K).

6.2.9 Determination of BMD

Table 4. Summary of Effects in P Generation *P. leucopus* Exposed to PFNA

Critical effect	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	BMD (mg/kg-d)	BMDL (mg/kg-d)	BMR	Model
Increased liver: body weight (males)	0.14	1.0	1.36	0.42	1 St. dev.	Frequentist unrestricted Power
Decreased serum T4 (males)	0.14	1.0	1.28	0.92	1 St. dev.	Frequentist restricted Exponential 2
Decreased PFC formation	1.0	3.0	3.60	2.31	1 St. dev.	Frequentist restricted exponential 2

6.3 Experiment 3: PFHxS

6.3.1 Analytical Verification of Dosing Solutions

Comparisons of the measured concentrations of PFHxS in solution to the nominal concentrations of the solutions revealed ~6% accuracy for the solutions containing 0.25, 1.0, and 2.0 mg/mL PFHxS at days 14 and 28 (Appendix D). Results were outside the pre-established acceptance of 70-130% accuracy level. The analytically verified concentrations of PFHxS revealed that 0.5.0 mg/mL PFHxS corresponded to ~70% of the nominal concentration and was reported as nominal (Appendix D). As such, the doses administered were reported as 1.6, 3.5, 7.0, and 14.0 mg/kg-day rather than the targeted doses of 2.5, 5.0, 10.0 and 20.0 mg/kg-day.

6.3.2 Clinical Observations and Body Weight

Clinical observations in the P generation were limited to wounds, malocclusions, and hair loss. Body weight of P generation males was unaffected by PFHxS ($F_{4, 107} = 0.341$, $p = 0.849$, Appendix E). Body weight of P generation females was unaffected by PFHxS ($F_{4, 116} = 0.962$, $p = 0.431$). Finally, maternal weight gain was unaffected by PFHxS ($F_{4, 96} = 0.545$, $p = 0.703$).

Clinical observations in the F1 generation were limited to wounds. Litter weights on PND0, 1, 4, 7, and 10 did not differ between PFHxS-treated and control animals ($F_{4, 63} = 0.649$, $p = 0.630$, Appendix E). The developmental landmark of pinna unfolding was unaffected by PFHxS ($F_{4, 74} = 1.481$, $p = 0.217$).

6.3.3 Reproduction and Fertility

Fertility was unaffected by PFHxS (Pearson χ^2 (4, $n = 120$) = 4.913, $p = 0.296$, Appendix F).

Pregnancy was affected by PFHxS (Pearson χ^2 (4, $n = 120$) = 11.814, $p = 0.019$).

Specifically, pregnancy was decreased in P generation animals exposed to 14.0 mg/kg-d PFHxS (Pearson χ^2 (1, n = 47) = 4.905, p = 0.027) compared to controls (Appendix F).

Total litter size ($F_{4, 98} = 1.295$, p = 0.277) and live litter size ($F_{4, 98} = 2.400$, p = 0.055) were unaffected by PFHxS (Appendix F). Stillbirths were increased in animals exposed to PFHxS compared to controls (number: $F_{4, 98} = 3.594$, p = 0.0009, percent per litter: $F_{4, 98} = 3.582$, p = 0.0009, Appendix F).

Additionally, total litter loss by PND1 was unaffected by PFHxS (Pearson χ^2 (4, n = 103) = 4.432, p = 0.351), Appendix F).

6.3.4 Necropsy and Organ Weights

Liver weights were increased in male P generation animals exposed to PFHxS compared to controls (liver: $F_{4, 108} = 8.72$, p < 0.001, Appendix G). Liver weights were increased in female P generation animals exposed to PFHxS compared to controls ($F_{4, 106} = 62.68$, p < 0.001, Appendix G). All other results in both sexes of P generation animals were not significant (p > 0.05) or biologically relevant (e.g., between treated groups rather than treated vs. control).

6.3.5 Sperm Analysis

Total sperm count was unaffected by PFHxS ($F_{4, 107} = 1.81$, p = 0.133), (Appendix H).

6.3.6 Hormone Analysis

Serum levels of sex hormones (i.e., estradiol, progesterone, and testosterone) were not evaluated in animals exposed to PFHxS due to negative data with the PFOS, PFNA, and 6:2 FTS experiments conducted herein.

Serum levels of thyroid were affected by exposure to PFHxS in P generation males (Wilks' Lambda = 0.586, $F_{8, 126} = 4.817$, p < 0.001) and females (Wilks' Lambda = 0.126, $F_{8, 140} = 31.731$, p < 0.001). This multivariate effect was due to an effect on T4 in both males ($F_{4, 64} = 7.190$, p < 0.001) and females ($F_{4, 71} = 85.551$, p < 0.001). T4 levels were decreased in males exposed to 7 and 14 mg/kg-day PFHxS and all treated females compared to controls (Appendix I).

6.3.7 Histopathology

Livers in males exposed to 14.0 mg/kg-day PFHxS were identical to control animals. Livers in females were unaffected by 14.0 mg/kg-day PFHxS (Pearson χ^2 (1, n = 19) = 0.532, p = 0.466).

6.3.8 Immune Assay

The effect of PFHxS on formation of PFCs differed in males and females. In males, PFC formation was decreased ($F_{4, 42.407} = 12.654$, p < 0.001) in the 14.0 mg/kg-day PFHxS group compared to all other groups. In females, PFC formation was decreased ($F_{4, 39.041} = 11.040$, p <

0.001) in the 7 and 14 mg/kg-day PFHxS groups compared to all other groups. In females, the 7.0 and 14.0 mg/kg-day PFHxS groups did not differ from one another (Appendix K).

6.3.9 Determination of BMD

Table 5. Summary of Effects in P Generation *P. maniculatus* Exposed to PFHxS

Critical effect	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	BMD (mg/kg-d)	BMDL (mg/kg-d)	BMR	Model
Increased stillbirths	7.0	14.0	13.4	5.72	10%	Nested logistic (isc+ilc+)
Increased liver: body weight (females)	n/a	1.6	1.27	0.81	1 St. dev.	Bayesian Hill
Decreased serum T4 (males)	3.5	7.0	7.39	3.33	1 St. dev.	Bayesian Hill
Decreased PFC formation (males)	7.0	14.0	8.79	6.12	1 St. dev.	Bayesian Hill
Decreased PFC formation (females)	7.0	14.0	6.91	4.36	1 St. dev.	Bayesian Hill

6.4 Experiment 4: 6:2 FTS

6.4.1 Analytical Verification of Dosing Solutions

Comparisons of the measured concentrations of 6:2 FTS in solution to the nominal concentrations of the solutions revealed 76-100% accuracy for the solutions containing 0.02, 0.1, 0.5, and 2.5 mg/mL 6:2 FTS at days 6, 20, and 35. As such, the doses administered were reported as nominal: 0.2, 1.0, 5.0 and 25 mg/kg-day 6:2 FTS, (Appendix D). Occurrence of contaminants in the 6:2 FTS dosing solutions are described in APHC (2021) and Narizzano et al. (2021).

6.4.2 Clinical Observations and Body Weight

Clinical observations in the P generation were limited to wounds, malocclusions, and hair loss. Body weight of P generation males was unaffected by 6:2 FTS ($F_{4, 100} = 0.584$, $p = 0.675$, Appendix E). Body weight of P generation females was unaffected by 6:2 FTS ($F_{4, 115} = 0.297$, $p = 0.879$). Finally, maternal weight gain was unaffected by 6:2 FTS ($F_{4, 65} = 0.577$, $p = 0.680$).

Clinical observations in the F1 generation were limited to wounds. Litter weights on PND0, 1, 4, 7, and 10 did not differ between 6:2 FTS-treated and control animals ($F_{4, 50} = 1.039$, $p = 0.397$, Appendix E). The developmental landmark of pinna unfolding was unaffected by 6:2 FTS ($F_{4, 51} = 0.428$, $p = 0.788$).

6.4.3 Reproduction and Fertility

Fertility (Pearson χ^2 (4, n = 117) = 1.381, p = 0.847) and pregnancy (Pearson χ^2 (4, n = 72) = 4.682, p = 0.321) were unaffected by 6:2 FTS (Appendix F).

Total litter size ($F_{4, 67} = 1.291$, p = 0.282) and live litter size ($F_{4, 67} = 0.908$, p = 0.464) were unaffected by 6:2 FTS (Appendix F).

Additionally, total litter loss by PND1 was unaffected by 6:2 FTS (Pearson χ^2 (4, n = 72) = 3.520, p = 0.475, Appendix F).

6.4.4 Necropsy and Organ Weights

Spleen weights were increased in male P generation animals exposed to 5 mg/kg-day 6:2 FTS ($F_{4, 100} = 2.98$, p = 0.023) compared to controls and 0.2 mg/kg-day 6:2 FTS (Appendix G). All other results in both sexes of P generation animals were not significant (p > 0.05).

6.4.5 Sperm Analysis

Sperm count could not be normalized; therefore, a Kruskal-Wallis nonparametric test was performed. Total sperm count was unaffected by 6:2 FTS ($H_4 = 3.22$, p = 0.522, Appendix H).

6.4.6 Hormone Analysis

Serum levels of sex hormones (i.e., estradiol, progesterone, and testosterone) were unaffected by 6:2 FTS exposure in P generation males (Wilks' Lambda = 0.909, $F_{12, 177.575} = 0.546$, p = 0.882) and females (Wilks' Lambda = 0.899, $F_{12, 182.848} = 0.624$, p = 0.820).

Serum levels of thyroid hormones were unaffected by 6:2 FTS exposure in P generation males (Wilks' Lambda = 0.943, $F_{8, 136.000} = 0.508$, p = 0.849) and females (Wilks' Lambda = 0.952, $F_{8, 140.000} = 0.435$, p = 0.899, Appendix I).

6.4.7 Histopathology

Livers in males (Pearson χ^2 (1, n = 46) = 0.013, p = 0.909), and females (Pearson χ^2 (1, n = 39) = 0.208, p = 0.648) were unaffected by 25 mg/kg-day 6:2 FTS. Spleens and thymuses in animals exposed to 25 mg/kg-day 6:2 FTS were identical to control animals.

6.4.8 Immune Assay

The effect of 6:2 FTS on formation of PFCs differed in males and females, with males producing more PFCs, in general, than females. In males, PFC formation was decreased ($F_{4, 40.298} = 36.679$, p < 0.001) in the 5.0 and 25.0 mg/kg-d 6:2 FTS groups compared to all other groups (Appendix K). In females, PFC formation was decreased ($F_{4, 31.344} = 28.680$, p < 0.001) in the 5.0 and 25.0 mg/kg-d 6:2 FTS groups compared to all other groups.

6.4.9 Determination of BMD

Table 6. Summary of Effects in P Generation *P. leucopus* Exposed to 6:2 FTS

Critical effect	NOAEL (mg/kg-d)	LOAEL (mg/kg-d)	BMD (mg/kg-d)	BMDL (mg/kg-d)	BMR	Model
Decreased PFC formation (males)	1.0	5.0	4.06	2.63	1 St. dev.	Bayesian Hill
Decreased PFC formation (females)	1.0	5.0	3.72	2.26	1 St. dev.	Bayesian Hill

6.5 Derivation of Serum-Based PODs

Table 7 presents protective critical effects for each PFAS investigated. Effects were considered protective, based on the biological relevance of the health effect (i.e., detrimental effects on reproduction, development, behavioral effects that could result in poor survival or predator avoidance, etc.) rather than the lowest BMDL value (Mayfield and Skall, 2018; Williams et al., 2015). Then, using the ratio of PFAS in serum (ng/mL) to the dose (mg/kg-day) delivered (Narizzano et al., 2021) serum-based PODs were calculated.

Table 7. Summary of PODs for *Peromyscus* Exposed to Four PFAS

Chemical	Protective Critical Effect	BMDL	PFAS (ng/mL)/dose (mg/kg-day) ^a	Serum-based POD (ng/mL)
PFOS	Increased total litter loss by PND1	0.12	47000.0	5640
PFHxS	Increased stillbirths per litter	5.72	1745.0	9982
PFNA	Decreased PFC formation	2.31	22583.3	52167
6:2 FTS	Decreased PFC formation	2.63 (male) 2.26 (female)	1.0	2.63 (male) 2.26 (female)

Legend:

^a Narizzano et al., 2021

7 DISCUSSION

PFOS did not affect reproductive performance (mating, sperm count, and fertility); however, reproductive outcome, as demonstrated by increased number of stillborn pups and total litter loss by PND1 were affected. These findings were consistent with studies by Luebker et al. (2005) and Grasty et al. (2003) in rats and Abbott et al. (2009) in mice. Notably, total litter loss occurred in 100% and 41% of *Peromyscus* exposed to 5.0 and 1.0 mg/kg-d PFOS, respectively. Our findings are consistent with Lau et al., (2003) who observed 100%, 50%, and negligible mortality in rat pups exposed prenatally to 5, 3, and 1 mg/kg-day. In contrast, Butenhoff et al., (2009a) noted no significant effects on reproductive outcomes or litter parameters in rats

exposed up to 1 mg/kg-day PFOS. A BMDL of 0.12 mg/kg-day was derived for PFOS based on total litter loss.

This BMDL for total litter loss expressed as circulating PFOS in serum in pregnant dams (with continuing exposure) is 5,640 ng/mL. This serum-specific POD relies on 28-day kinetics data from Narizzano et al. (2021) which suggests PFOS serum concentrations may continue to rise beyond 28 days of exposure. As serum concentration is the numerator in the ratio, if serum concentration is rising, the serum-specific POD may be higher than our estimate here. Toxicity is a function of accumulation of PFOS in target tissues—serum-specific PODs from nonsteady state serum concentrations need to be taken in context of bioaccumulation processes. For instance, in the absence of complete tissue kinetics, it remains unclear if high serum PFOS concentrations per unit dose are a snapshot into active kinetic processes or directly mimic target tissue concentrations.

Neither fertility nor pregnancy was affected by the doses of PFNA administered herein. This may be explained by the fact that administered doses were lower than what was targeted (i.e., 0.03, 0.14, 1.0, and 3.0 mg/kg-day vs. 0.04, 0.2, 1.0, and 5.0 mg/kg). Das et al. (2015) reported that >5.0 mg/kg-day PFNA induced lethality in inbred mice. The formation of PFCs, however, was decreased in animals exposed to 3 mg/kg-day PFNA; hence a BMDL of 2.31 mg/kg-day for nonreproductive/development effects. Furthermore, other immune endpoints have been negatively affected by PFNA at similar doses to those tested here, supporting the biological relevance of the observed effect (Fang et al., 2009; 2010; 2012a, b; 2008; Feng et al., 2009, 2010; Rockwell et al., 2013).

PFNA showed a steady-state serum concentration reached in 28 days, so it is likely that the serum-specific POD developed from immune effects in this study and the serum: dose ratio of Narizzano of 52000 ng/mL is potentially representative of serum concentrations in this study. Notably, this is an order of magnitude above the serum-specific PODs for other PFAS in this study even with similar magnitude BMDLs. The membrane-partitioning coefficient of PFNA is the second highest value in the PFAS of this study—PFNA data points to supporting the hypothesis of incomplete PFOS saturation in Narizzano et al. (2021), and that PFAS with high membrane distribution coefficients require high circulating serum concentrations to observe toxic effects.

PFHxS did not affect fertility, reproduction, total litter size, live litter size, or total litter loss, which is consistent with studies in laboratory rats and mice (Butenhoff et al., 2009b; Chang et al., 2018). However, stillbirths were increased in animals exposed to PFHxS compared to controls, hence a BMDL of 5.72 mg/kg-day. Of note, live litter size was reduced by 1 and 3 mg/kg-day PFHxS in mice (Chang et al., 2018) whereas this was unaffected in the current study (i.e., the highest dose (14.0 mg/kg-day PFHxS) was also the No observed adverse effect level (NOAEL)).

Expressing this reproductive effect as a serum-specific POD based on ratios in Narizzano et al. (2021) leads to an estimate of 9000 ng/mL. Importantly, in Narizzano et al., (2021) PFHxS (oral gavage) was observed to be steady state at day 28 so the animals in this study potentially had similar circulating serum concentrations. This serum-specific value is an order of magnitude

lower than PFNA and the reduction in membrane partitioning coefficient indicates this is reasonable. The log phospholipid membrane-water distribution coefficient (D_{mw}) is not, however, a whole log unit reduced. This indicates that process at the target tissue or excretion/metabolism processes also influence the relationship between toxicity, internal dosimetry, and dose delivered.

Oral exposures to 6:2 FTS did not affect fertility, reproduction, total litter size, live litter size, or total litter loss. However, the formation of PFCs was decreased in animals exposed to ≥ 5.0 mg/kg-day 6:2 FTS. As such, BMDLs of 2.63 and 2.26 mg/kg-day 6:2 FTS were derived for male and female *Peromyscus*, respectively, based on reduced immune response.

The estimation of serum-specific PODs in 6:2 FTS based on the data from Narizzano et al. (2021), lead to two main points. First, the very low serum: dose ratio (Narizzano et al., 2021) indicates that immune effects could occur at quite low circulating 6:2 FTS concentrations. The alternative hypotheses are that prior to day 28 and the activation of excretion or metabolic processes (see decrease in serum concentrations in Narizzano et al. 2021), 6:2 FTS has already reached toxic levels in target tissues or the metabolism/excretion of 6:2 FTS is the driver of immune effects. Based on the several orders of magnitude reduction in serum concentrations observed in Narizzano et al. (2021), it is possible that this serum-specific POD would increase. However, exposures in this study were much longer than in Narizzano et al. (2021), (i.e., 112 days vs. 28). As such, the reduced immune response after prolonged exposure may be associated with metabolites of 6:2 FTS or a function of adaptive excretion/metabolism process upregulation.

8 CONCLUSIONS

Endpoints affected by four PFAS in *Peromyscus* are largely consistent with studies previously conducted in inbred and outbred rats and mice. Specifically, prenatal exposure to high doses of PFOS caused neonatal mortality (BMDL 0.12 mg/kg-day PFOS), though growth and development were unaffected by low doses. Additionally, prenatal exposure to PFHxS caused increased stillbirths (BMDL 5.72 mg/kg-day PFHxS) and reduced immune response. At the doses tested, PFNA and 6:2 FTS did not markedly affect reproduction or development, but these experiments suggest they reduce immune response in *Peromyscus* (BMDL 2.31 mg/kg-d PFNA; BMDL 2.26–2.63 mg.kg-day 6:2 FTS). Finally, *Peromyscus* exposed to PFAAs, but not 6:2 FTS, exhibited decreased serum T4 levels and increased liver weights.

9 POINT OF CONTACT

Questions pertaining to this report should be referred to Dr. 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.0073458-18, Protocol No. 03-18-08-01: Reproductive and Developmental Toxicity of Per- and Polyfluoroalkyl Substances (PFAS) in *Peromyscus* spp., September 2018–October 2020, the following critical phases were inspected/audited Quality Systems and Regulatory Compliance Office (QSARC):

Study Specific Critical Phase Inspection/Audited	Date Inspected/Audited	Date reported to Management/SD
Study Protocol Good Laboratory Practice Standards and Animal Care Review	7/27/2018	7/27/2018
P Generation - Test System Facilities, Identification, Husbandry, Food/Water Monitoring, Enrichment	10/31/2018	11/06/2018
P Generation– Test Article Storage, Control, Dose Selection, Mixing, Labeling and Administration	10/31/2018	11/06/2018
P Gen - Compliance w protocol & test guideline, animal dose group composition & special husbandry	10/31/2018	11/06/2018
P Generation - Litter and Offspring Parameter Examinations	10/31/2018	11/06/2018
P Generation - Mating Co-Housing Procedures, observations, body weight and food/H2O consumption	10/31/2018	11/06/2018
Test System - Facilities, Husbandry, Feed and Water Supply and Veterinary Care	3/6/2019	3/14/2019
Test System ID, Body Wt & Food Consumption Procedures & Maintenance & Cal of Associated Equipment	3/6/2019	3/14/2019
Test Article and Immune Challenge Solution Administration Procedures Inspection and Document Review	3/06/2019	3/14/2019
Study Conduct and Record Keeping Good Documentation Procedures Review	3/06/2019	3/14/2019
Pre-Procedures, Anesthesia, and Blood Collection Procedures	3/13/2019	3/20/2019
Euthanasia, Gross Necropsy, Tissue Collection and Tissue Preservation Procedures	3/13/2019	3/20/2019
Sub Study Animal In-Life Endpoint Criteria Compliance	3/13/2019	3/20/2019
Maintenance and Calibration of Necropsy Related Equipment and Good Documentation Procedures	3/13/2019	3/20/2019

Toxicological Report No. S.0073458-18, September 2018–October 2020

Study Specific Critical Phase Inspection/Audited	Date Inspected/Audited	Date reported to Management/SD
Test System - Facilities, Husbandry, Feed and Water Supply and Veterinary Care	6/19/2019	6/26/2019
Test System Identification, Body Weight & Maintenance & Calibration of Associated Equipment	6/19/2019	6/26/2019
Study Conduct and Record Keeping Good Documentation Procedures Review	6/19/2019	6/26/2019
Test Substance - Mixing, Labeling, Administration via Oral Gavage & Initial Test System Observations	6/19/2019	6/26/2019
Test Substance - Mixing, Labeling, Administration via Oral Gavage & Initial Test System Observations	11/15/2019	11/22/2020
Pre and Post-procedural Provisions, Observations and Body Weight Determination	11/15/2019	11/22/2020
Maintenance and Calibration of Equipment and Good Documentation Practices	11/15/2019	11/22/2020
Test System Facilities, Identification, Husbandry, Feed and Water Supply & Enrichment	11/15/2019	11/22/2020
Necropsy Study Personnel Qualifications, Training Records Review and Good Documentation Procedures	1/15/2020	1/22/2020
Necropsy Procedures, Maintenance and Calibration of Equipment and Good Documentation Procedures	1/15/2020	1/22/2020
Test Article Specific Sub-study Endpoint Criteria Compliance	1/15/2020	1/22/2020
PFHxS - Test Article Storage, Control, Mixing, and Labeling	1/17/2020	1/24/2020
PFHxS - Test System Facilities, Identification, Husbandry, Feed and Water Supply & Enrichment	1/17/2020	1/24/2020
6:2 FTS - Pre and Post-procedural Provisions, Observations and Body Weight Determination	1/17/2020	1/24/2020
PFHxS - Maintenance and Calibration of Equipment and Good Documentation Practices	9/25/2020	9/27/2020
PFHxS - Test System Facilities, Identification, Husbandry, Feed and Water Supply & Enrichment	9/25/2020	9/27/2020
PFHxS - Maintenance and Calibration of Equipment and Good Documentation Practices	9/25/2020	9/27/2020
PFHxS - Test Article Storage, Control, Mixing, and Labeling	9/25/2020	9/27/2020
Necropsy Procedures, Maintenance and Calibration of Equipment and Good Documentation Procedures	10/2/2020	10/6/2020
Sub Study Animal In-Life Endpoint Criteria Compliance	10/02/2020	10/06/2020

Toxicological Report No. S.0073458-18, September 2018–October 2020

Study Specific Critical Phase Inspection/Audited	Date Inspected/Audited	Date reported to Management/SD
Sub Study Report and Raw Data Good Laboratory Practice Review	5/12/2021	5/17/2021
PFHxS - Test System Facilities, Identification, Husbandry, Feed and Water Supply & Enrichment	6/24/2021	7/01/2020
Necropsy Gross Macroscopic Pathology Exam, tissue collection and tissue preservation procedures and sub-study endpoint criteria	9/24/2021	9/24/2021
Histopathology Procedures and Protocol and SOP compliance	9/24/2021	9/24/2021
Necropsy Study Personnel Qualifications, Training Records Review and Good Documentation Procedures	9/24/2021	9/24/2021
Necropsy Procedures, Maintenance and Calibration of Equipment and Good Documentation Procedures	9/24/2021	9/24/2021
Final Study Report and Raw Data Good Laboratory Practice Review	12/09/2021	12/13/2021

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 of 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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Michael P. Kefauver
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 S. 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, Ph.D., 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 L. Kilby, Animal Health Technician.

In-Life Support: Taryn K. Brown, Biological Science Technician, TEV; Meredith E. Bohannon, Biologist, TEV; Lee C.B. Crouse, Biologist, TEV; Blas A. Guigni, Biologist, TEV; Theresa L. Hanna, Biological Science Technician, TEV; Jarod M. Hanson, Executive Officer, TOX; Allison M. Narizzano, Biologist, TEV; Caroline L. Procell, Biological Science Technician, HEF; Michael J. Quinn, Biologist, HEF; Mark R. Way, Biologist, TEV; HEF.

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

Appendix D
Analytical Verification of Dosing Solutions

Table D-1. Mean Analytical Concentrations of PFOS in Water

	Nominal (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	<2.0e ⁻⁹	--	3.05e ⁻⁹	--	--	--	0
	0.02	0.017	83.5	0.021	105	0.02	94.5	0.2
	0.1	0.091	91.0	0.107	107	0.01	99.0	1.0
	0.5	0.342	68.4	0.479	95.8	0.41	82.1	5.0

Dosing solutions were prepared and shipped immediately to Eurofins Eaton Analytical for initial sample verification. Samples for stability verification were held at U.S. Army Public Health Center for 2 weeks and sent to Eurofins. Day 14: Samples were held for 1 (control) or 2 (PFOS) weeks prior to being extracted or diluted, respectively, and analyzed. Day 28: Samples were held an additional week at Eurofins prior to being extracted (control) or diluted (PFOS) and another week prior to being analyzed.

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

	Nominal (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	4.33e ⁻⁹	--	9.23e ⁻⁹	--	6.78e ⁻⁹	--	0
	0.004	0.002	59.0	0.003	68.5	0.003	63.8	0.03
	0.02	0.013	63.5	0.015	74.5	0.014	69.0	0.14
	0.1	0.067	66.8	0.076	75.8	0.071	71.3	1.0
	0.5	0.260	52.0	0.322	64.4	0.291	58.2	3.0

Dosing solutions were prepared and shipped immediately to Eurofins Eaton Analytical for initial sample verification. Samples for stability verification were held at U.S. Army Public Health Center for 2 weeks and sent to Eurofins. Day 14: Samples were held for 1 (control) or 2 (PFNA) weeks prior to being extracted or diluted, respectively, and analyzed. Day 28: Samples were held an additional week at Eurofins prior to being extracted (control) or diluted (PFNA) and another week prior to being analyzed.

Table D-3. Mean Analytical Concentrations of PFHxS in Water

	Nominal (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 H x S	0	8.02e ⁻⁸	--	2.95e ⁻⁸	--	6.78e ⁻⁹	--	0
	0.025	0.151	60.4	0.171	68.4	0.161	64.4	1.6
	0.5	0.343	68.6	0.374	74.8	0.359	71.7	3.5
	1.0	0.617	61.7	0.721	72.1	0.669	66.9	7.0
	2.0	1.31	65.5	1.46	73.0	1.386	69.3	14.0

Dosing solutions were prepared and shipped immediately to Eurofins Eaton Analytical for initial sample verification. Samples for stability verification were held at U.S. Army Public Health Center for 2 weeks and sent to Eurofins. Day 14: Samples were held for 1 (control) or 2 (PFHxS) weeks prior to being extracted or diluted, respectively, and analyzed. Day 28: Samples were held an additional week at Eurofins prior to being extracted (control) or diluted (PFHxS) and another week prior to being analyzed.

Table D-4. Mean Analytical Concentrations of 6:2 FTS in Water

	Nominal (mg/mL)	Measured (mg/mL) Day 6	Accuracy (%) Day 6	Measured (mg/mL) Day 20	Accuracy (%) Day 20	Measured (mg/mL) Day 35	Accuracy (%) Day 35	Average (mg/mL)	Average Accuracy (%)	Dose (mg/kg- day)
6:2 FTS	0	<LOQ	--	<LOQ	--	<LOQ	--	<LOQ	--	0
	0.02	0.020	100	0.020	100.0	0.019	95.0	0.020	98.3	0.2
	0.1	0.96	96	0.88	88.0	0.89	89.0	0.091	91.0	1.0
	0.5	--	--	0.46	92.0	0.43	86.0	0.445	89.0	5.0
	2.5	2.20	88.8	1.90	76.0	1.90	76.0	2.00	80.0	25

Dosing solutions were prepared and shipped immediately to 3M. Samples were held for 1 weeks prior to being diluted and analyzed. Samples for stability verification were held an additional 2 and 4 weeks at 3M prior to being diluted and analyzed.

Appendix E
Body Weights

Table E-1. Body Weight (g) of P Generation Males Over 20 Weeks of Exposure to PFOS

Week of exposure	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day
Week 0	22.51 ± 0.66, N=31	22.71 ± 0.65, N=31	22.54 ± 0.68, N=31	22.03 ± 0.51, N=31
Week 1	22.13 ± 0.60, N=29	22.65 ± 0.61, N=31	22.09 ± 0.60, N=31	21.10 ± 0.47, N=31
Week 2	21.97 ± 0.58, N=29	22.30 ± 0.59, N=31	21.65 ± 0.57, N=31	20.57 ± 0.44, N=31
Week 3	21.70 ± 0.52, N=29	22.05 ± 0.61, N=31	21.84 ± 0.58, N=31	20.58 ± 0.38, N=31
Week 4	21.48 ± 0.52, N=29	21.62 ± 0.60, N=31	21.46 ± 0.58, N=31	20.06 ± 0.34, N=31
Week 5	21.59 ± 0.48, N=29	21.40 ± 0.52, N=30	21.39 ± 0.46, N=31	20.26 ± 0.30, N=31
Week 6	21.39 ± 0.48, N=29	20.70 ± 0.48, N=30	21.19 ± 0.44, N=31	19.89 ± 0.30, N=31
Week 7	21.11 ± 0.46, N=29	20.82 ± 0.45, N=30	20.78 ± 0.44, N=31	19.79 ± 0.33, N=31
Week 8	21.24 ± 0.43, N=29	20.75 ± 0.42, N=30	20.86 ± 0.43, N=30	19.64 ± 0.32, N=31
Week 9	20.91 ± 0.53, N=29	20.48 ± 0.48, N=30	20.71 ± 0.40, N=30	19.41 ± 0.33, N=31
Week 10	21.31 ± 0.47, N=27	20.81 ± 0.49, N=30	20.98 ± 0.38, N=30	20.17 ± 0.29, N=28
Week 11	21.27 ± 0.44, N=27	20.47 ± 0.42, N=30	20.56 ± 0.42, N=29	19.91 ± 0.31, N=27
Week 12	21.37 ± 0.45, N=27	20.71 ± 0.51, N=28	20.64 ± 0.39, N=28	19.96 ± 0.32, N=27
Week 13	21.10 ± 0.46, N=26	20.56 ± 0.48, N=27	20.34 ± 0.36, N=27	19.86 ± 0.31, N=27
Week 14	21.07 ± 0.40, N=26	20.19 ± 0.53, N=26	20.12 ± 0.48, N=26	19.65 ± 0.31, N=26
Week 15	20.78 ± 0.45, N=26	20.01 ± 0.48, N=24	20.36 ± 0.42, N=24	19.49 ± 0.29, N=26
Week 16	21.23 ± 0.44, N=26	20.09 ± 0.49, N=24	20.57 ± 0.41, N=24	19.76 ± 0.35, N=25
Week 17	20.91 ± 0.46, N=26	20.00 ± 0.53, N=22	20.30 ± 0.39, N=23	19.89 ± 0.35, N=24
Week 18	20.95 ± 0.51, N=23	20.05 ± 0.55, N=22	20.44 ± 0.37, N=23	19.92 ± 0.34, N=24
Week 19	20.75 ± 0.58, N=22	20.23 ± 0.52, N=22	20.32 ± 0.39, N=22	20.29 ± 0.32, N=24
Week 20	21.18 ± 0.57, N=19	20.42 ± 0.57, N=20	20.92 ± 0.44, N=19	20.44 ± 0.35, N=23

Note:

Mean ± SEMs

Table E-2. Body Weight (g) of P Generation Females Over 20 Weeks of Exposure to PFOS

Week of exposure	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day
Week 0	19.67 ± 0.55, N=31	19.24 ± .047, N=31	20.24 ± 0.56, N=31	20.04 ± 0.50, N=31
Week 1	19.43 ± 0.51, N=31	19.03 ± 0.46, N=31	19.88 ± 0.55, N=31	19.62 ± 0.49, N=31
Week 2	19.37 ± 0.53, N=31	19.00 ± 0.47, N=31	19.49 ± 0.56, N=31	19.15 ± 0.48, N=31
Week 3	19.00 ± 0.51, N=30	18.82 ± 0.45, N=31	19.55 ± 0.55, N=31	18.88 ± 0.48, N=31
Week 4	19.67 ± 0.55, N=31	19.24 ± 0.47, N=31	20.24 ± 0.56, N=31	20.04 ± 0.50, N=31
Week 5	19.43 ± 0.51, N=31	19.03 ± 0.46, N=31	19.88 ± 0.55, N=31	19.62 ± 0.49, N=31
Week 6	19.37 ± 0.53, N=31	19.00 ± 0.47, N=31	19.49 ± 0.56, N=31	19.15 ± 0.48, N=31
Week 7	19.00 ± 0.51, N=30	18.82 ± 0.45, N=31	19.55 ± 0.55, N=31	18.88 ± 0.48, N=31
Week 8	18.76 ± 0.47, N=30	18.35 ± 0.43, N=31	19.11 ± 0.49, N=31	18.46 ± 0.44, N=31
Week 9	19.34 ± 0.43, N=30	18.91 ± 0.38, N=31	19.28 ± 0.46, N=31	18.86 ± 0.37, N=31
Week 10	19.15 ± 0.41, N=30	18.65 ± 0.41, N=31	19.48 ± 0.48, N=30	18.79 ± 0.38, N=31
Week 11	19.10 ± 0.44, N=30	18.84 ± 0.48, N=31	19.77 ± 0.55, N=30	18.87 ± 0.42, N=30
Week 12	19.43 ± 0.56, N=30	18.84 ± 0.54, N=31	19.86 ± 0.59, N=29	19.31 ± 0.53, N=29
Week 13	19.18 ± 0.51, N=30	19.15 ± 0.72, N=30	19.97 ± 0.57, N=29	18.84 ± 0.40, N=27
Week 14	19.19 ± 0.49, N=30	19.52 ± 0.63, N=30	20.64 ± 0.69, N=29	19.45 ± 0.45, N=25
Week 15	19.40 ± 0.52, N=29	19.07 ± 0.58, N=30	20.21 ± 0.67, N=29	19.52 ± 0.49, N=25
Week 16	19.46 ± 0.54, N=29	19.18 ± 0.67, N=28	19.87 ± 0.56, N=27	19.71 ± 0.56, N=25
Week 17	19.77 ± 0.66, N=28	18.80 ± 0.61, N=28	19.89 ± 0.60, N=26	19.15 ± 0.43, N=25
Week 18	19.21 ± 0.40, N=27	18.43 ± 0.40, N=26	20.05 ± 0.76, N=25	19.20 ± 0.51, N=24
Week 19	19.73 ± 0.51, N=27	18.59 ± 0.52, N=25	20.28 ± 0.85, N=24	19.93 ± 0.56, N=24
Week 20	20.20 ± 0.64, N=27	18.99 ± 0.46, N=25	20.29 ± 0.74, N=24	19.82 ± 0.46, N=23

Note:

Mean ± SEMs

Table E-3. Body Weight (g) of Dams Exposed to PFOS during Gestation

Gestation day (GD)	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day
GD0	16.80 ± 0.95, N=3	17.85 ± 0.55, N=2	20.50 ± 0.99, N=4	18.40, N=1
GD1	19.43 ± 0.63, N=6	21.25 ± 2.55, N=2	17.60, N=1	19.00 ± 1.42, N=3
GD2	18.37 ± 0.92, N=3	17.60 ± 0.31, N=3	19.68 ± 0.67, N=4	17.80, N=1
GD3	N/A	19.18 ± 0.51, N=4	20.15 ± 1.04, N=4	18.15 ± 1.15, N=2
GD4	19.27 ± 0.87, N=6	18.95 ± 0.67, N=4	19.63 ± 0.41, N=4	19.90, N=1
GD5	19.17 ± 1.07, N=3	N/A	18.38 ± 1.43, N=4	17.25 ± 1.15, N=2
GD6	16.90, N=1	18.24 ± 0.51, N=5	19.90 ± 0.63, N=6	N/A
GD7	16.97 ± 0.79, N=3	18.40 ± 0.00, N=2	20.18 ± 0.63, N=4	19.40, N=1
GD8	21.17 ± 0.80, N=6	21.55 ± 2.15, N=2	17.40, N=1	19.80 ± 1.72, N=3
GD9	19.23 ± 1.10, N=3	18.15 ± 0.39, N=4	19.76 ± 0.72, N=5	18.50, N=1
GD10	15.70, N=1	19.97 ± 0.58, N=3	20.32 ± 0.83, N=5	18.50 ± 0.60, N=2
GD11	20.48 ± 1.71, N=4	19.53 ± 0.52, N=4	21.13 ± 0.57, N=4	22.60, N=1
GD12	20.37 ± 1.20, N=3	22.45 ± 2.55, N=2	20.27 ± 0.89, N=6	18.20 ± 0.70, N=2
GD13	18.90, N=1	20.00 ± 0.34, N=4	21.80 ± 1.24, N=4	N/A
GD14	20.70 ± 1.99, N=4	20.50 ± 0.70, N=2	22.63 ± 0.76, N=4	20.00 ± 0.70, N=2
GD15	22.37 ± 0.66, N=6	24.13 ± 1.79, N=3	22.43 ± 1.39, N=4	21.80 ± 1.47, N=3
GD16	22.43 ± 1.34, N=3	21.08 ± 0.31, N=4	22.48 ± 0.64, N=4	20.00, N=1
GD17	17.80, N=1	23.87 ± 1.13, N=3	23.70 ± 0.82, N=6	18.60, N=1
GD18	24.17 ± 1.21, N=6	24.68 ± 1.50, N=5	24.57 ± 1.80, N=3	24.20, N=1
GD19	24.40 ± 0.94, N=5	21.50, N=1	21.70 ± 0.50, N=5	22.70 ± 1.60, N=2
GD20	23.60, N=1	24.23 ± 0.31, N=4	26.68 ± 1.28, N=4	19.70, N=1
GD21	24.93 ± 2.32, N=4	27.10 ± 1.07, N=5	27.88 ± 1.32, N=6	24.40, N=1
GD22	26.82 ± 0.82, N=6	33.00 ± 3.20, N=2	26.28 ± 1.75, N=4	25.30 ± 1.18, N=3
GD23	27.53 ± 2.08, N=3	26.10 ± 0.70, N=4	25.98 ± 1.70, N=5	22.10, N=1

Note:

Mean ± SEMs

Table E-4. Average Pup Weight (g) and Day of Pinna Unfolding and Eye Opening in Pups of Dams Exposed to PFOS

Postnatal day (PND)	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day
PND0	1.60 ± 0.05, N=13	1.80 ± 0.07, N=11	1.80 ± 0.13, N=12
PND4	2.50 ± 0.11, N=12	2.81 ± .013, N=11	2.79 ± 0.15, N=9
PND7	3.45 ± 0.14, N=12	3.72 ± 0.21, N=10	3.91 ± 0.21, N=9
PND10	4.41 ± 0.20, N=12	4.82 ± 0.14, N=10	4.96 ± 0.36, N=9
PND14	5.24 ± 0.34, N=12	5.76 ± 0.18, N=11	6.53 ± 0.51, N=8
PND17	6.21 ± 0.35, N=10	6.48 ± 0.26, N=11	7.38 ± 0.56, N=9
PND20	6.97 ± 0.40, N=11	7.80 ± 0.43, N=11	8.45 ± 0.69, N=9
PND25	9.10 ± 0.41, N=11	9.59 ± 0.34, N=10	10.35 ± 0.68, N=9
Milestone	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day
Pinna unfolding	2.83 ± 0.24, N=12	2.82 ± 0.33, N=11	3.44 ± 0.29, N=9
Eye opening	12.75 ± 0.58, N=12	12.00 ± 0.40, N=11	14.11 ± 1.71, N=9

Note:

Mean ± SEMs

Table E-5. Body Weights (g) of P Generation Males Over 16 Weeks of Exposure to PFNA

Week of exposure	0 mg/kg-day	0.03 mg/kg-day	0.14 mg/kg-day	1.0 mg/kg-day	3.0 mg/kg-day
Week 0	21.66 ± 0.52, N=25	21.65 ± 0.58, N=25	21.37 ± 0.53, N=25	21.11 ± 0.64, N=25	20.35 ± 0.53, N=25
Week 1	21.66 ± 0.56, N=24	21.56 ± 0.59, N=25	21.38 ± 0.55, N=25	21.03 ± 0.64, N=25	20.46 ± 0.50, N=25
Week 2	21.64 ± 0.53, N=23	21.54 ± 0.60, N=25	21.45 ± 0.52, N=25	20.89 ± 0.60, N=25	20.46 ± 0.49, N=25
Week 3	21.74 ± 0.53, N=23	21.59 ± 0.62, N=24	21.23 ± 0.47, N=25	20.84 ± 0.62, N=25	20.42 ± 0.51, N=25
Week 4	21.73 ± 0.54, N=23	21.88 ± 0.61, N=24	21.38 ± 0.48, N=25	20.90 ± 0.56, N=25	20.64 ± 0.49, N=25
Week 5	21.20 ± 0.41, N=23	20.89 ± 0.51, N=24	20.87 ± 0.45, N=24	20.19 ± 0.51, N=25	20.30 ± 0.45, N=25
Week 6	21.36 ± 0.38, N=22	21.08 ± 0.48, N=24	20.96 ± 0.41, N=25	20.56 ± 0.50, N=25	20.46 ± 0.45, N=25
Week 7	21.50 ± 0.37, N=23	21.13 ± 0.45, N=24	21.08 ± 0.40, N=25	20.69 ± 0.50, N=24	20.67 ± 0.44, N=25
Week 8	21.24 ± 0.38, N=23	20.93 ± 0.44, N=24	21.03 ± 0.42, N=25	20.58 ± 0.49, N=24	20.50 ± 0.43, N=25
Week 9	21.54 ± 0.40, N=23	21.29 ± 0.46, N=24	21.04 ± .049, N=25	21.07 ± 0.52, N=22	20.76 ± 0.45, N=25
Week 10	21.96 ± 0.43, N=23	21.62 ± 0.55, N=24	21.58 ± 0.49, N=24	21.23 ± 0.54, N=22	20.89 ± 0.43, N=25
Week 11	22.37 ± 0.46, N=23	21.75 ± 0.60, N=24	21.87 ± 0.51, N=23	21.25 ± 0.58, N=22	21.16 ± 0.44, N=25
Week 12	22.56 ± 0.51, N=23	21.73 ± 0.64, N=24	22.00 ± 0.50, N=23	21.38 ± 0.56, N=22	21.11 ± 0.46, N=25
Week 13	22.45 ± 0.52, N=23	21.68 ± 0.63, N=24	21.83 ± 0.50, N=22	21.30 ± 0.58, N=22	21.02 ± 0.43, N=25
Week 14	22.40 ± 0.51, N=23	21.71 ± 0.66, N=23	21.79 ± 0.48, N=22	21.04 ± 0.59, N=22	20.95 ± 0.43, N=25
Week 15	22.39 ± 0.51, N=23	21.62 ± 0.58, N=24	21.78 ± 0.47, N=22	21.27 ± 0.61, N=22	20.79 ± 0.43, N=25
Week 16	22.42 ± 0.52, N=22	21.80 ± 0.54, N=24	21.83 ± 0.50, N=22	21.53 ± 0.59, N=22	20.96 ± 0.43, N=25

Note:

Mean ± SEMs

Table E-6. Body Weight (g) of P Generation Females Exposed to PFNA Prior to Establishing Mating Pairs

Week of exposure	0 mg/kg-day	0.03 mg/kg-day	0.14 mg/kg-day	1.0 mg/kg-day	3.0 mg/kg-day
Week 0	19.00 ± 0.43, N=25	19.18 ± 0.62, N=25	18.94 ± 0.59, N=25	18.55 ± 0.47, N=25	18.95 ± 0.46, N=25
Week 1	18.88 ± 0.44, N=25	18.78 ± 0.64, N=25	18.60 ± 0.52, N=25	18.42 ± 0.47, N=25	18.71 ± 0.46, N=25
Week 2	18.90 ± 0.39, N=25	18.92 ± 0.56, N=25	18.70 ± 0.52, N=25	18.40 ± 0.42, N=25	18.98 ± 0.44, N=25
Week 3	18.70 ± 0.42, N=25	18.88 ± 0.54, N=25	18.85 ± 0.52, N=25	18.18 ± 0.43, N=25	19.05 ± 0.50, N=24
Week 4	18.76 ± 0.40, N=25	18.96 ± 0.50, N=25	18.71 ± 0.52, N=25	18.32 ± 0.40, N=25	19.10 ± 0.49, N=24

Note:

Mean ± SEMs

Table E-7. Body Weight (g) of Dams Exposed to PFNA during Gestation

Gestation day (GD)	0 mg/kg-day	0.03 mg/kg-day	0.14 mg/kg-day	1.0 mg/kg-day	3.0 mg/kg-day
GD0	18.59 ± 0.36, N=21	19.09 ± 0.61, N=20	19.28 ± 0.60, N=19	18.04 ± 0.32, N=20	19.05 ± 0.41, N=15
GD1	18.53 ± 0.32, N=21	18.78 ± 0.49, N=20	19.06 ± 0.56, N=20	17.98 ± 0.34, N=20	18.82 ± 0.37, N=16
GD2	18.54 ± 0.36, N=21	18.82 ± 0.45, N=20	19.00 ± 0.54, N=20	18.08 ± 0.33, N=20	18.91 ± 0.42, N=17
GD3	18.40 ± 0.33, N=21	18.84 ± 0.46, N=20	19.13 ± 0.53, N=20	18.23 ± 0.37, N=20	18.94 ± 0.41, N=17
GD4	18.38 ± 0.30, N=21	18.80 ± 0.41, N=20	19.19 ± 0.49, N=20	18.51 ± 0.36, N=20	18.98 ± 0.43, N=17
GD5	18.64 ± 0.30, N=21	18.92 ± 0.44, N=20	19.07 ± 0.51, N=20	18.51 ± 0.35, N=20	19.20 ± 0.42, N=17
GD6	18.79 ± 0.32, N=21	18.97 ± 0.43, N=20	19.10 ± 0.54, N=20	18.62 ± 0.35, N=20	19.26 ± 0.40, N=17
GD7	18.80 ± 0.33, N=21	19.00 ± 0.44, N=20	19.29 ± 0.50, N=20	18.75 ± 0.36, N=20	19.32 ± 0.42, N=17
GD8	19.15 ± 0.34, N=20	19.11 ± 0.46, N=20	19.53 ± 0.53, N=20	18.83 ± 0.37, N=20	19.37 ± .043, N=17
GD9	19.26 ± 0.33, N=21	19.43 ± 0.46, N=20	19.77 ± 0.50, N=20	19.10 ± 0.39, N=20	19.66 ± 0.46, N=17
GD10	19.36 ± 0.35, N=21	19.63 ± 0.46, N=20	19.94 ± 0.46, N=20	19.17 ± 0.40, N=20	19.73 ± 0.40, N=17
GD11	19.55 ± 0.31, N=21	19.88 ± 0.50, N=20	20.24 ± 0.48, N=20	19.49 ± 0.39, N=20	20.00 ± 0.49, N=17
GD12	20.00 ± 0.34, N=21	20.22 ± 0.46, N=20	20.54 ± 0.53, N=20	19.71 ± 0.40, N=20	20.58 ± 0.47, N=17
GD13	20.67 ± 0.34, N=21	20.89 ± 0.47, N=20	21.22 ± 0.55, N=20	20.29 ± 0.40, N=20	21.09 ± 0.54, N=17
GD14	21.24 ± 0.38, N=21	21.29 ± 0.53, N=20	21.83 ± 0.55, N=20	20.85 ± 0.41, N=20	21.86 ± 0.52, N=17
GD15	21.67 ± 0.41, N=21	21.82 ± 0.57, N=20	22.44 ± 0.59, N=20	21.48 ± 0.39, N=20	22.56 ± 0.47, N=17
GD16	22.14 ± 0.38, N=21	22.36 ± 0.56, N=20	23.03 ± 0.59, N=20	21.86 ± 0.43, N=20	22.91 ± 0.46, N=17
GD17	22.69 ± 0.36, N=21	22.74 ± 0.58, N=20	23.16 ± 0.57, N=20	22.43 ± 0.41, N=20	23.52 ± 0.46, N=17
GD18	23.39 ± 0.44, N=21	23.36 ± 0.65, N=20	23.76 ± 0.58, N=20	22.75 ± 0.40, N=20	24.05 ± 0.49, N=17
GD19	24.08 ± 0.44, N=21	23.90 ± 0.66, N=20	24.58 ± 0.61, N=20	23.51 ± 0.42, N=20	24.79 ± 0.50, N=17
GD20	24.87 ± 0.49, N=21	24.73 ± 0.70, N=20	25.25 ± 0.66, N=20	24.16 ± 0.45, N=20	26.06 ± 0.59, N=17
GD21	25.86 ± 0.53, N=21	25.33 ± 0.75, N=20	26.05 ± 0.68, N=20	25.01 ± 0.50, N=20	26.84 ± 0.59, N=17
GD22	26.68 ± 0.57, N=21	25.97 ± 0.80, N=20	26.57 ± 0.89, N=20	25.98 ± 0.56, N=20	27.85 ± 0.65, N=17
GD23	26.97 ± 0.59, N=21	25.77 ± 0.96, N=20	26.66 ± 0.91, N=20	26.37 ± 0.55, N=20	28.07 ± 0.68, N=17

Note:

Mean ± SEMs

Table E-8. Average Pup Weight (g) and Day of Pinna Unfolding in Pups of Dams Exposed to PFNA

Postnatal day (PND)	0 mg/kg-day	0.03 mg/kg-day	0.14 mg/kg-day	1.0 mg/kg-day	3.0 mg/kg-day
PND0	1.77 ± 0.05, N=20	1.73 ± 0.07, N=19	1.62 ± 0.05, N=16	1.60 ± 0.05, N=19	1.73 ± 0.06, N=12
PND1	1.96 ± 0.07, N=19	1.94 ± 0.10, N=18	1.88 ± 0.07, N=15	1.76 ± 0.05, N=18	1.88 ± 0.06, N=12
PND4	2.84 ± 0.11, N=19	2.85 ± 0.16, N=17	2.73 ± 0.13, N=15	2.66 ± 0.09, N=18	2.81 ± 0.16, N=12
PND7	3.96 ± 0.14, N=19	3.91 ± 0.24, N=17	3.94 ± 0.21, N=15	3.74 ± 0.13, N=18	3.75 ± 0.19, N=12
PND10	5.03 ± 0.16, N=19	4.79 ± 0.28, N=18	5.06 ± 0.27, N=15	4.83 ± 0.20, N=18	4.70 ± 0.25, N=12
Milestone	0 mg/kg-day	0.03 mg/kg-day	0.14 mg/kg-day	1.0 mg/kg-day	3.0 mg/kg-day
Pinna unfolding	3.53 ± 0.28, N=19	3.47 ± 0.24, N=17	3.07 ± 0.27, N=14	4.33 ± 0.29, N=18	4.42 ± 0.29, N=12

Note:

Mean ± SEMs

Table E-9. Body Weight (g) of P Generation Males Over 16 Weeks of Exposure to PFHxS

Week of exposure	0 mg/kg-day	1.6 mg/kg-day	3.5 mg/kg-day	7.0 mg/kg-day	14.0 mg/kg-day
Week 0	18.39 ± 0.25, N=24	18.28 ± 0.30, N=25	18.65 ± 0.24, N=25	18.24 ± 0.33, N=25	18.17 ± 0.35, N=25
Week 1	17.99 ± 0.30, N=24	17.89 ± 0.33, N=25	18.09 ± 0.29, N=24	18.02 ± 0.37, N=25	17.92 ± 0.33, N=25
Week 2	18.00 ± 0.27, N=24	17.89 ± 0.34, N=25	18.19 ± 0.28, N=24	18.13 ± 0.34, N=25	18.02 ± 0.35, N=25
Week 3	18.06 ± 0.26, N=23	17.96 ± 0.36, N=25	18.25 ± 0.30, N=24	18.16 ± 0.36, N=25	17.98 ± 0.32, N=25
Week 4	17.93 ± 0.31, N=23	18.19 ± 0.39, N=25	18.30 ± 0.33, N=24	18.32 ± 0.33, N=25	18.21 ± 0.32, N=25
Week 5	18.06 ± 0.25, N=23	18.10 ± 0.35, N=25	18.43 ± 0.23, N=24	18.08 ± 0.24, N=25	18.32 ± 0.29, N=25
Week 6	18.13 ± 0.22, N=24	18.15 ± 0.35, N=25	18.19 ± 0.24, N=24	17.97 ± 0.27, N=25	18.00 ± 0.25, N=25
Week 7	18.18 ± 0.25, N=24	18.06 ± 0.33, N=25	18.48 ± 0.24, N=24	18.05 ± 0.26, N=25	18.14 ± 0.27, N=25
Week 8	18.27 ± 0.24, N=24	18.07 ± 0.33, N=25	18.53 ± 0.25, N=23	18.04 ± 0.27, N=24	18.16 ± 0.25, N=25
Week 9	18.41 ± 0.21, N=24	18.25 ± 0.31, N=25	18.73 ± 0.27, N=22	18.10 ± 0.28, N=24	18.28 ± 0.27, N=25
Week 10	18.54 ± 0.23, N=24	18.48 ± 0.30, N=25	18.74 ± 0.30, N=21	18.24 ± 0.27, N=24	18.56 ± 0.29, N=25
Week 11	18.59 ± 0.23, N=24	18.51 ± 0.32, N=25	18.97 ± 0.29, N=21	18.47 ± 0.25, N=24	18.50 ± 0.27, N=24
Week 12	18.64 ± 0.25, N=24	18.69 ± 0.33, N=25	18.79 ± 0.25, N=21	18.63 ± 0.24, N=24	18.57 ± 0.29, N=24
Week 13	18.73 ± 0.27, N=24	18.66 ± 0.33, N=25	18.90 ± 0.29, N=21	18.52 ± 0.23, N=24	18.75 ± 0.27, N=24
Week 14	18.70 ± 0.22, N=24	18.61 ± 0.32, N=24	18.92 ± 0.28, N=21	18.48 ± 0.22, N=23	18.79 ± 0.30, N=24
Week 15	18.60 ± 0.25, N=23	18.66 ± 0.34, N=24	18.91 ± 0.27, N=21	18.45 ± 0.23, N=23	18.75 ± 0.26, N=24
Week 16	18.76 ± 0.26, N=23	18.83 ± 0.30, N=24	19.05 ± 0.25, N=20	18.60 ± 0.22, N=23	18.92 ± 0.28, N=24

Note:

Mean ± SEMs

Table E-10. Body Weight (g) of P Generation Females Exposed to PFHxS Prior to Establishing Mating Pairs

Week of exposure	0 mg/kg-day	1.6 mg/kg-day	3.5 mg/kg-day	7.0 mg/kg-day	14.0 mg/kg-day
Week 0	16.42 ± 0.27, N=25	16.72 ± 0.28, N=25	16.27 ± 0.33, N=25	16.26 ± 0.36, N=25	15.96 ± 0.23, N=24
Week 1	16.15 ± 0.28, N=25	16.72 ± 0.33, N=25	15.82 ± 0.28, N=25	16.22 ± 0.36, N=24	15.77 ± 0.25, N=24
Week 2	16.28 ± 0.27, N=24	16.64 ± 0.28, N=25	15.98 ± 0.27, N=25	16.44 ± 0.34, N=24	16.03 ± 0.25, N=24
Week 3	16.34 ± 0.31, N=24	16.84 ± 0.28, N=25	16.17 ± 0.30, N=25	16.61 ± 0.39, N=24	16.28 ± 0.24, N=24
Week 4	16.50 ± 0.35, N=24	16.94 ± 0.31, N=25	16.42 ± 0.30, N=25	16.85 ± 0.37, N=24	16.60 ± 0.27, N=24

Note:

Mean ± SEMs

Table E-11. Body Weight (g) of Dams Exposed to PFHxS during Gestation

Gestation day (GD)	0 mg/kg-day	1.6 mg/kg-day	3.5 mg/kg-day	7.0 mg/kg-day	14.0 mg/kg-day
GD0	16.92 ± 0.34, N=22	17.20 ± 0.28, N=23	16.94 ± 0.29, N=24	17.07 ± 0.40, N=19	17.01 ± 0.33, N=15
GD1	16.88 ± 0.33, N=22	17.21 ± 0.29, N=23	16.96 ± 0.28, N=24	17.04 ± 0.39, N=19	16.88 ± 0.31, N=15
GD2	16.99 ± 0.34, N=22	17.41 ± 0.31, N=23	16.90 ± 0.31, N=24	17.06 ± 0.40, N=19	16.89 ± 0.36, N=15
GD3	17.11 ± 0.33, N=22	17.36 ± 0.30, N=23	16.90 ± 0.33, N=24	17.12 ± 0.38, N=19	17.07 ± 0.35, N=15
GD4	17.14 ± 0.33, N=22	17.43 ± 0.29, N=23	17.28 ± 0.29, N=24	17.27 ± 0.37, N=19	17.29 ± 0.44, N=15
GD5	17.22 ± 0.35, N=22	17.58 ± 0.29, N=23	17.32 ± 0.30, N=24	17.35 ± 0.38, N=19	17.32 ± 0.38, N=15
GD6	17.44 ± 0.35, N=22	17.78 ± 0.28, N=23	17.31 ± 0.29, N=24	17.46 ± 0.37, N=19	17.47 ± 0.39, N=15
GD7	17.61 ± 0.32, N=22	17.84 ± 0.30, N=23	17.56 ± 0.29, N=24	17.49 ± 0.34, N=19	17.69 ± 0.33, N=15
GD8	17.60 ± 0.35, N=22	17.97 ± 0.31, N=23	17.67 ± 0.29, N=24	17.55 ± 0.34, N=19	17.81 ± 0.39, N=15
GD9	17.88 ± 0.32, N=22	18.04 ± 0.32, N=23	17.78 ± 0.28, N=24	17.74 ± 0.31, N=19	18.03 ± 0.39, N=15
GD10	17.96 ± 0.36, N=22	18.35 ± 0.30, N=23	17.78 ± 0.28, N=24	17.86 ± 0.34, N=19	18.04 ± 0.40, N=15
GD11	18.19 ± 0.34, N=22	18.58 ± 0.28, N=23	18.05 ± 0.32, N=24	18.21 ± 0.34, N=19	18.33 ± 0.41, N=15
GD12	18.40 ± 0.36, N=22	18.84 ± 0.29, N=23	18.37 ± 0.30, N=24	18.44 ± 0.33, N=19	18.53 ± 0.42, N=15
GD13	18.79 ± 0.38, N=22	19.29 ± 0.28, N=23	18.78 ± 0.31, N=24	18.75 ± 0.34, N=19	18.91 ± 0.41, N=15
GD14	19.10 ± 0.38, N=22	19.66 ± 0.31, N=23	19.18 ± 0.34, N=24	19.19 ± 0.32, N=19	19.43 ± 0.41, N=15
GD15	19.61 ± 0.40, N=22	20.28 ± 0.32, N=23	19.64 ± 0.34, N=24	19.66 ± 0.35, N=19	19.77 ± 0.40, N=15
GD16	20.05 ± 0.43, N=22	20.73 ± 0.32, N=23	19.96 ± 0.38, N=24	20.13 ± 0.38, N=19	20.21 ± 0.44, N=15
GD17	20.42 ± 0.45, N=22	21.52 ± 0.34, N=23	20.41 ± 0.38, N=24	20.51 ± 0.37, N=19	20.76 ± 0.45, N=15
GD18	20.84 ± 0.48, N=22	21.86 ± 0.37, N=23	21.08 ± 0.42, N=24	21.12 ± 0.40, N=19	21.29 ± 0.43, N=15
GD19	21.52 ± 0.54, N=22	22.59 ± 0.37, N=23	21.70 ± 0.44, N=24	21.82 ± 0.45, N=19	21.83 ± 0.43, N=15
GD20	22.22 ± 0.58, N=22	23.30 ± 0.42, N=23	22.19 ± 0.46, N=24	22.62 ± 0.48, N=19	22.48 ± 0.43, N=15
GD21	22.72 ± 0.58, N=22	24.01 ± 0.45, N=23	22.88 ± 0.49, N=24	23.21 ± 0.47, N=19	23.34 ± 0.43, N=15
GD22	23.45 ± 0.66, N=22	24.93 ± 0.51, N=23	23.55 ± 0.56, N=24	23.90 ± 0.51, N=19	24.03 ± 0.45, N=15
GD23	24.01 ± 0.71, N=22	25.70 ± 0.52, N=23	24.16 ± 0.58, N=24	24.55 ± 0.50, N=19	24.46 ± 0.56, N=15

Note:

Mean ± SEMs

Table E-12. Average Pup Weight (g) and Day of Pinna Unfolding in Pups of Dams Exposed to PFHxS

Postnatal day (PND)	0 mg/kg-day	1.6 mg/kg-day	3.5 mg/kg-day	7.0 mg/kg-day	14.0 mg/kg-day
PND0	1.69 ± 0.04, N=20	1.66 ± 0.04, N=21	1.72 ± 0.04, N=21	1.70 ± 0.05, N=16	1.61 ± 0.05, N=9
PND1	1.89 ± 0.05, N=18	1.87 ± 0.05, N=21	1.91 ± 0.05, N=21	1.89 ± 0.06, N=16	1.68 ± 0.10, N=8
PND4	2.66 ± 0.11, N=14	2.72 ± 0.15, N=20	2.62 ± 0.09, N=20	2.76 ± 0.09, N=14	2.64 ± 0.18, N=5
PND7	3.78 ± 0.15, N=13	3.88 ± 0.13, N=20	3.58 ± 0.12, N=20	3.79 ± 0.12, N=14	3.44 ± 0.29, N=5
PND10	4.64 ± 0.17, N=13	4.84 ± 0.15, N=18	4.43 ± 0.14, N=19	4.68 ± 0.12, N=14	4.36 ± 0.28, N=5
Milestone	0 mg/kg-day	1.6 mg/kg-day	3.5 mg/kg-day	7.0 mg/kg-day	14.0 mg/kg-day
Pinna unfolding	2.31 ± 0.38, N=16	2.48 ± 0.31, N=21	1.90 ± 0.41, N=21	1.67 ± 0.32, N=15	1.29 ± 0.61, N=7

Note:

Mean ± SEMs

Table E-13. Body Weight (g) of P Generation Males over 16 Weeks of Exposure to 6:2 FTS

Week of exposure	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day	25.0 mg/kg-day
Week 0	21.52 ± 0.43, N=25	21.00 ± 0.58, N=24	20.65 ± 0.46, N=23	21.31 ± 0.58, N=22	22.02 ± 0.60, N=20
Week 1	21.33 ± 0.43, N=25	20.91 ± 0.57, N=24	20.84 ± 0.45, N=23	21.23 ± 0.62, N=22	21.92 ± 0.64, N=20
Week 2	21.33 ± 0.40, N=25	20.57 ± 0.59, N=24	20.93 ± 0.46, N=23	21.16 ± 0.60, N=22	22.49 ± 0.60, N=20
Week 3	21.40 ± 0.44, N=25	20.93 ± 0.52, N=24	20.97 ± 0.47, N=23	21.22 ± 0.64, N=22	22.53 ± 0.57, N=20
Week 4	21.62 ± 0.41, N=25	20.88 ± 0.55, N=24	21.10 ± 0.47, N=23	21.47 ± 0.63, N=22	22.61 ± 0.57, N=20
Week 5	20.79 ± 0.35, N=25	20.26 ± 0.56, N=24	20.79 ± 0.45, N=23	20.60 ± 0.62, N=22	21.94 ± 0.53, N=20
Week 6	20.88 ± 0.33, N=25	20.27 ± 0.51, N=24	20.51 ± 0.38, N=23	20.63 ± 0.62, N=22	21.79 ± 0.53, N=20
Week 7	20.60 ± 0.36, N=25	20.24 ± 0.50, N=24	20.43 ± 0.41, N=23	20.59 ± 0.54, N=21	21.43 ± 0.58, N=20
Week 8	20.88 ± 0.36, N=25	20.23 ± 0.52, N=24	20.37 ± 0.44, N=22	20.45 ± 0.57, N=20	21.50 ± 0.54, N=20
Week 9	20.94 ± 0.39, N=25	20.58 ± 0.53, N=24	20.77 ± 0.43, N=22	20.91 ± 0.57, N=20	21.84 ± 0.54, N=20
Week 10	21.27 ± 0.41, N=24	20.87 ± 0.56, N=24	21.01 ± 0.45, N=22	21.02 ± 0.53, N=20	21.92 ± 0.56, N=20
Week 11	21.45 ± 0.42, N=24	20.83 ± 0.57, N=24	21.09 ± 0.45, N=22	20.97 ± 0.57, N=20	21.93 ± 0.51, N=20
Week 12	21.62 ± 0.42, N=24	20.54 ± 0.60, N=24	21.20 ± 0.41, N=21	21.08 ± 0.61, N=20	21.95 ± 0.50, N=20
Week 13	21.56 ± 0.44, N=24	20.80 ± 0.59, N=23	21.13 ± 0.45, N=21	20.87 ± 0.67, N=20	21.96 ± 0.56, N=19
Week 14	21.53 ± 0.45, N=24	20.71 ± 0.58, N=23	21.34 ± 0.42, N=21	21.19 ± 0.63, N=20	21.84 ± 0.54, N=20
Week 15	21.71 ± 0.47, N=23	20.84 ± 0.60, N=23	21.22 ± 0.52, N=21	21.34 ± 0.64, N=20	22.07 ± 0.58, N=19
Week 16	21.89 ± 0.46, N=23	21.03 ± 0.62, N=23	21.63 ± 0.54, N=21	21.38 ± 0.69, N=20	22.28 ± 0.66, N=20

Note:

Mean ± SEMs

Table E-14. Body Weight (g) of P Generation Females Exposed to 6:2 FTS Prior to Establishing Mating Pairs

Week of exposure	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day	25.0 mg/kg-day
Week 0	19.14 ± 0.60, N=25	18.82 ± 0.46, N=24	18.67 ± 0.45, N=23	19.43 ± 0.43, N=24	19.36 ± 0.44, N=25
Week 1	19.01 ± 0.54, N=25	18.62 ± 0.45, N=24	18.41 ± 0.47, N=23	18.93 ± 0.41, N=24	19.09 ± 0.43, N=25
Week 2	18.92 ± 0.53, N=25	18.64 ± 0.45, N=24	18.62 ± 0.45, N=23	19.12 ± 0.40, N=24	19.03 ± 0.42, N=25
Week 3	18.82 ± 0.48, N=25	18.48 ± 0.45, N=24	18.52 ± 0.44, N=23	19.00 ± 0.37, N=24	18.62 ± 0.38, N=24
Week 4	19.00 ± 0.53, N=25	18.60 ± 0.44, N=24	18.63 ± 0.47, N=23	19.08 ± 0.36, N=24	18.73 ± 0.39, N=24

Note:

Mean ± SEMs

Table E-15. Body Weight (g) of Dams Exposed to 6:2 FTS during Gestation

Gestation day (GD)	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day	25.0 mg/kg-day
GD0	18.87 ± 0.57, N=18	19.34 ± 0.58, N=12	19.06 ± 0.54, N=13	19.22 ± 0.42, N=14	18.58 ± 0.63, N=13
GD1	18.68 ± 0.59, N=18	19.33 ± 0.50, N=12	19.11 ± 0.60, N=13	18.89 ± 0.42, N=14	18.23 ± 0.60, N=13
GD2	18.75 ± 0.57, N=18	19.33 ± 0.53, N=12	19.11 ± 0.60, N=13	18.95 ± 0.40, N=14	18.38 ± 0.65, N=13
GD3	18.86 ± 0.59, N=18	19.39 ± 0.52, N=12	18.99 ± 0.59, N=13	18.94 ± 0.37, N=14	18.42 ± 0.55, N=13
GD4	18.87 ± 0.59, N=18	19.38 ± 0.44, N=12	19.06 ± 0.58, N=13	19.13 ± 0.38, N=14	18.36 ± 0.54, N=13
GD5	19.11 ± 0.55, N=18	19.47 ± 0.40, N=12	19.03 ± 0.60, N=13	19.17 ± 0.46, N=14	18.52 ± 0.57, N=13
GD6	18.94 ± 0.56, N=18	19.64 ± 0.48, N=12	19.05 ± 0.61, N=13	19.26 ± 0.39, N=14	18.62 ± 0.56, N=13
GD7	19.05 ± 0.57, N=18	19.63 ± 0.45, N=12	19.24 ± 0.67, N=13	19.34 ± 0.40, N=14	18.68 ± 0.60, N=13
GD8	19.12 ± 0.53, N=18	19.78 ± 0.44, N=12	19.37 ± 0.60, N=13	19.49 ± 0.46, N=14	18.66 ± 0.50, N=13
GD9	19.13 ± 0.56, N=18	20.02 ± 0.52, N=12	19.46 ± 0.56, N=13	19.56 ± 0.43, N=14	18.92 ± 0.58, N=13
GD10	19.52 ± 0.51, N=18	20.19 ± 0.52, N=12	19.57 ± 0.52, N=13	19.24 ± 0.69, N=14	19.04 ± 0.54, N=13
GD11	19.69 ± 0.50, N=18	20.34 ± 0.50, N=12	19.88 ± 0.54, N=13	19.93 ± 0.48, N=14	19.18 ± 0.55, N=13
GD12	20.26 ± 0.52, N=18	20.77 ± 0.47, N=12	20.09 ± 0.57, N=13	20.45 ± 0.46, N=14	19.25 ± 0.55, N=13
GD13	20.78 ± 0.49, N=18	21.38 ± 0.55, N=12	20.31 ± 0.52, N=13	20.84 ± 0.49, N=14	19.93 ± 0.59, N=13
GD14	21.26 ± 0.51, N=18	21.51 ± 0.53, N=12	21.14 ± 0.52, N=13	21.75 ± 0.54, N=14	20.62 ± 0.64, N=13
GD15	21.74 ± 0.56, N=18	22.35 ± 0.49, N=12	21.73 ± 0.54, N=13	22.11 ± 0.51, N=14	20.92 ± 0.69, N=13
GD16	22.15 ± 0.62, N=18	22.44 ± 0.50, N=12	21.98 ± 0.49, N=13	22.74 ± 0.55, N=14	21.44 ± 0.71, N=13
GD17	22.64 ± 0.60, N=18	22.88 ± 0.53, N=12	22.42 ± 0.46, N=13	23.04 ± 0.51, N=14	21.84 ± 0.72, N=13
GD18	23.01 ± 0.65, N=18	23.25 ± 0.50, N=12	22.97 ± 0.56, N=13	23.32 ± 0.50, N=14	22.20 ± 0.69, N=13
GD19	23.92 ± 0.71, N=18	23.76 ± 0.58, N=12	23.82 ± 0.56, N=13	24.36 ± 0.59, N=14	23.22 ± .072, N=13
GD20	24.57 ± 0.72, N=18	24.74 ± 0.68, N=12	24.67 ± 0.52, N=13	25.29 ± 0.62, N=14	23.86 ± 0.78, N=13
GD21	24.98 ± 0.87, N=18	25.71 ± 0.62, N=12	25.78 ± 0.55, N=13	26.36 ± 0.69, N=14	24.59 ± 0.79, N=13
GD22	25.97 ± 0.90, N=18	26.03 ± 0.70, N=12	26.91 ± 0.63, N- 13	27.24 ± 0.77, N=14	25.32 ± 0.97, N=13
GD23	26.25 ± 0.97, N=18	26.12 ± 0.65, N=12	26.58 ± 0.74, N=13	27.35 ± 0.84, N=14	25.89 ± 1.04, N=13

Note:

Mean ± SEMs

Table E-16. Average Pup Weight (g) and Day of Pinna Unfolding in Pups of Dams Exposed to 6:2 FTS

Postnatal day (PND)	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day	25.0 mg/kg-day
PND0	1.64 ± 0.06, N=15	1.75 ± 0.05, N=9	1.66 ± 0.04, N=13	1.68 ± 0.05, N=15	1.73 ± 0.05, N=11
PND1	1.84 ± 0.06, N=14	1.96 ± 0.07, N=8	1.77 ± 0.04, N=13	1.86 ± 0.07, N=13	1.80 ± 0.09, N=11
PND4	2.69 ± 0.10, N=14	2.84 ± 0.12, N=8	2.50 ± 0.11, N=13	2.70 ± 0.15, N=13	2.43 ± 0.12, N=9
PND7	3.85 ± 0.17, N=14	3.83 ± 0.16, N=8	3.59 ± 0.16, N=13	3.76 ± 0.19, N=12	3.42 ± 0.19, N=8
PND10	4.84 ± 0.18, N=14	4.91 ± 0.17, N=8	4.62 ± 0.21, N=13	4.75 ± 0.20, N=12	4.45 ± 0.20, N=8
Milestone	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day	25.0 mg/kg-day
Pinna unfolding	2.79 ± 0.26, N=14	2.88 ± 0.30, N=8	3.18 ± 0.23, N=11	3.08 ± 0.33, N=13	3.20 ± 0.29, N=10

Note:

Mean ± SEMs

Appendix F
Reproduction and Fertility

Table F-1. Reproductive Outcomes in *Peromysus* Exposed to PFOS

Endpoints		0 mg/kg-day	0.2 mg/kg-day	1 mg/kg-day	5 mg/kg-day
Mating pairs (#)		30	31	30	30
Pregnant animals (#)		13	12	17	6
Delivered live litter (#)		13	12	12	1*
Total litter loss (#)		0	1	7*	6**
Stillbirths (%) ¹		9.6 ± 6.7	10.4 ± 5.6	32.4 ± 11.3	83.3.7 ± 16.7***
Live litter size (#) ¹		2.4 ± 0.3	2.7 ± 0.4	1.8 ± 0.4	0.5 ± 0.5*
Total litter size (#) ¹		2.7 ± 0.3	2.9 ± 0.4	2.4 ± 0.3	1.7 ± 0.4

Notes:

¹Mean ± SEMs

Asterisks denote significant differences from controls by Pearson χ^2 (pregnancy, fertility, total litter loss: $p < 0.025^{**}$, $p < 0.05^*$ with Bonferroni-Holm corrections) or ANOVA (stillbirths, live litter size, total litter size: $p < 0.05^*$, $p < 0.001^{***}$).

All other results were not significant ($p > 0.05$).

Table F-2. Reproductive Outcomes in *Peromysus* Exposed to PFNA

Endpoints	0 mg/kg-day	0.03 mg/kg-day	0.14 mg/kg-day	1.0 mg/kg-day	3.0 mg/kg-day
Mating pairs (#)	23	24	25	25	23
Pregnant animals (#)	21	20	20	20	17
Delivered live litter (#)	20	19	16	19	12
Total litter loss (#)	2	2	5	2	5
Stillbirths (%) ¹	6.3 ± 7.2	13.9 ± 7.4	26.7 ± 7.4	18.3 ± 7.4	30.7 ± 8.0
Live litter size (#) ¹	2.9 ± 0.3	2.1 ± 0.3	2.1 ± 0.3	2.6 ± 0.3	2.5 ± 0.3
Total litter size (#) ¹	3.0 ± 0.3	2.5 ± 0.3	2.8 ± 0.3	3.0 ± 0.3	3.3 ± 0.3

Notes:

¹Mean ± SEMs

No results were significant ($p > 0.05$).

Table F-3. Reproductive Outcomes in *Peromysus* Exposed to PFHxS

Endpoints	0 mg/kg-day	1.6 mg/kg-day	3.5 mg/kg-day	7.0 mg/kg-day	14.0 mg/kg-day
Mating pairs (#)	24	25	25	23	23
Pregnant animals (#)	23	24	24	20	19
Delivered live litter (#)	22	23	24	19	15*
Total litter loss (#)	4	2	3	3	5
Stillbirths (%) ¹	12.3 ± 6.5 ^a	15.6 ± 6.6 ^a	13.9 ± 6.9 ^a	19.3 ± 8.6 ^{ab}	51.7 ± 11.6 ^b
Live litter size (#) ¹	2.1 ± 0.2	2.3 ± 0.3	2.0 ± 0.3	1.9 ± 0.3	1.3 ± 0.3
Total litter size (#) ¹	2.4 ± 0.2	2.7 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.0 ± 0.2

Notes:

¹Mean ± SEMs

Asterisks denote significant differences from controls by Pearson χ^2 (pregnancy, fertility, total litter loss: $p < 0.05^*$).

Letters denote significant differences by ANOVA (stillbirths, live litter size, total litter size: $p < 0.05$).

All other results were not significant ($p > 0.05$).

Table F-4. Reproductive Outcomes in *Peromysus* Exposed to 6:2 FTS

Endpoints	0 mg/kg-day	0.2 mg/kg-day	1.0 mg/kg-day	5.0 mg/kg-day	25.0 mg/kg-day
Mating pairs (#)	25	23	23	23	23
Pregnant animals (#)	18	13	15	15	14
Delivered live litter (#)	16	9	13	15	11
Total litter loss (#)	4	4	1	2	2
Stillbirths (%) ¹	16.7 ± 8.1	29.2 ± 13.0	11.9 ± 7.5	17.4 ± 6.3	21.8 ± 10.6
Live litter size (#) ¹	2.2 ± 0.4	1.7 ± 0.4	2.6 ± 0.3	2.5 ± 0.3	2.2 ± 0.4
Total litter size (#) ¹	2.4 ± 0.3	2.1 ± 0.2	2.8 ± 0.3	3.0 ± 0.3	2.5 ± 0.4

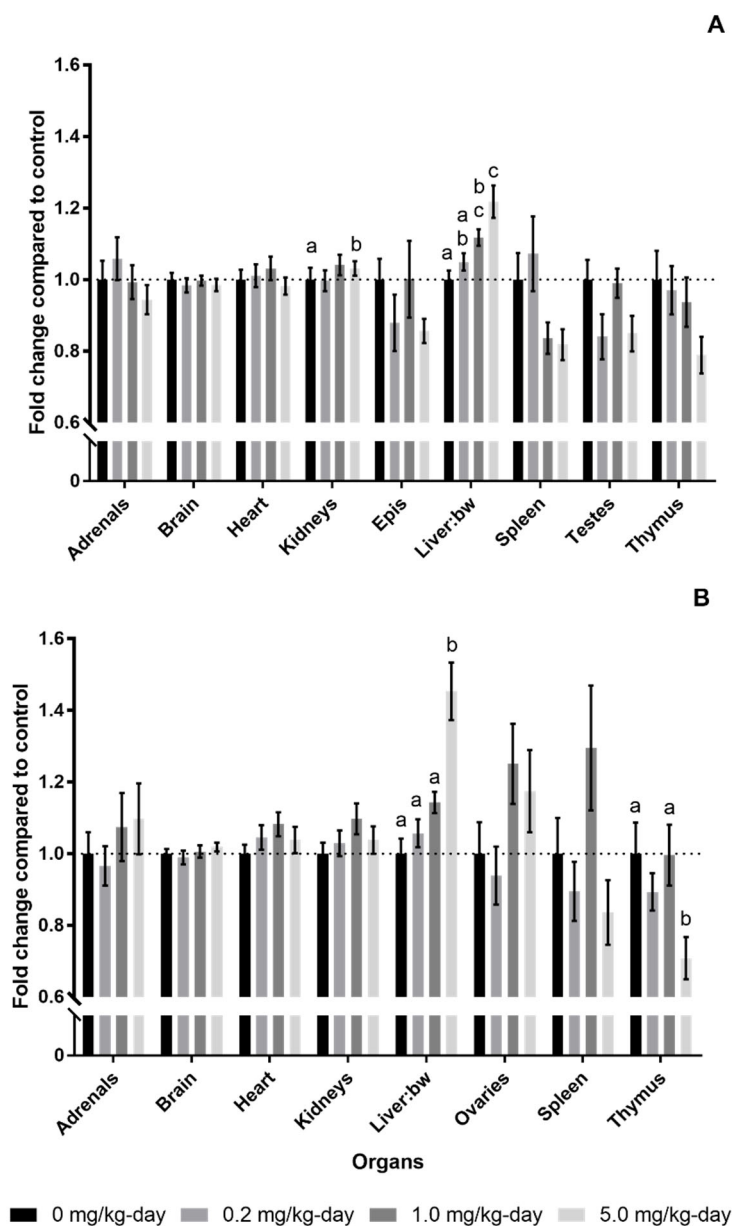
Notes:

¹Mean ± SEMs

No results were significant ($p > 0.05$).

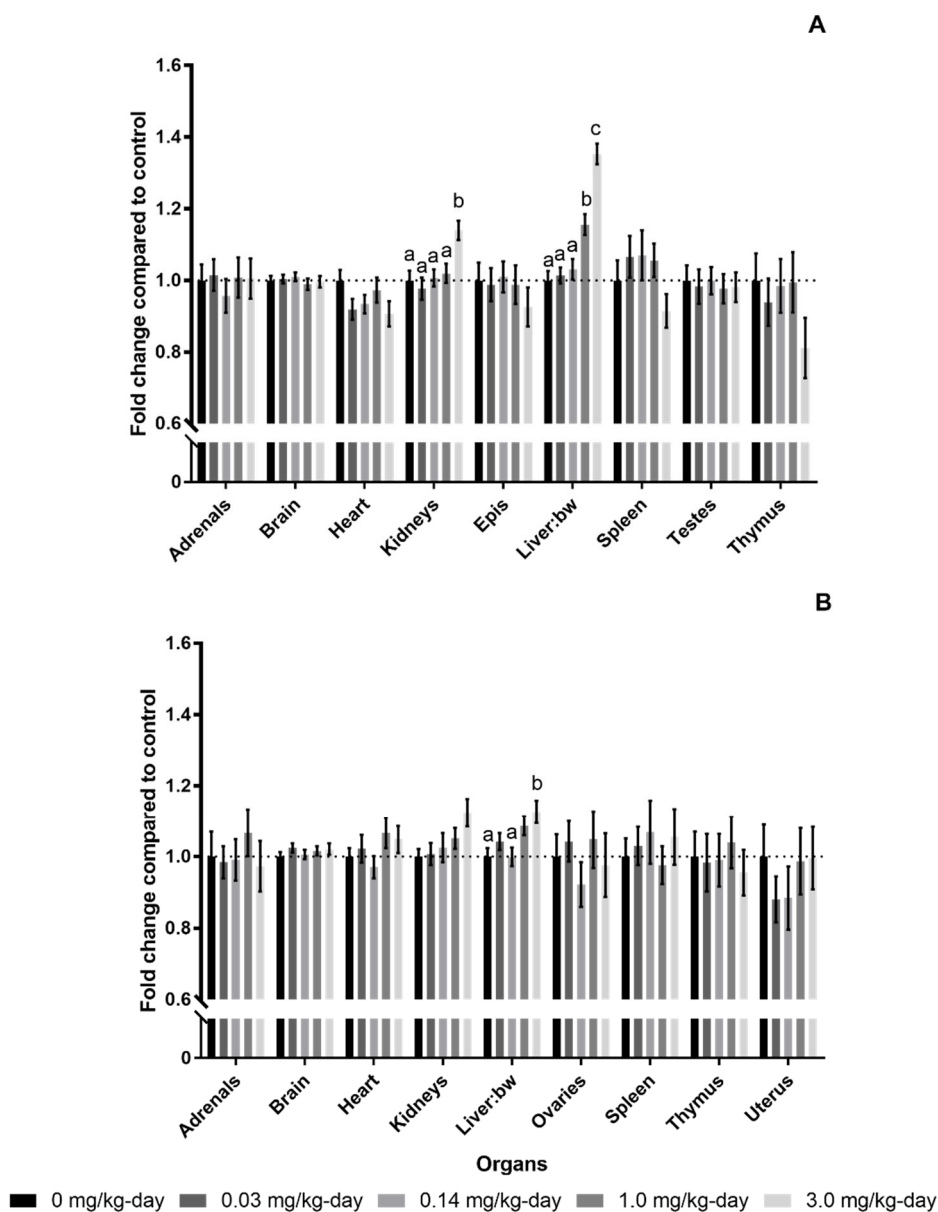
Appendix G
Organ Weights

Figure G-1. Organ Weights of P Generation White-Footed Mice Exposed to PFOS



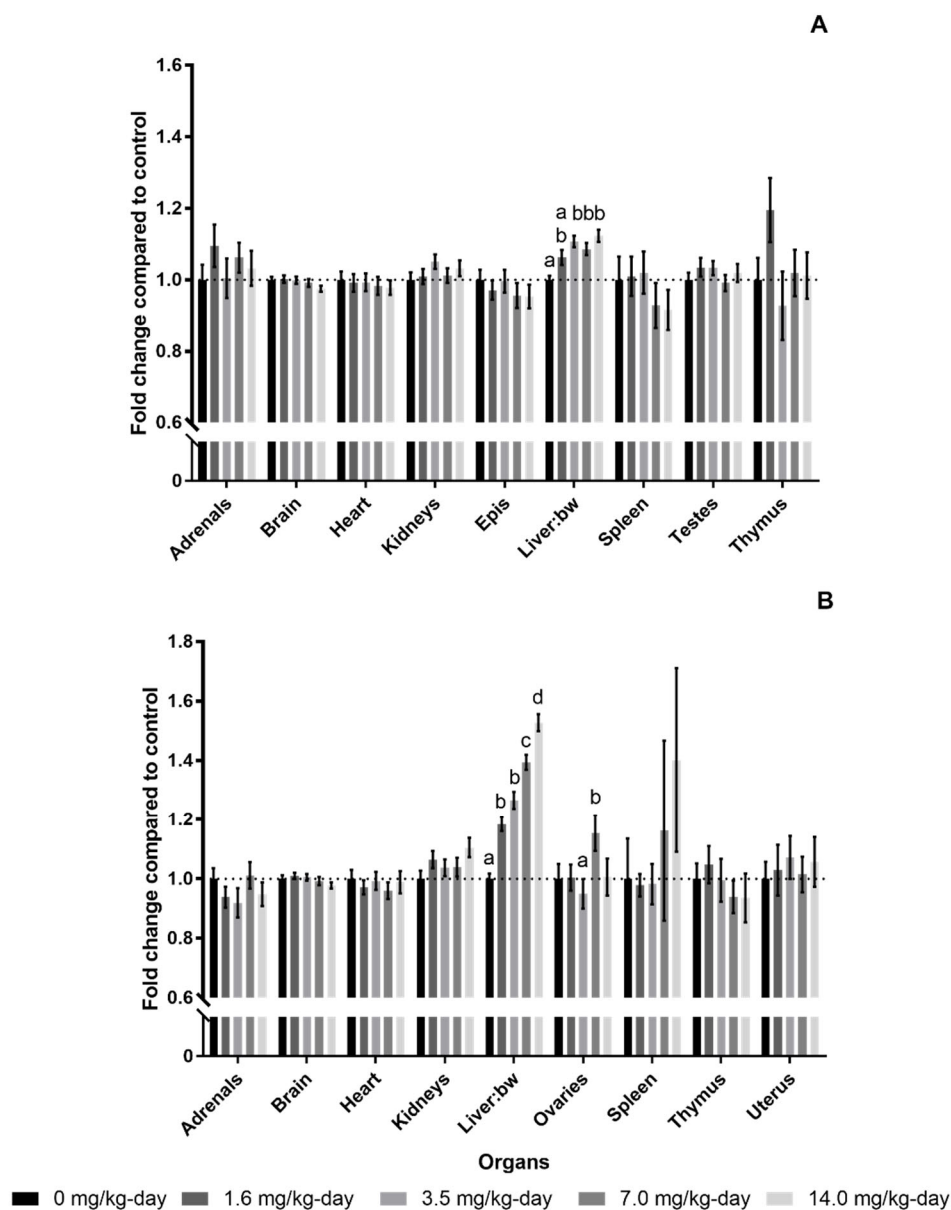
Graph and error bars represent mean and SEMs, respectively, of results obtained from P generation male (A, N=24-29/sex/dose) and female (B, N =16-31/sex/dose) white-footed mice exposed to PFOS. Data are presented as fold change compared to control. Males exposed to PFOS had increased liver and kidney weights. Females exposed to PFOS had increased liver and decreased thymus weights. All other results were not significantly different ($p > 0.05$).

Figure G-2. Organ Weights of P Generation White-Footed Mice Exposed to PFNA



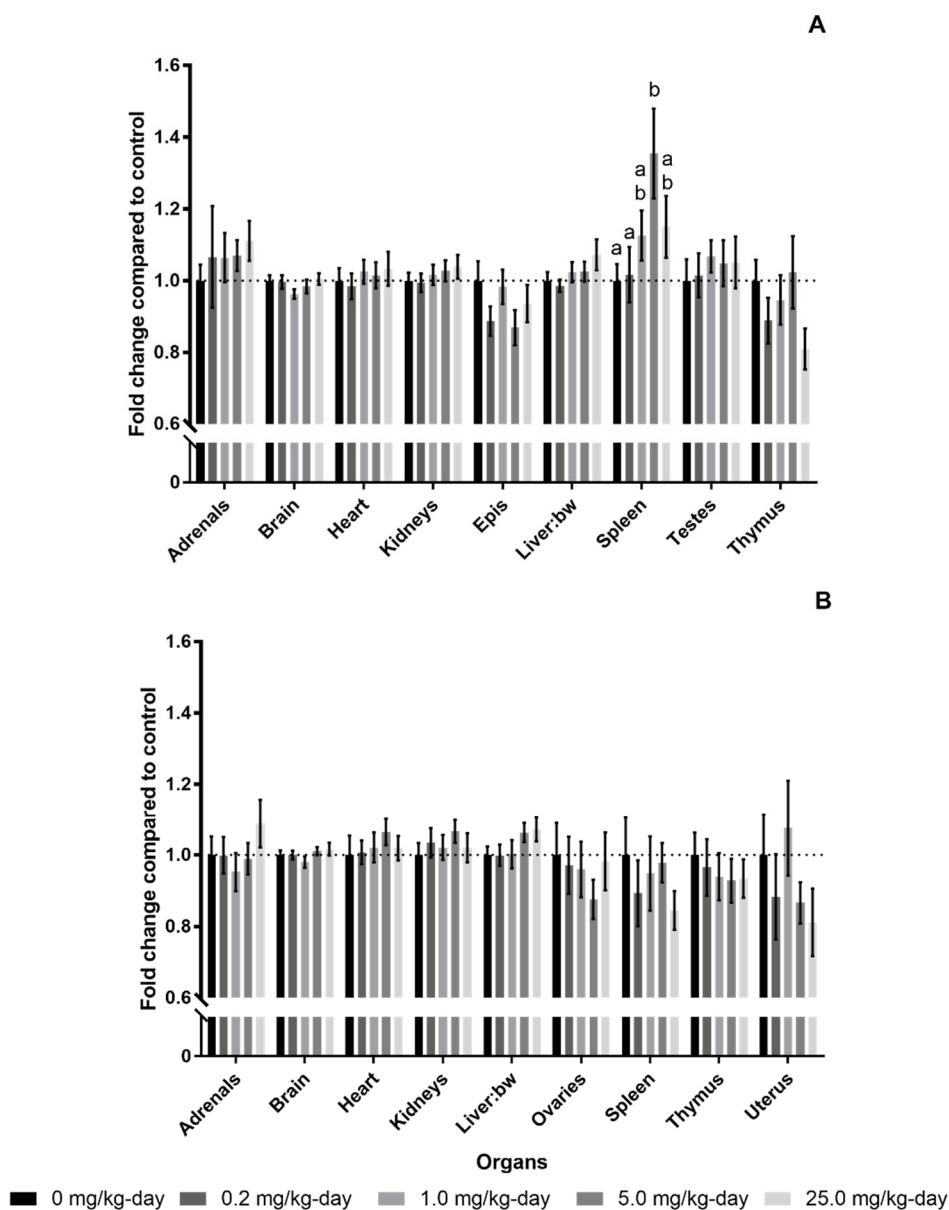
Graph and error bars represent mean and SEMs, respectively, of results from P generation male (A, N=22-25/sex/dose) and female (B, N=21-24/sex/dose) white-footed mice exposed to PFNA. Data are presented as fold change compared to control. Males exposed to PFNA had increased kidney and liver weights. Females exposed to PFNA had increased liver weights. All other results were not significantly different ($p > 0.05$).

Figure G-3. Organ Weights of P Generation White-Footed Mice Exposed to PFHxS



Graph and error bars represent mean and SEMs, respectively, of results from P generation male (A, N=21-24/sex/dose) and female (B, N=16-24/sex/dose) deer mice exposed to PFHxS. Data are presented as fold change compared to control. Animals exposed to PFHxS had increased liver and ovarian weights. All other results were not significantly different ($p > 0.05$).

Figure G-4. Organ Weights of P Generation White-Footed Mice Exposed to 6:2 FTS

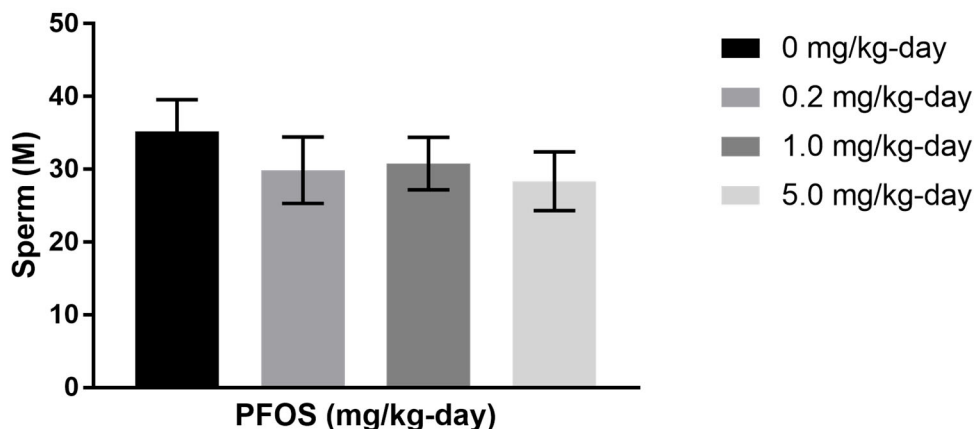


Graph and error bars represent mean and SEMs, respectively, of results from P generation male (A, N=19-23/sex/dose) and female (B, N=17-22/sex/dose) white-footed mice exposed to 6:2 FTS. Data are presented as fold change compared to control. Males exposed to 6:2 FTS had increased spleen weights. All other results were not significantly different ($p > 0.05$).

APPENDIX H

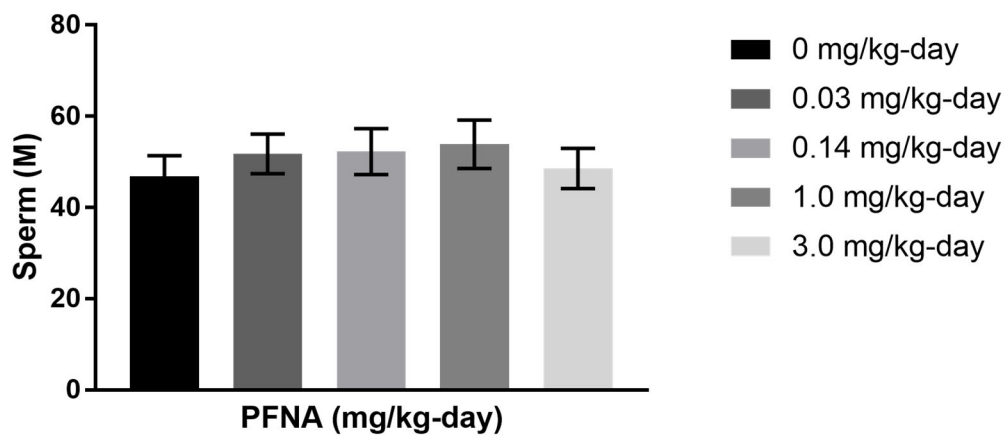
Sperm Analysis

Figure H-1. Sperm Count in P Generation White-Footed Mice Exposed to PFOS



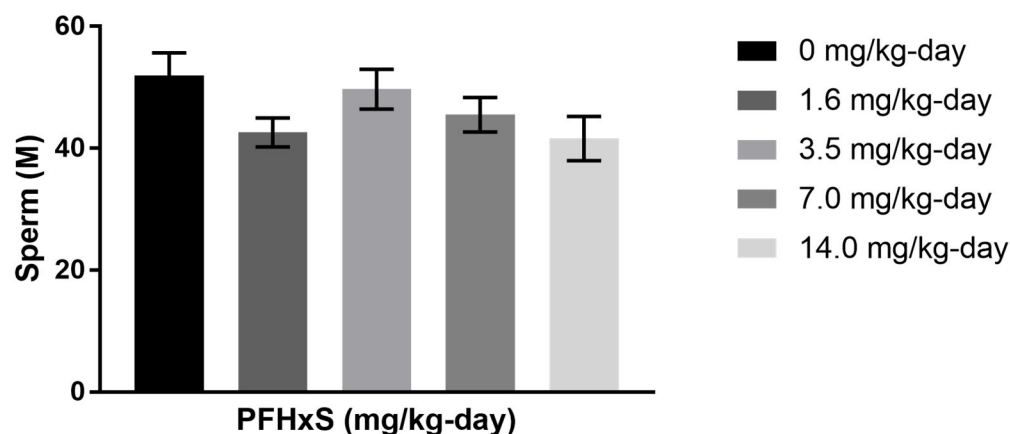
Graph and error bars represent mean and SEM, respectively, of results obtained from P generation males (N=23-28/dose) exposed to PFOS. Data are presented per million cells. All results were not significant ($p > 0.05$).

Figure H-2. Sperm Count in P Generation White-Footed Mice Exposed to PFNA



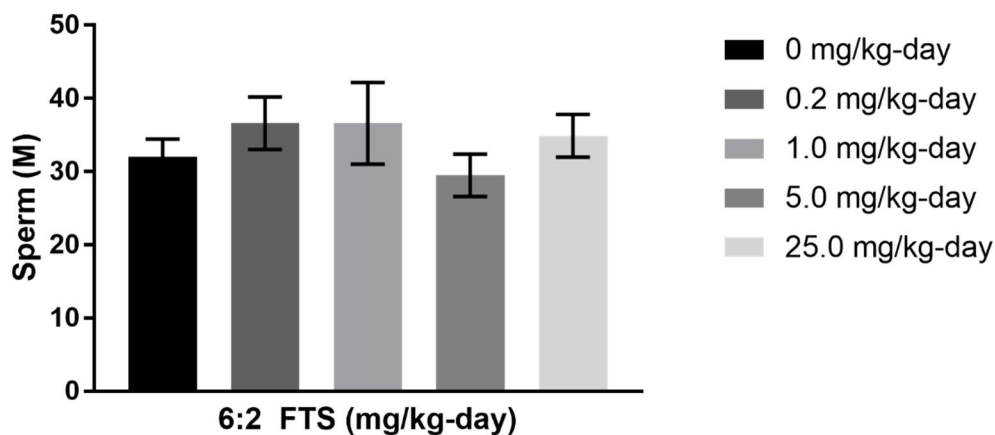
Graph and error bars represent mean and SEM, respectively, of results obtained from P generation males (N=21-24/dose) exposed to PFNA. Data are presented per million cells. All results were not significant ($p > 0.05$).

Figure H-3. Sperm Count in P Generation Deer Mice Exposed to PFHxS



Graph and error bars represent mean and SEM, respectively, of results obtained from P generation males (N=21-24/dose) exposed to PFHxS. Data are presented per million cells. All results were not significant ($p > 0.05$).

Figure H-4. Sperm Count in P Generation White-Footed Mice Exposed to 6:2 FTS

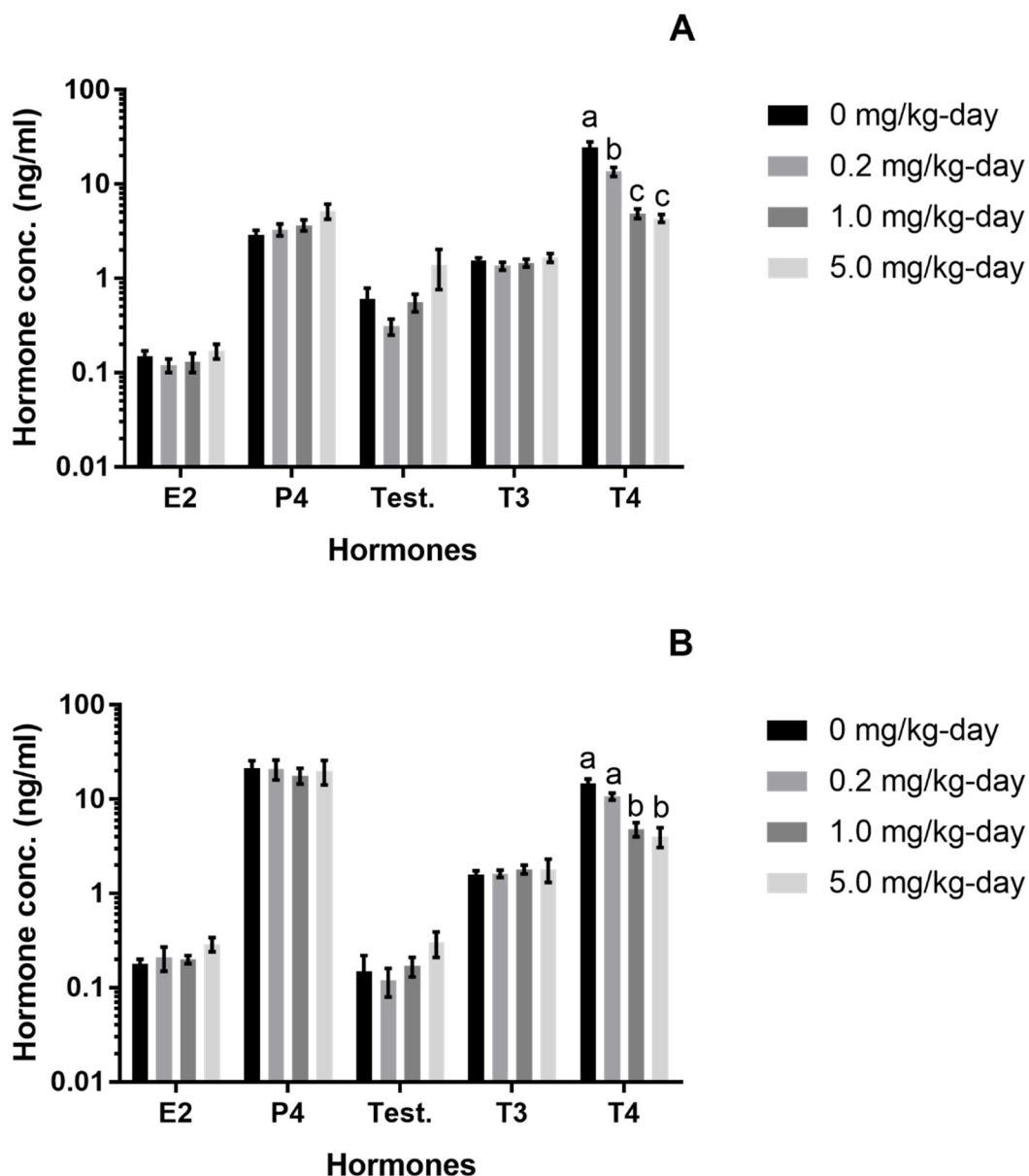


Graph and error bars represent mean and SEM, respectively, of results obtained from P generation males (N=20-23/dose) exposed to 6:2 FTS. Data are presented per million cells. All results were not significant ($p > 0.05$).

APPENDIX I

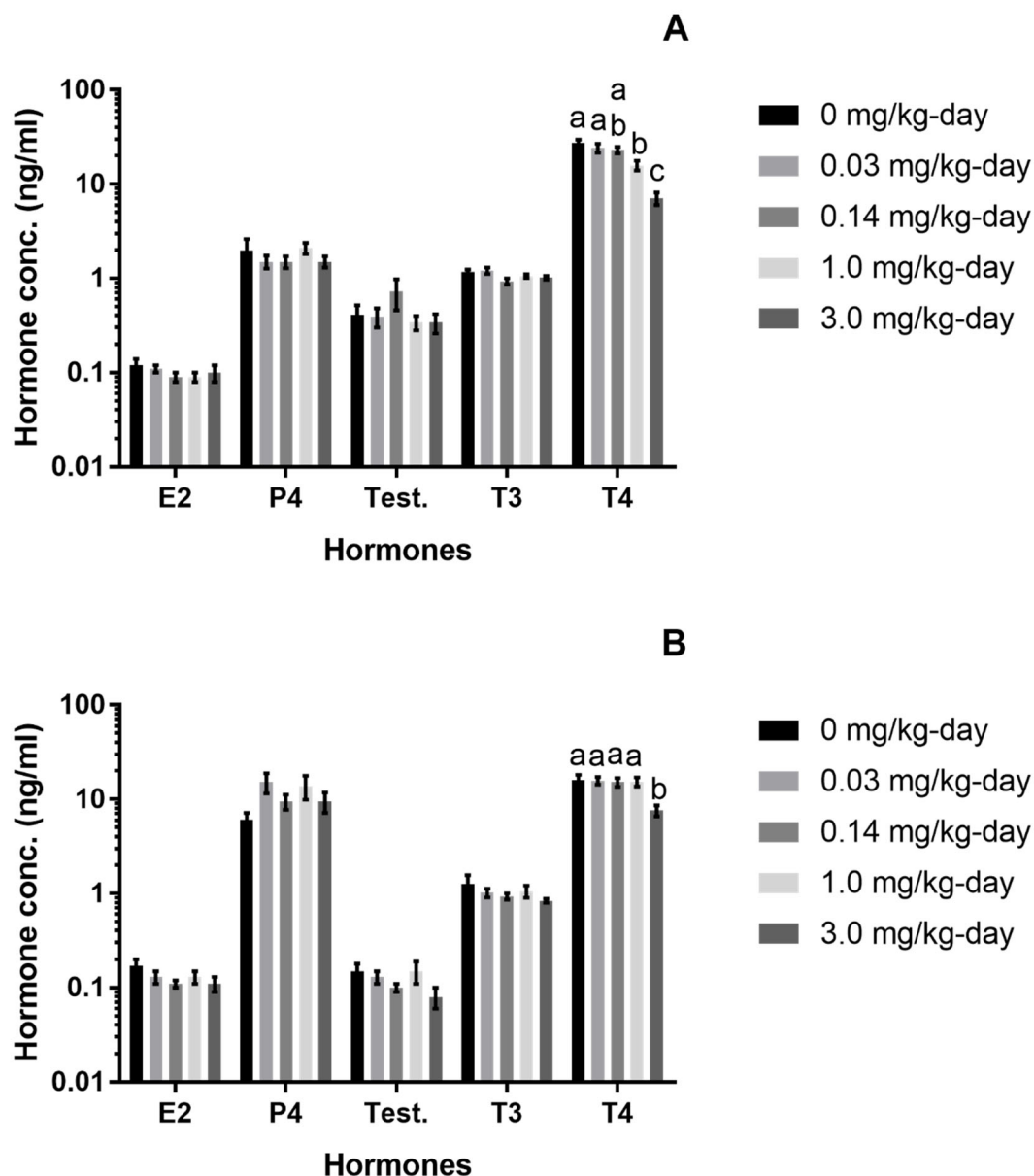
Hormone Analysis

Figure I-1. Hormones in P Generation White-Footed Mice Exposed to PFOS



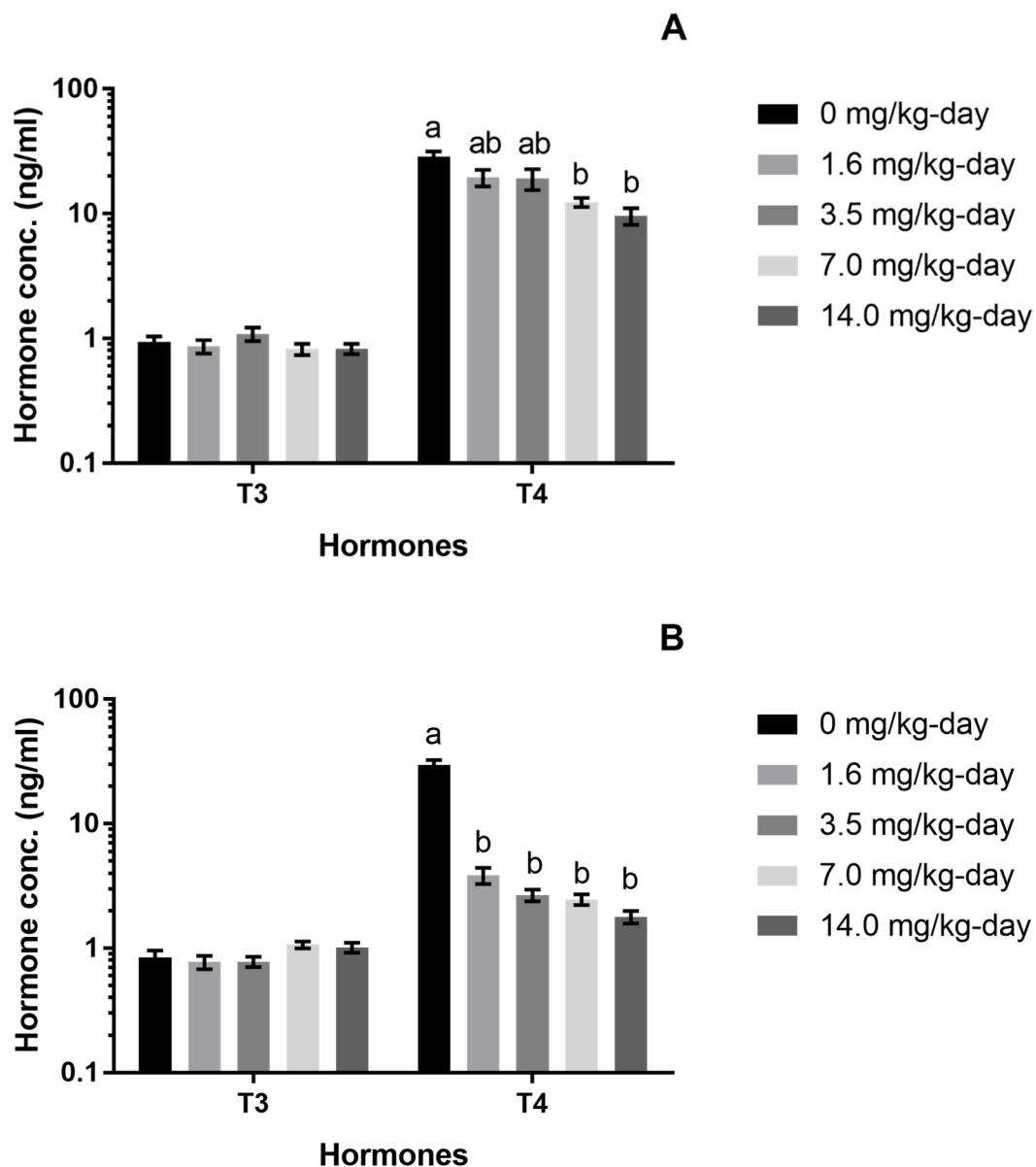
Graph and error bars represent mean and SEMs, respectively, of results from P generation male (A, N=9-18/dose) and female (B, N=14-20/dose) white-footed mice exposed to PFOS. Male and females exposed to PFOS had decreased T4 levels. For all other hormones, results obtained from exposed and nonexposed animals were not significantly different ($p > 0.05$).

Figure I-2. Hormones in P Generation White-Footed Mice Exposed to PFNA



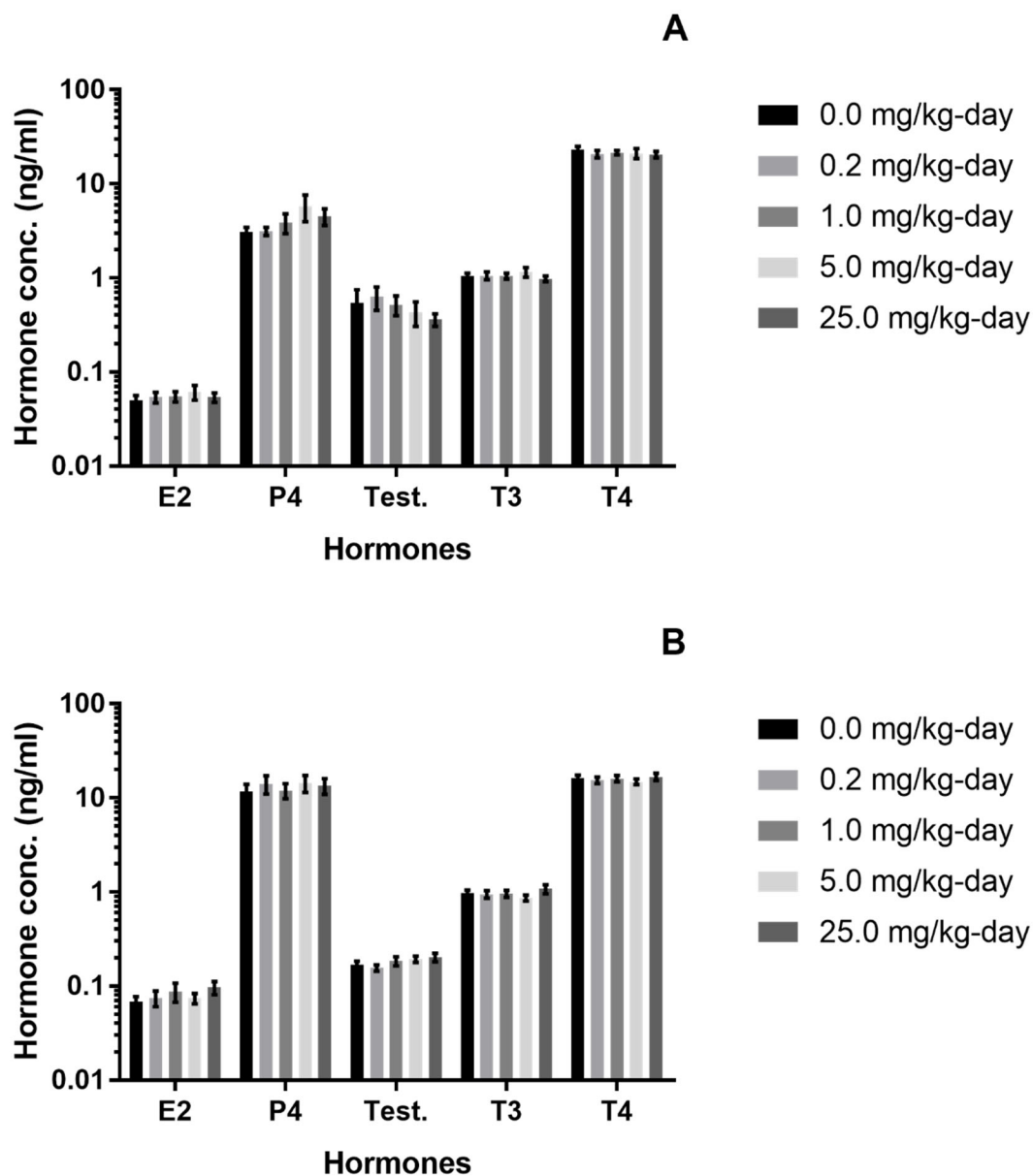
Graph and error bars represent mean and SEMs, respectively, of results from P generation male (A, N=18-24/dose) and female (B, N=15-23/dose) white-footed mice exposed to PFNA. Male and females exposed to PFNA had decreased T4 levels. For all other hormones, results obtained from exposed and nonexposed animals were not significantly different ($p > 0.05$).

Figure I-3. Hormones in P Generation Deer Mice Exposed to PFHxS



Graph and error bars represent mean and SEMs, respectively, of results from P generation male (A, N=13-18/dose) and female (B, N=12-20/dose) deer mice exposed to PFHxS. Male and females exposed to PFHxS had decreased T4 levels. T3 levels from exposed and nonexposed animals were not significantly different ($p > 0.05$).

Figure I-4. Hormones in P Generation White-Footed Mice Exposed to 6:2 FTS



Graph and error bars represent mean and SEMs, respectively, of results from P generation male (A, N=14-15/dose) and female (B, N=15-16/dose) white-footed mice exposed to 6:2 FTS. Results obtained from exposed and nonexposed animals were not significantly different ($p > 0.05$).

APPENDIX J

Pathology

Table J-1. Frequency of Histopathology in P Generation Animals Exposed to PFOS

Tissue	0 mg/kg-day	0.2 mg/kg-day	1 mg/kg-day	5 mg/kg-day
Liver				
Male	2/30	3/27	9/31*	15/23**
Female	7/29	13/30	13/31	21/22*
Spleen				
Male	10/27	---	---	4/23
Female	18/29	---	---	17/22
Kidneys				
Male	2/27	---	---	0/23
Female	3/29	---	---	4/22
Bone marrow				
Male	0/25	---	---	0/23
Female	0/29	---	---	0/22
Thymus				
Male	0/19	---	---	0/21
Female	1/22	---	---	0/20
Lymph nodes				
Male	1/20	---	---	0/19
Female	0/13	---	---	0/13

Notes:

Asterisks (*) denote significant differences by Pearson χ^2 (Bonferroni-Holm adjusted $p < 0.025^{**}$, $p < 0.05^*$). All other results were not significant by Pearson χ^2 ($p > 0.05$).

Table J-2. Liver Findings in P Generation Animals Exposed to PFOS

Finding	Frequency in 0.0 mg/kg-day	Frequency in 5.0 mg/kg-day	Pearson χ^2	DF	N	p-value
Hepatocellular hypertrophy						
Male	2/27	13/23	41.26	1	50	<0.0001
Female	4/29	21/22	33.382	1	51	<0.0001
Vacuolation, finely vacuolated, cytoplasm, single (individual), hepatocytes						
Male	0/27	15/23	25.115	1	50	<0.0001
Female	9/29	19/22	15.467	1	51	<0.0001
Vacuolar degeneration, lipid-type						
Male	0/27	0/23	n/a	n/a	50	n/a
Female	0/29	21/22	47.059	1	51	<0.0001
Vacuolar degeneration, lipid-type, diffuse						
Male	0/27	0/23	n/a	n/a	50	n/a
Female	0/29	7/22	10.695	1	51	0.001
Vacuolar degeneration, lipid-type, multifocal						
Male	2/27	15/23	18.497	1	50	<0.0001
Female	7/29	21/22	25.697	1	51	<0.0001
Karyomegaly						
Male	1/27	9/23	9.742	1	50	0.002
Female	1/29	21/22	43.172	1	51	<0.0001
Necrosis, hepatocellular, single cell						
Male	0/27	7/23	9.555	1	50	0.002
Female	3/29	20/22	32.794	1	51	<0.0001
Necrosis, hepatocellular, multifocus						
Male	1/27	0/23	0.869	1	50	0.351
Female	0/29	1/22	1.345	1	51	0.246
Infiltrates, neutrophilic						
Male	0/27	3/23	3.747	1	50	0.053
Female	0/29	0/22	n/a	n/a	51	n/a
Infiltrates, lymphocytic						
Male	1/27	5/23	3.826	1	50	0.050
Female	3/29	14/22	15.987	1	51	<0.0001
Periportal, lymphoid follicular hyperplasia						
Male	1/27	1/23	0.013	1	50	0.908
Female	0/29	0/22	n/a	n/a	51	n/a
Rarefaction, hepatocellular (vacuolation, glycogen)						
Male	9/27	17/23	8.194	1	50	0.004
Female	4/29	15/22	15.831	1	51	<0.0001
Hepatic lobular atrophy						
Male	0/27	0/23	n/a	n/a	50	n/a
Female	0/29	1/22	1.345	1	51	0.246
Cytoplasmic inclusions, hepatocyte (plasma influx/congestion droplets)						
Male	1/27	3/23	1.472	1	50	0.225
Female	3/29	4/22	0.649	1	51	0.421
Pigment, intrahistiocytic						
Male	2/27	0/23	1.775	1	50	0.183
Female	0/29	0/22	n/a	n/a	51	n/a
Abscess, chronic, multifocal, with fibrosi						
Male	0/27	0/23	n/a	n/a	50	n/a
Female	1/29	0/22	0.774	1	51	0.379
Bacteria, intrabscess						
Male	0/27	0/23	n/a	n/a	50	n/a

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Female	1/29	0/22	0.774	1	51	0.379
Perotinitis, capsule inflammation and fibrosis						
Male	0/27	0/23	n/a	n/a	50	n/a
Female	1/29	0/22	0.774	1	51	0.379

Notes:

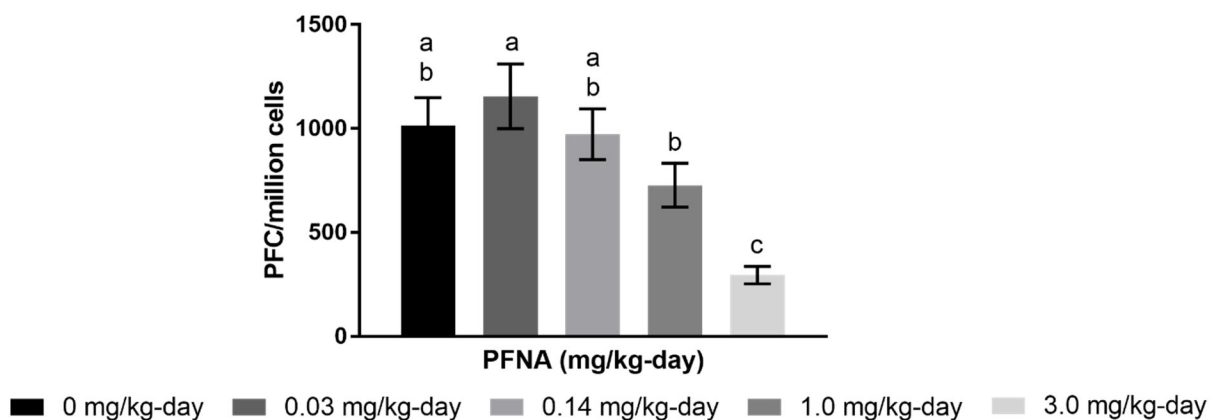
Asterisks denote significant differences by Pearson χ^2 .

All other results were not significant by Pearson χ^2 ($p > 0.05$).

APPENDIX K

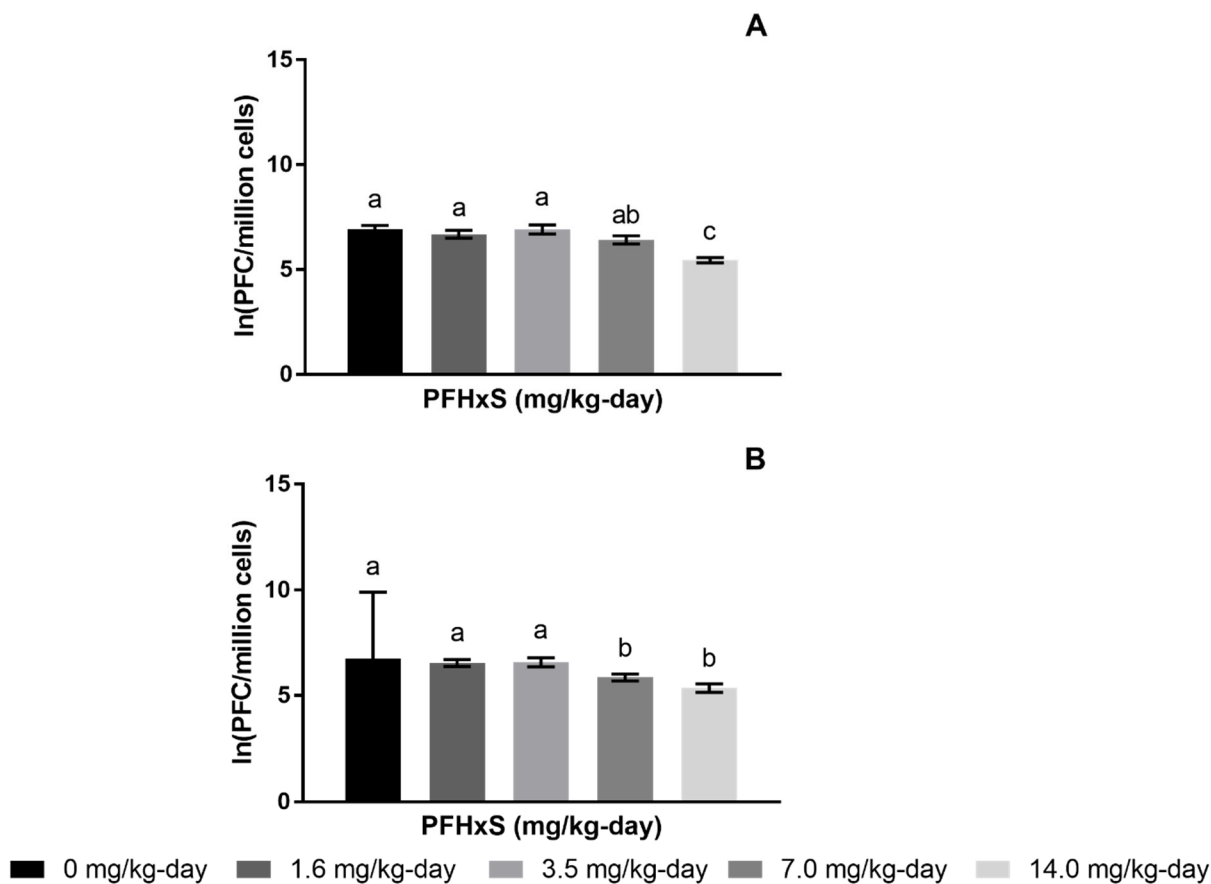
Immune Assay

Figure K-1. Plaque Forming Cells in P Generation White-Footed Mice Exposed to PFNA



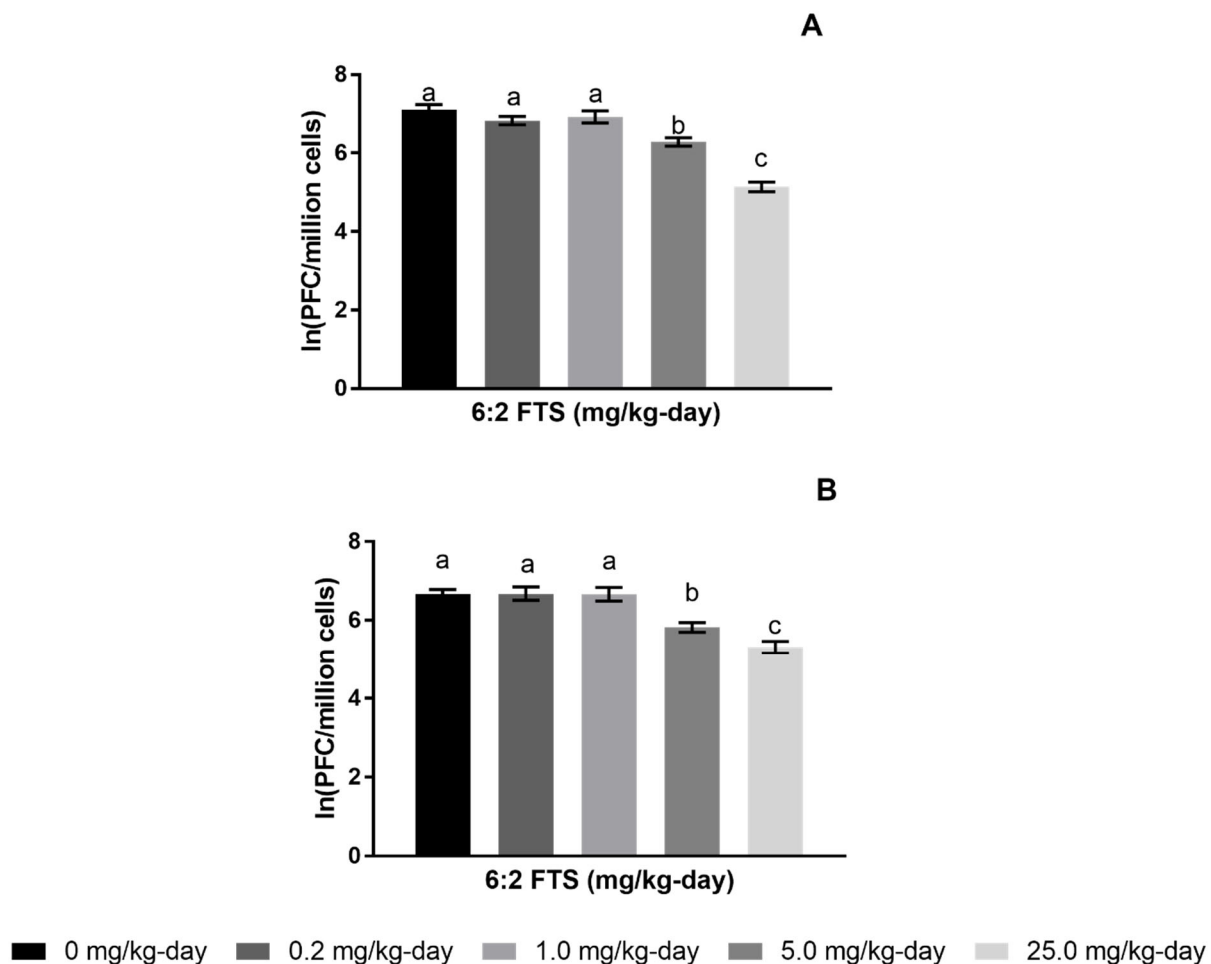
Graph and error bars represent mean and SEM, respectively, of results obtained from P generation animals (N=33-36/dose) exposed to PFNA. Data are presented as PFCs per million cells. Formation of PFCs was reduced in animals exposed to.

Figure K-2. Plaque Forming Cells in P Generation Deer Mice Exposed to PFHxS



Graph and error bars represent mean and SEM, respectively, of results obtained from P generation males (A, N=20-24/dose) and females (B, N=18-25/dose) exposed to PFHxS. Data are presented as ln(PFCs per million cells). Formation of PFCs was reduced in animals exposed to PFHxS.

Figure K-3. Plaque Forming Cells in P Generation White-Footed Mice Exposed to 6:2 FTS



Graph and error bars represent mean and SEM, respectively, of results obtained from P generation males (A, N=18-21/dose) and females (B, N=19-22/dose) exposed to 6:2 FTS. Data are presented as ln(PFCs per million cells). Formation of PFCs was reduced in animals exposed to PFHxS.

APPENDIX L

Protocol and Modifications

ARMY PUBLIC HEALTH CENTER
PROTOCOL REVIEW AND APPROVAL FORM

Test Type: 03

IACUC protocol number: 18-08-01

TITLE: Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

1. PRINCIPAL INVESTIGATOR/ STUDY DIRECTOR

Printed Name (First, MI, Last)

Title/ Division/ Program/ Directorate

Allison, M, Jackovitz

Biologist/Toxicity Evaluation/Toxicology

PHONE: 410-436-8772

EMAIL: [REDACTED]

2018-07-18

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.07.18 10:12:25 -04'00'

Date

Signature

(SD/PI will sign here before submitting protocol to the IACUC office)

2. SCIENTIFIC/ PEER REVIEW

Printed Name (First, MI, Last)

Title/ Division/ Program/ Directorate

Emily N. Reinke

Biologist, Health Effects Division, Toxicology

PHONE: 410-436-2896

EMAIL: [REDACTED]

2018-07-18

REINKE.EMILY.NICOLE.1447560620

Digitally signed by REINKE.EMILY.NICOLE.1447560620
Date: 2018.07.18 10:05:01 -04'00'

Date

Signature

3. STATISTICAL REVIEW

Printed Name (First, MI, Last)

Title/ Division/ Program/ Directorate

Robyn Lee-Stubbs

USAMRICD

PHONE: 410-436-5322

EMAIL: [REDACTED]

2018-07-10

LEE-STUBBS.ROBYN.BELLEK.1267390289

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Date: 2018.07.10 11:00:15 -04'00'

Date

Signature

4. ATTENDING VETERINARIAN REVIEW

Printed Name (First, MI, Last)

Title/ Division/ Program/ Directorate

LTC Krinon Moccia

Chief, RSD, USAMRICD

PHONE: 410-436-1131

EMAIL: [REDACTED]

2018-07-11

MOCCIA.KRINON.DODEKA.1167935563

Digitally signed by MOCCIA.KRINON.DODEKA.1167935563
Date: 2018.07.11 14:30:40 -04'00'

Date

Signature

5. IACUC APPROVAL

Date: 2018-08-13

Printed Name (First, MI, Last)

Signature

Kristin Newkirk

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.08.13 12:27:41 -04'00'

6. INSTITUTIONAL OFFICIAL REVIEW

Printed Name (First, MI, Last)

Signature

John J. Resta

RESTA.JOHN.J.1229129305

Digitally signed by RESTA.JOHN.J.1229129305
Date: 2018.08.15 15:40:04 -04'00'

DOD IACUC Protocol

1.0 General Information

Please enter the full title of your study:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Please enter the "Protocol Number" you would like to use to reference the protocol:

Note: This field allows you to enter an abbreviated version of the Protocol Title to quickly identify this protocol.

2.0 Add Site(s)

2.1 List site(s) associated with this study:

	Primary Dept?	Department Name
+	☐	
-	☒	Army: APHC (Army Public Health Center)

3.0 Assign project personnel access to the project

3.1 Please add a Principal Investigator for the study:

Allison M. Jackovitz

Select PI status, if applicable

For IACUC use only

DOD E-IACUC protocol format
Version 2.0, 10/2017

IACUC number (03 -) 18-08-01

IACUC approval date 13 August 2018

IACUC expiration date 13 August 2021

3.2 If applicable, please select the Research Staff personnel:

A) Additional Investigators

	Name	Role
+	Michael Quinn, Jr.	Co-Investigator
-		
+	Jarod Hanson	Associate Investigator
-		

B) Research Support Staff

	Name	Role
+	James Cox	Team Member
-		
+	Lee Crouse	Team Member
-		
+	Theresa Hanna	Team Member
-		
+	Emily Lent	Team Member
-		
+	Caroline Procell	Team Member
-		
+	Thomas Sussan	Team Member
-		
+	Mark Way	Team Member
-		

3.3 Please add a Protocol Contact:

The Protocol Contact(s) will receive all important system notifications along with the Principal Investigator. (i.e. The protocol contact(s) are typically either the Protocol Coordinator or the Principal Investigator themselves).

Michael Quinn

4.0 Funding

4.1 Would you like to state your funding information?

- ☒ Yes
- ☐ No

	Funding Source	Fund Code	Grant title(s)
--	----------------	-----------	----------------

For IACUC use only

IACUC number	(03 -) 18-08-01
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DOD E-IACUC protocol format
Version 2.0, 10/2017

IACUC approval date	13 August 2018
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IACUC expiration date	13 August 2021
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+		
-		

The table below is used for **other** funding sources

	Other Funding Source	Other Funding Code	Grant title(s)
+	Strategic Environmental Research and Development Program (SERDP)	ER-2625	Development of Toxicity Data to Support Toxicity Reference Values for Perfluorinated Chemicals
-			

5.0 Animal(s) Used

5.1 List the animals used on this protocol:

***Note: Each species should be entered into a separate chart.**

Animal Species	White-footed mouse	
Scientific Name (genus/species)	Peromyscus leucopus	
Is Species USDA-regulated?	☒ Click if Yes	
Strain, if applicable		
Sex	Male/Female	
Age:	Adults, as available from vendor	
Weight	Appropriate for age	
Wildlife?	☐ Click if Yes	
Laboratory?	☒ Click if Yes	
Stock #/Order Code	USDA license number 56-R-0003	
Special Care	N/A	
Special Conditions	All animals will be placed on Animal Use Protocol Number 18-04-01 when they arrive from the vendor. After an extended acclimation period, animals will be transferred to this protocol.	
Unique anatomical features/ phenotype:	N/A	
USDA pain category	USDA Category	Number requested
	B	
	C	
	D	1,488
	E	
Total Number Requested:	1488	

For IACUC use only

IACUC number (03 -) 18-08-01

DOD E-IACUC protocol format
Version 2.0, 10/2017

IACUC approval date 13 August 2018

IACUC expiration date 13 August 2021

Animal Species	White-footed mouse	
Scientific Name (genus/species)	Peromyscus leucopus	
Is Species USDA-regulated?	☒ Click if Yes	
Strain, if applicable		
Sex	Male/Female	
Age:	Pups	
Weight	Appropriate for age	
Wildlife?	☐ Click if Yes	
Laboratory?	☒ Click if Yes	
Stock #/Order Code	N/A, these animals will be offspring of adults.	
Special Care	N/A	
Special Conditions	These animals will be produced in-housed. The number of animals requested assumes 100% mating success and 5 pups per litter. According to the vendor, about 80% of pairs will successfully breed and virgin females have litters of about 1-2 pups. If more than 3,720 pups are produced, a Modification Request will be submitted to the IACUC to use the additional animals. Since data will be collected on pups, pups will be counted as study animals prior to weaning.	
Unique anatomical features/ phenotype:	N/A	
USDA pain category	USDA Category	Number requested
	B	
	C	3,720
	D	
	E	
Total Number Requested:	3720	

Add another Species:

+

-

For IACUC use only

DOD E-IACUC protocol format
Version 2.0, 10/2017

IACUC number (03 -) 18-08-01

IACUC approval date 13 August 2018

IACUC expiration date 13 August 2021

6.0 Study Personnel Qualifications and Training

6.1 All personnel performing observations, procedures, euthanasia, and/or manipulations described in the protocol must be identified and appropriately trained and qualified to perform these procedures.

	Name	Qualifications	Procedures Performed	Training
☐	Allison Jackovitz	B.S. Biology, 8+ years animal research experience	Handling and observations	6/4/09; 2/18/15; 3/12/15; 12/8/16; 12/20/16; 12/22/16; 1/24/17
☐				
☐			Oral gavage	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18
☐				
☐			IP injections	2/18/15; 3/12/15; 12/20/16; 9/6/17; 9/13/17
☐				
☐			CO2 anesthesia/euthanasia	3/12/15; 7/6/17; 8/30/17; 9/6/17; 9/13/17
☐				
☐			Cervical dislocation	7/6/17; 9/6/17; 9/13/17
☐				
☐	James Cox	M.S., Biology, 7+ years animal research experience	Handling and observations	7/6/17; 8/30/17; 9/6/17; 9/13/17
☐				
☐	Lee Crouse	M.S., Environmental Sciences, 19+ years animal research experience	Handling and observations	12/20/16
☐				
☐	Theresa Hanna	ALAT, 16+ years animal research experience	Handling and observations	6/30/11; 2/12/15; 2/18/15; 2/25/15 3/12/15; 12/12/16; 12/20/16
☐				
☐	Jarod Hanson	DVM, Ph.D., Infectious Diseases/Virology, 24+ years animal experience	Handling and observations	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18
☐				
☐			Oral gavage	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18
☐				
☐	Emily Lent	Ph.D., Natural Resources and Environmental Studies, M.S., Wildlife Biology, 19+ years animal research experience	Handling and 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
☐				
☐	Caroline Procell	B.S., Biology, 3+ years animal experience	Handling and observations	12/20/16; 12/22/16; 1/24/17
☐				
☐			IP injections	12/20/16
☐				
☐			CO2 anesthesia/euthanasia	8/30/17; 9/13/17
☐				
☐			Cervical dislocation	8/30/17; 9/13/17

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☐			Decapitation and exsanguination	8/30/17
☐				
☐				
☐	Michael Quinn	Ph.D., Animal Science 19+ years animal research experience	Handling and 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
☐				
☐			Oral gavage	12/20/16; 12/22/16; 3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18
☐				
☐			IP injections	6/30/11; 2/25/15; 3/12/15; 12/20/16; 9/6/17
☐				
☐			CO2 anesthesia/euthanasia	7/6/17; 8/30/17; 9/6/17
☐				
☐			Cervical dislocation	7/6/17; 8/30/17; 9/6/17
☐				
☐			Decapitation and exsanguination	7/6/17; 8/30/17; 9/6/17
☐				
☐	Thomas Sussan	Ph.D., Biochemistry, Cellular, and Molecular Biology, 18+ years animal research experience	Handling and observations	9/6/17; 9/13/17
☐				
☐			Oral gavage	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18
☐				
☐			CO2 anesthesia/euthanasia	9/6/17; 9/13/17
☐				
☐			Cervical dislocation	9/6/17; 9/13/17
☐				
☐			Decapitation and exsanguination	9/6/17; 9/13/17
☐				
☐				
☐	Mark Way	B.S., Biology 18+ years animal research experience	Handling and 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
☐				
☐			Oral gavage	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18
☐				
☐			IP injections	2/12/15; 2/18/15; 2/25/15; 3/12/15; 12/20/16; 8/30/17; 9/6/17
☐				
☐			CO2 anesthesia/euthanasia	6/24/09; 2/12/15; 3/12/15; 7/6/17; 8/30/17; 9/6/17
☐				
☐			Cervical dislocation	9/6/17; 9/13/17
☐				
☐			Decapitation and exsanguination	9/6/17; 9/13/17

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6.2 Please identify the emergency contact(s) for animal care/disposition (or other critical needs) for this protocol:

	Name	Telephone Number(s)
+	Allison Jackovitz	[REDACTED]
-		work: 410-436-8772
+	Michael Quinn	[REDACTED]
-		work: 410-436-8772

6.3 Does this protocol involve the use of animals for human medical training?

- ☐ Yes
☒ No

7.0 Objective/Hypothesis/Background/Relevance

7.1 Objective/Hypothesis

State the objective of this protocol and/or the hypothesis to be accepted or rejected.

The main objective of reproductive and developmental toxicity studies are to assess the possible effects on reproduction and/or development, either at an early stage of assessing toxicological properties of chemicals or on chemicals of concern (OECD 2011, OECD 2016). The purpose of this study, which includes six separate experiments with six chemicals, is to evaluate the effects of exposure to per- and polyfluoroalkyl substances (PFAS) prior to mating, during gestation, and through weaning on male and female reproductive performance, mating behavior, conception, and prenatal and postnatal development of offspring.

7.2 Non-Technical Synopsis

Provide a brief, narrative description of the proposal that is easily understood by a high school graduate. Include a brief description of animal use in your description.

PFAS are compounds manufactured for use in paints, cleaning agents, non-stick cookware and food containers, and water-impermeable products. Concerns about PFAS stem from their ubiquitous presence in the environment, widespread reports of general toxicity, and the resistance of these compounds to degradation. Data from this study will be used to derive Toxicity Reference Values (TRVs). TRVs are critical components of environmental risk assessments that help determine if the risk of environmental exposure is acceptable.

In this study, six PFAS will be administered daily to male and female white-footed mice (*Peromyscus leucopus*) in six separate experiments to evaluate the effects of combined pre- and postnatal exposure to PFAS on development and reproduction. Parental generation animals will be dosed with the test compounds by oral gavage for 10-12 weeks (based on sex). Fetuses and offspring will only be exposed to the test compounds through pregnancy and maternal transfer (lactation) and will be maintained on the study through puberty. All animals will be monitored for body weight changes and signs of toxicity. Immune function will be evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood will be collected from anesthetized animals via decapitation and truck blood collection.

Blood will be analyzed for chemical concentration, hormones, etc., as available. Selected tissues will be weighed and processed for histopathology. Immunological organs (thymus and spleen) may also be processed and evaluated for immune effects. This study will provide information about the effects of PFAS on the adult male and female reproductive system. Examination of physical and functional development following combined pre- and postnatal exposure is expected to identify specific target organs in the offspring and may reveal effects not seen with more abbreviated exposures.

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7.3 Background

Include a brief statement of the requirement or need for the information being sought. A description of the general approach should be provided. The literature or the experience that led to the proposal may be briefly reviewed, reference cited. Acronyms and abbreviations must be spelled out on first-time use. Standard formatting for all scientific taxa and agents applies.

PFAS are environmentally persistent, bioaccumulative, and have growing evidence of toxicity in animals. In 2016, the U.S. Environmental Protection Agency (USEPA) published lifetime drinking water Health Advisory values of 0.2 and 0.4 µg/L for perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA), respectively. These recommended levels are 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 PFAS have emerged in commerce (e.g., perfluorohexanesulfonate (PFHxS), perfluorobutanesulfonate (PFBS), etc.), 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 PFAS 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, few comprehensive tests for any PFAS have been undertaken with a wild mammalian species. Toxicity data from this series of experiments 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. Myriad laboratory animal studies and human epidemiological studies suggest the transfer of PFAS via lactation (Butenhoff *et al.*, 2009).

This study will evaluate the effects of PFAS exposure prior to mating, during gestation, and through weaning on male and female reproductive performance, mating behavior, conception, and prenatal and postnatal development of offspring. The study will include six separate experiments with six PFAS commonly found at Department of Defense (DoD) impacted sites.

7.4 Military Relevance

Provide a brief and succinct reason for the research with regard to military needs and mission requirements.

PFAS are key ingredients in highly efficient fire suppressant agents known as Aqueous Film Forming Foams (AFFF). AFFFs have been used by the DoD since approximately 1970 for fire-training and emergency response activities. PFAS 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 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. TRVs and a general increased understanding of these unique chemicals will assist in the development of appropriate site-specific risk assessment and decisions related to mitigation of exposures and/or future cleanup.

8.0 Literature Search for Duplication

8.1 Is a literature search required ?

- ☒ Yes
- ☐ No -- This protocol uses animals solely for training purposes or breeding colonies and therefore is duplicative by nature.

Literature search details:

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* Date of search should be within 6 months of protocol submission to IACUC.

** **Results of Search:** Based on the literature search, indicate why this study is not unnecessarily duplicative. Provide written narrative, not just a list of articles found.

*** **Add a new row** for each source necessary.

	Source Searched	Date of Search	Dates covered by Search	Search Strategy for Keywords	Results of Search
+					
-					
	PubMed	8 JUNE 2018	1900-2018	PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid AND reproduction and rats; PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid AND reproduction and mice; PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid AND development and rats; PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid AND development and mice; PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid AND peromyscus	A PubMed search for PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid generated 2637 publications. Of these, 13 publications provide data on PFOS, rats, and reproductive toxicity and 4 publications provide data on PFOS, mice, and reproductive toxicity. Additionally, 17 publications provide data on PFOS, rats, and developmental toxicity and 15 publications provide data on PFOS, mice, and developmental toxicity. Most of the publications retrieved were mechanistic in nature and outside the realm of the proposed study. However, one of these publications (Fuentes et al., 2006) investigated the effects of concurrent PFOS exposure and stress in pregnant CD-1 mice. Fuentes et al., saw a significant general effect of restraint on body weight gain. Although restraint of this sort will not occur in this study, it is worth considering the impact of stress from routine handling and procedures, given that the white-footed mouse is a wild-type species and, thus, is prone to stress. Sample sizes have been adjusted accordingly. Importantly, none of the publications retrieved reported data on peromyscus exposed to PFOS, thus eliminating the possibility of duplication of effort.

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[illegible]

				sulfonate AND development and mice; PFBS or perfluorobutane sulfonate AND peromyscus	lactation. F1 generation animals were also directly exposed to PFBS via oral gavage post-weaning. F2 generation animals were not directly exposed to PFBS. Feng et al., (2017) exposed mice via oral gavage to PFBS during gestation. F1 generation animals were weaned and PND21 and maintained through PND60, similar to what is proposed in this study. Importantly, none of the publications retrieved reported data on peromyscus exposed to PFBS, thus eliminating the possibility of duplication of effort.
+ -	PubMed	8 JUNE 2018	1900-2018	FTS or tridecafluorooctane-1-sulphonic acid	A PubMed search for FTS or tridecafluorooctane-1-sulphonic acid generated 0 publications.
+ -					
	PubMed	8 JUNE 2018	1900-2018	PFNA or perfluorononanoic acid AND reproduction and rats; PFNA or perfluorononanoic acid AND reproduction and mice PFNA or perfluorononanoic acid AND development and rats; PFNA or perfluorononanoic acid AND development and mice; PFNA or perfluorononanoic acid AND peromyscus	A PubMed search for PFNA or perfluorononanoic acid generated 821 publications. Of these, 0 publications provide data on PFNA, rats, and reproductive toxicity and 0 publication provide data on PFNA, mice, and reproductive toxicity. Additionally, 0 publications provide data on PFNA, rats, and developmental toxicity and 2 publications provide data on PFNA, mice, and developmental toxicity. One publication provides data that serves as the basis for the potential utility of the work proposed here. For example, Das et al., (2015) exposed CD-1 mice via oral gavage to PFNA from GD1-17 and then examined fetuses. Importantly, neither of the publications retrieved reported data on peromyscus exposed to PFBS, thus eliminating the possibility of duplication of effort.

+ -				
	PubMed	20 JUNE 2018	1900-2018	peromyscus OR wild mouse AND toxicity
				A PubMed search for toxicity studies with peromyscus and other wild-type species revealed that significant treatment-related differences are especially hard to detect. For example, Biser et al., (2004) investigated the effects of heavy metals on immunocompetence of white-footed mouse. He found that differences between animals for spleen weight were due to age, not contamination. This is a concern for this study, since the age range on animals from the breeder can be quite wide (e.g., 3-12 months of age). In addition, statistical analysis showed that variation across individuals (i.e., error variance) comprised 57% of the overall variance for both parameters. Therefore, any differences in immune parameters due to contamination differences at the sites would have to be very large in order to detect. Range finding studies conducted prior to this study support the need a robust sample size to detect treatment-related effects.
+ -				
	DTIC	20 JUNE 2018	1992-2018	PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid
				A PubMed search also revealed challenges with using flow cytometry for immunologic assays with Peromyscys, specifically that cross-reactivity between the Mus epitope and its peromyscus counterpart was insufficient.
+ -				
	DTIC	20 JUNE 2018	1992-2018	PFOA or perfluorooctanoate or perfluorooctanoic acid
				A DTIC search for PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid generated 7 hits, most of which were related to remediating contaminated sites and the use of AFFFs. All of these topics are outside the realm of the proposed study, thus eliminating the possibility of duplication of effort. A DTIC search for PFOA or perfluorooctanoate or perfluorooctanoic acid generated 59 hits, most of which were related to remediating contaminated sites, the use of AFFFs, in vitro work, and the mechanism of PFAS toxicity. All of these topics are outside the realm of the proposed study, thus eliminating the possibility of duplication of effort.

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+ -	DTIC	20 JUNE 2018	1992-2018	PFHxS or perfluorohexane sulfonate	A DTIC search for PFHxS or perfluorohexane sulfonate generated 3 hits, all three of which were generated in previous searches.
+ -	DTIC	20 JUNE 2018	1992-2018	PFBS or perfluorobutane sulfonate	A DTIC search for PFBS or perfluorobutane sulfonate generated 3 hits, all three of which were generated in previous searches.
+ -	DTIC	20 JUNE 2018	1992-2018	FTS or tridecafluorooctane-1-sulphonic acid	A DTIC search for FTS or tridecafluorooctane-1-sulphonic acid generated 0 hits.
+ -	DTIC	20 JUNE 2018	1992-2018	PFNA or perfluorononanoic acid	A DTIC search for PFNA or perfluorononanoic acid generated 3 hits, all three of which were generated in previous searches.
+ -					There are 58 active projects with NIH funding for PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid. Several of these projects were related to remediating contaminated sites. Several other projects were related to the human health effects of exposure to PFAS. Most of these projects were related to the mechanism of PFAS toxicity (e.g., PPAR-alpha and NRF2 activation). All of these projects are outside the realm of the proposed study, thus eliminating the possibility of duplication of effort.
	NIH RePORTER	20 JUNE 2018	1985-2018 (active projects)	PFOS or perfluorooctanesulfonate or perfluorooctane sulfonic acid	
+ -					There are 126 active projects with NIH funding for PFOA or perfluorooctanoate or perfluorooctanoic acid. Several of these projects were related to remediating contaminated sites. Most of these projects were related to the human health effects of gestational exposure to PFAS. One project, led by Dr. Suzanne Fenton, is investigating the mammary gland as a sensitive end point to the effects of endocrine disruptors. Dr. Fenton was consulted in the design of this study and came to APHC to train personnel on mammary gland mounting. One project, led by Dr. Grace Kissling, is identifying and evaluating sources of variability in rodent studies with endocrine disrupting chemicals, including PFOA. Another project, led by Dr.
	NIH RePORTER	20 JUNE 2018	1985-2018 (active projects)	PFOA or perfluorooctanoate or perfluorooctanoic acid	

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+	-	NIH RePORTER	20 JUNE 2018	1985-2018 (active projects)	PFHxS or perfluorohexane sulfonate	Milton Hejtmancik, is a two-year study with rats exposed to PFOA via diet to evaluate chronic toxicity and carcinogenicity. All of these projects are outside the realm of the proposed study, thus eliminating the possibility of duplication of effort.
						There are 15 active projects with NIH funding for PFHxS or perfluorohexane sulfonate. Several of these projects were related to remediating contaminated sites. Several other projects were related to the human health effects of exposure to PFAS. All of these projects are outside the realm of the proposed study, thus eliminating the possibility of duplication of effort.
+	-	NIH RePORTER	20 JUNE 2018	1985-2018 (active projects)	PFBS or perfluorobutane sulfonate	There are 2 active projects with NIH funding for PFBS or perfluorobutane sulfonate. Both projects are led by Dr. Liping Feng. Dr. Feng is looking at the effects of PFBS exposure on maternal and child health in Shanghai. This project is outside the realm of the proposed study, thus eliminating the possibility of duplication of effort.
+	-	NIH RePORTER	20 JUNE 2018	1985-2018 (active projects)	FTS or tridecafluorooctane-1-sulphonic acid	There are 0 active projects with NIH funding for FTS or tridecafluorooctane-1-sulphonic acid.
+	-	NIH RePORTER	20 JUNE 2018	1985-2018 (active projects)	PFNA or perfluorononanoic acid	There are 15 active projects with NIH funding for PFHxS or perfluorohexane sulfonate. Several of these projects were related to remediating contaminated sites. Several other projects were related to the human health effects of exposure to PFAS. All of these projects are outside the realm of the proposed study, thus eliminating the possibility of duplication of effort.

9.0 Materials and Methods

9.1 Experimental Design and General Procedures:

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Provide a complete description of the proposed use of animals, allowing the reader to follow an animal through the course of the study from start to finish. It should include a summary table and/or flow chart of the experimental groups and/or breeding scheme(s). Outline the scientific plan and direction of experimentation. If several experiments or sequential studies are to be included in the protocol, describe the experimental design of each separate experiment. The number requested must equal the minimum number required to complete the study yet be sufficient to yield meaningful results. Technical methodology (e.g. injections, surgical methods, etc.) need not be described in this section.

A. ADMINISTRATION OF TEST SUBSTANCES AND EXPOSURE MODE: This study includes six separate experiments with six test articles: PFOS, PFOA, PFHxS, PFBS, tridecafluorooctane-1-sulphonic acid (6:2 FTS), and perfluorononanoic acid (PFNA). Chemical/physical properties of the six chemicals are included in a table below. The experiments may be run separately, concurrently, or with an overlap, depending on animal, facility, and staff availability.

The test substances will be prepared based on their unique chemical and physical properties, which may include dissolving the test article in water, suspending the test article in corn oil, or dissolving the test article in an appropriate solvent to create a stock solution and then dissolving or suspending the stock solution in an appropriate vehicle to create a solution or suspension, respectively. The frequency with which dosing solutions or suspensions are prepared will depend on the test article.

Although the solvent and vehicle used for the six test articles may be different, each test article will be prepared consistently throughout exposure. Examples of appropriate solvents are Tween, methanol, ethanol, etc. Examples of appropriate vehicles are filtered water, deionized water, PFAS-free water, corn oil, etc. 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). So long as not more than a quarter of the median lethal dose (LD50) is used, solvents can safely be used without confounding results and compromising animal lives and data integrity. Forming a uniform solution or suspension is superior for delivering the intended dosage accurately and consistently. Neat test material will be stored at room temperature (e.g., 20±5°C). Concentration verification and stability analysis may be conducted.

Dosing solutions or suspensions will be mixed or rocked during dosing, to prevent the test substance from partitioning (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 (2014). Doses will be calculated according to the most recent body weight measurement. Dose levels will be based on the literature and data from previously conducted range finding studies, taking into account that there may be differences in sensitivity between pregnant and non-pregnant animals. Dose selection is based on the ultimate objective of being able to detect reproductive and development effects, if present. To that end, it is recommended that "the highest dose level should be chosen with the aim of inducing toxic effects, but not death or severe suffering" (OECD 2016). Oral gavage will be performed as described in 15.17.

Given the sensitivity of the species involved, the Study Director/Principal Investigator (SD/PI) attempted to reduce handling and invasive procedures in a previously conducted range finding study. As such, in the first portion of the aforementioned range finding, three test articles (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 for subsequent experiments. Instead, 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. 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 exposure route was refined to oral gavage, which is the only other physiologically relevant exposure route. Since PFAS 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.

B. EXPOSURE DURATION: A diagram of the exposure scheme is included below. The exposure scheme will be repeated for each of the six test articles. Each test article will have its own control group.

Parental (P) generation males will be dosed daily with the test article for at least 10 weeks: four weeks prior to mating, the variable time to mating and conception (up to 12 weeks), the duration of pregnancy (about three weeks), and after the mating period (as needed to ensure at least 10 weeks of dosing). P generation females will be dosed daily with the test article throughout the study: four weeks prior to mating, the variable time to mating and conception (up to 12 weeks), the duration of pregnancy (about three weeks), and through weaning (approximately postnatal day (PND) 21-28). Fetuses and pups will be exposed via maternal transfer. At weaning, dams will be removed from the study; however, First (F1) generation animals will be

maintained through puberty (approximately PND60).

C. CONTROLS: P generation males will be dosed daily with the vehicle control for at least 10 weeks: four weeks prior to mating, the variable time to mating and conception (up to 12 weeks), the duration of pregnancy (about three weeks), and after the mating period (as needed to ensure at least 10 weeks of dosing). P generation females will be dosed daily with the vehicle control throughout the study: four weeks prior to mating, the variable time to mating and conception (up to 12 weeks), the duration of pregnancy (about three weeks), and through weaning (approximately PND 21-28). Fetuses and pups will be exposed via maternal transfer. At weaning, dams will be removed from the study; however, F1 generation animals will be maintained through puberty (approximately PND60).

D. STUDY DESIGN: The P generation will be comprised of four groups of 31-sexually mature males and four groups of sexually mature females (see table below). There will be three test groups and one control group. The F1 generation will be comprised of animals generated by the P generation. P generation 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, the differences in the mean body weights and age between groups within each sex will be as small as possible. Cages will be arranged in racks randomly to minimize possible effects due to cage placement.

ANIMAL IDENTIFICATION: Animals will be identified by cage cards according to QSARC 804 (APHC, 2017a). An identification number or feature (e.g., the last 3 digits of the animal number or a colored marker) may be marked on the tail of each animal with a water-insoluble marker. F1 generation animals will not be individually identified until weaning.

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 dosing. 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, 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). Once daily, 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.

BODY WEIGHT AND FOOD CONSUMPTION: P generation animals will be weighed at the start of test compound administration, at least weekly thereafter, and at termination. F1 generation animals will be weighed at weaning, at least weekly thereafter, and at termination. Animals will be weighed in accordance with QSARC 812 (2018). Food consumption will be monitored at least weekly for all animals by weighing the food hopper. Food consumption will be measured per cage.

BREEDING: One male and one female mating (not between siblings) will be used in this study. All animals will be virgins. After four weeks of pre-mating dosing, one male will be placed in the home cage of one female from the same dose group. Unlike traditional rodent species, females cannot be examined for the presence of sperm or a vaginal plug in this species. Therefore, Day 0 of pregnancy cannot be confirmed in white-footed mouse. As such, 1:1 matings will be maintained (e.g., animals will be co-housed) until pups are born or 15 weeks have elapsed. If there are insufficient males, for example due to male death before mating, then male(s) which have already mated may be paired (1:1) with a second female(s) such that all females are paired. If there are insufficient females, females will not be paired with a second male.

LITTERS: Each litter will be examined as soon as possible after delivery to establish the number of pups, stillbirths, live births, runts (pups that are significantly smaller than corresponding control pups), and the presence of gross abnormalities. Live pups will be counted and litters weighed within 24 hours of parturition (day 0 or 1 post-partum) and at least on day 4 and 13 post-partum. Abnormal behavior in the offspring will be recorded. Classic developmental landmarks (e.g., ear opening, eye opening, nipple retention) will be observed, as possible. Unlike reproductive and/or developmental toxicity testing with traditional rodent species, the white-footed mouse has small litters of between one and five pups, therefore, litter sizes will not be adjusted. Litters will be maintained with dams through weaning.

F1 GENERATION ANIMALS: F1 generation animals will be same-sex group-housed without receiving the test article between weaning and puberty (approximately PND 60).

IMMUNE CHALLENGE: About one week prior to euthanasia, P and F1 generation animals will be administered 0.1 mL of foreign RBCs suspended in sterile saline via IP injection to evaluate immune function, as described 15.17.

Do you wish to add a supporting image?

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☒ Yes

☐ No

Table of Chemical/Physical Properties of Six PFAS						
CHEMICAL	PFOA	PFOA	PFHxS	PFBS	6:2 FTS	PFNA
Name	Perfluorooctane sulfonate	perfluorooctanoate	Perfluorohexane sulfonate	Perfluorobutane sulfonate	tridecafluorooctane-1-sulphonic acid	perfluorononanoic acid
CAS#	2795-39-3	335-67-1	3871-99-6	29420-49-3	27619-97-2	375-95-1
Physical State	White powder	White powder	White powder	White powder	White powder	White powder
Molecular Formula	C ₈ HF ₁₇ O ₃ S	C ₈ HF ₁₅ O ₂	C ₆ F ₁₃ O ₃ S	C ₄ HF ₉ O ₃ S	C ₈ H ₅ F ₁₃ O ₃ S	C ₉ HF ₁₇ O ₂
Molecular Weight	499 g/mol	413 g/mol	399 g/mol	299 g/mol	428 g/mol	463 g/mol
Solubility	0.6 g/L	9.5 g/L	--	46 g/L	--	<9.5 g/L
Proposed solvent		MeOH	n/a		MeOH	MeOH
Proposed vehicle			Deionized water		Deionized water	Deionized water

Caption

STUDY DESIGN: Diagram of Exposure to PFAS				
	Pre-mating Exposure	Mating Exposure	Post-Mating Exposure	
F0 males	4 weeks	up to 12 weeks	Pregnancy: ~3 weeks	Lactation: as needed
F0 females	4 weeks	up to 12 weeks	Pregnancy: ~3 weeks	Lactation: ~3 weeks
	F1 males and females	in-utero	Pre-weaning	

Caption

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Breakdown of Animals for Six Experiments with Six PFAS						
CHEMICAL	PFOS	PFOA	PFHxS	PFBS	6:2 FTS	PFNA
Parental generation	Males Females	Males Females	Males Females	Males Females	Males Females	Males Females
Vehicle control	31 31	31 31	31 31	31 31	31 31	31 31
Dose 1	31 31	31 31	31 31	31 31	31 31	31 31
Dose 2	31 31	31 31	31 31	31 31	31 31	31 31
Dose 3	31 31	31 31	31 31	31 31	31 31	31 31
	TOTAL = 124 124	TOTAL = 124 124	TOTAL = 124 124	TOTAL = 124 124	TOTAL = 124 124	TOTAL = 124 124
Estimated # pups*	Males Females	Males Females	Males Females	Males Females	Males Females	Males Females
Vehicle control	78 78	78 78	78 78	78 78	78 78	78 78
Dose 1	78 78	78 78	78 78	78 78	78 78	78 78
Dose 2	78 78	78 78	78 78	78 78	78 78	78 78
Dose 3	78 78	78 78	78 78	78 78	78 78	78 78
	TOTAL = 312 312	TOTAL = 312 312	TOTAL = 312 312	TOTAL = 312 312	TOTAL = 312 312	TOTAL = 312 312

Pup* estimation based on 100% success between mated pairs, 5 pups per litter, and a sex ratio of 1:1.

Caption

Add another Image



9.2 List potential adverse sequelae that could occur during the natural course of the proposed animal experiments:

Given that the white-footed mouse is a wild-type species and prone to stress, it is not unexpected for some animals (e.g., fewer than 10% of the total animals) to die during handling and/or procedures, including oral gavage.

9.3 Data Analysis:

Describe the statistical test(s) or strategy intended to evaluate the data. Include the statistical methodology used to determine group size and total number of animals. A power-based assessment of the sample size is the preferable method of determining the minimum number to yield significant results. Be certain to include animals necessary for controls or technique development.

Current reproductive toxicity test guidelines state that, "for all but the rarest events (such as malformations, abortions, total litter loss), evaluation of between 16 to 20 litters for rodents and rabbits tends to provide a degree of consistency between studies. Below 17 litters per evaluation, between study results become inconsistent, above 20-24 litters per group consistency and precision is not greatly enhanced" (ICH 2005). Developmental toxicity studies using a sample size of 20 litters and evaluating all fetuses reportedly have the power to detect an increased incidence of malformations of 5 to 12 times above the control levels, an increase of 3 to 6 times the *in utero* death rate, and a decrease of 0.15 to 0.25 times the fetal weight (OECD 2008). In order to produce the desired 20 litters, 31 pairs will be mated. The expected rate of mating with this species is 80%. The expected success rate of mating with this species is also 80%.

Furthermore, a literature search for toxicity studies with peromyscus and other wild-type species revealed that significant treatment-related differences are especially hard to detect. For example, Biser *et al.*, (2004) investigated the effects of heavy metals on immunocompetence of white-footed mouse. He found that differences between animals for spleen weight were due to age, not contamination. This is a concern for this study, since the age range on animals from the breeder can be quite wide (e.g., 3-12 months of age). In addition, statistical analysis showed that variation across individuals (i.e., error variance) comprised 57% of the overall variance for both parameters. Therefore, any differences in immune parameters due to

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contamination differences at the sites would have to be very large in order to detect. Range finding studies conducted prior to this study support the need for a robust sample size to detect treatment-related effects.

Data will be reported individually and summarized in tabular form. Statistical significance is defined as a $p < .05$. Continuous data will be analyzed using a one-way Analysis of Variance (ANOVA) with dose group as the main effect. All organ weights will be analyzed by Analysis of Covariance (ANCOVA) using final body weight and age (as appropriate) as the covariate. Mean pup body weight per litter will be calculated and then analyzed with one-way ANOVA. Weekly body weight and food consumption data may be analyzed using repeated measures ANOVA or one-way ANOVA to determine dose effect. When statistically significant main effects are observed, post hoc tests will be used to compare pairs of dose groups and dose groups to the control group; Tukey's multiple comparison test if the variance of the groups is similar and Dunnett's T3 test if the variances are unequal. Variance equality will be determined by Levene's test. If the data are not normally distributed, the data may be transformed appropriately prior to ANOVA/ANCOVA, or analyzed using a nonparametric Kruskal-Wallis test. Non-parametric analysis will be the method of last resort since it does not allow analysis of co-variation.

Appropriate statistical tests (e.g., Chi-square, Fisher's exact, and regression analysis) will be used to determine significant differences for nominal or count data (e.g., litter size, malformation frequency, etc.). When possible, appropriate statistical analysis, such as Chi-square analysis, will be applied to histology results.

9.4 Laboratory Animals Required and Justification:

Animal (In Vivo) Justification:

Provide a rationale for the use of animals.

The objectives of this study are to evaluate the effects of PFAS exposure prior to mating, during gestation, and through weaning on male and female reproductive performance, mating behavior, conception, and prenatal and postnatal development of offspring. There are no appropriate animal substitutes (e.g., computer models, tissue/cell cultures) for the data that will be 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.

9.5 Animal Model and Species Justification:

Provide scientific justification for choice of animal model. Describe which physiological and morphological characteristics this animal possesses that make it the best possible model.

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). The genus *Peromyscus* is the most widespread and geographically variable of the North American rodents (Godin, 1977). Although using a wild-type animal model presents statistical challenges, they also give studies more power if effects are detected. If effects are seen with a wild-type animal model, they are likely real and population effects, not effects due to a single genetic trait or influence.

9.6 Source/Vendor:

Are all suppliers USDA-approved or are animals obtained from within the facility?

- ☒ Yes
☐ No

9.7 Does this protocol involve Field Studies or Field Research?

- ☐ Yes
☒ No

10.0 Refinement, Reduction, Replacement (3Rs)

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10.1 Refinement, Reduction, Replacement (3Rs):

Investigators are required to consider the 3 Rs when preparing an animal use research protocol. In the section below, describe all provisions in this protocol that refine, reduce, or replace the use of animals. Discuss what provisions were considered and why they were not chosen. You must address each section below.

Refinement:

Refinement refers to modifications of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress.

Every attempt will be made to socially house all animals. In instances of incompatibility and aggression between cage mates that cannot be remedied, animals may be separated and housed individually. If there are insufficient males or females to ensure 1:1 matings, an extra animal may be individually housed. All animals will be provided with a certified plastic-free form of enrichment (e.g., Nestlet) in their cage/pen. Individually housed animals may be provided with extra enrichment if it is plastic-free and PFAS-free.

Reduction:

Reduction involves strategies for obtaining comparable levels of information from the use of fewer animals or for maximizing the information obtained from a given number of animals(without increasing pain or distress) so that in the long run fewer animals are needed to acquire the same scientific information. This approach relies on an analysis of experimental design, applications of newer technologies, the use of appropriate statistical methods, and control of environmentally related variability in animal housing and study areas.

Animals on Animal Use Protocol Number 18-04-01 may be transferred to this protocol. The minimum number of animals for valid statistical analysis is being used.

Replacement:

Replacement refers to methods that avoid using animals. The term includes absolute replacements (i.e., replacing animals with inanimate systems such as computer programs) as well as relative replacements (i.e., replacing animals such as vertebrates with animals that are lower on the phylogenetic scale).

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

11.0 Procedures with Potential to Cause Pain or Distress

11.1 Which of the following Categories apply to procedures in this Protocol?

Check if used	USDA category	Category Description
☐	Category B	Number of animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but not yet used for such purposes
☐	Category C	Number of animals upon which teaching, research, experiments, or tests are conducted involving no pain, distress, or use of pain-relieving drugs.
☒	Category D	Number of animals upon which experiments, teaching, research, surgery, or tests are conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs were used.

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Check if used	USDA category	Category Description
☐	Category E	Number of animals upon which experiments, teaching, research, surgery, or tests are conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs would have adversely affected the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.

11.2 List all procedures that are anticipated to cause more than slight or momentary pain or distress (Category D or E) in animals assigned to this protocol.

Animals will be deeply anesthetized with CO₂, euthanized via decapitation, and trunk blood will be collected via exsanguination.

11.3 Literature Search for Alternatives to Procedures with Potential to Cause Pain or Distress:

* Add a new row for each source necessary.

	Source Searched	Date of Search	Dates covered by Search	Search Strategy for Keywords	Results of Search
+					
-					
	ALTBIB AnimAlt-ZEBET Agricola BIOSIS PubMed	9-10 MAY 2018 at Animal Welfare Act Workshop 25 June 2018	all years covered by databases	mouse or rat AND decapitation AND exsanguination AND pain or distress or alternatives	These literature searches identified hundreds of references pertaining to alternatives to painful procedures. However, no acceptable alternative to the painful or distressful procedures (e.g., decapitation and exsanguination) in this study were found. Although other methods exist for blood collection (e.g., saphenous vein, tail vein) for the laboratory rat and mouse, none of these alternative methods would allow collection of a sufficient volume of blood to perform the required analysis, particularly with the model species in this study. In a previous range finding study, the blood volume required could not be collected via the originally proposed method (i.e., cardiac puncture) despite the literature suggesting otherwise and many training sessions with the Study Staff and Veterinary Medical Office personnel. Anesthesia will be provided prior to decapitation and exsanguination.

11.5 Pain/Distress Relief or Prevention

Describe the methods or strategies intended to relieve or prevent pain or distress if the study will cause more than slight or

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momentary pain or distress. Specify agents to be used and when these agents will be given (pre-emptive or postprocedural). Include all agents that will be used for chemical restraint.

Analgesic	
Species	
Strain, if applicable	
Analgesic drug	
Route	
Dose	
Volume	
Frequency	
Duration	
Monitoring	
Reason for administration	

Add another Analgesic drug:

+

-

Use space below for additional comments or descriptions of **analgesics**, such as drug formulation, if required.

Anesthetic/Sedative/Tranquilizer	
Species	White-footed mouse
Strain, if applicable	
Anesthetic drug	Carbon dioxide (CO2)
Route	
Dose	
Volume	10-30% of the chamber volume will be replaced per minute
Frequency	
Duration	
Monitoring	Non-response to strong toe-pinch and to palpebral stimulation

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Reason for administration	Anesthesia prior to euthanasia via decapitation

Add another Anesthetic drug:

+

-

Use space below for additional comments or descriptions of **anesthetics**, such as drug formulation, if required.

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 in accordance with QSARC 800 (APHC, 2017b). 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.

11.6 Other Methods to Prevent or Alleviate Pain/Distress:

Examples include acupuncture, hydrotherapy, hot/cold packs, and removal from environment to prevent or alleviate distress:

N/A. No other procedures are expected to cause pain or distress.

Will a Pain and Distress Scoresheet be used as part of this project?

- ☐ Yes
- ☒ No

12.0 Paralytics

12.1 Will the project employ the use of neuromuscular blockades or paralytics?

- ☐ Yes
- ☒ No

13.0 Restraint Devices

13.1 Will the project employ the use of restraint devices?

- ☐ Yes
- ☒ No

14.0 Surgery

14.1 Will the project involve Surgery?

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☐ Yes

☒ No

15.0 Animal Manipulations

15.1 Injections

Injection	
Species	Peromyscus leucopus
Substance	Foreign red blood cell suspension
Route	IP
Dose	
Volume	0.1 mL
Frequency	Once, about one week prior to euthanasia
Expected effect	Incite immune response in ex vivo assay
Possible side effect	None

Add another Injected Substance:

+

-

15.2 Are any non-pharmaceutical grade compounds used in this protocol?

☒ Yes

☐ No

Provide scientific justification, including availability of human or animal formulations, and describe clinical use of all non-pharmaceutical grade formulations. Consider grade, purity, sterility, pH, pyrogenicity, osmolality, stability, site and route of administration, formulation, compatibility, and pharmacokinetics of the chemical or substance to be administered, as well as animal welfare and scientific issues relating to its use.

The compounds being tested are not available in pharmaceutical grade composition. The manufacturer will provide a statement of purity and stability, which may be verified by Army Public Health Center Laboratory Sciences if the techniques are developed and validated.

15.3 Are you using adjuvants?

☐ Yes

☒ No

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15.6 Will antibody production be performed?

- ☐ Yes
☒ No

15.9 Will you collect samples from living animals?

- ☐ Yes
☒ No

15.11 Will the project employ the use of genotyping?

- ☐ Yes
☒ No

15.12 Will the project employ the use of breeding?

- ☒ Yes
☐ No

For animals bred under this protocol, describe the breeding plan, including weaning age, age of retirement, explanation of breeding numbers, disposition of unwanted or retired animals, and how overcrowding will be prevented.

In order to produce the desired 20 litters, 31 pairs will be mated. The expected rate of mating with this species is 80%. The expected success rate of mating with this species is also 80%. One male and one female mating (not between siblings) will be used in this study. All animals will be virgins.

After four weeks of pre-mating dosing, one male will be placed in the home cage of one female from the same dose group. Unlike traditional rodent species, females cannot be examined for the presence of sperm or a vaginal plug in this species. Therefore, Day 0 of pregnancy cannot be confirmed in white-footed mouse. As such, 1:1 matings will be maintained (e.g., animals will be co-housed) until pups are born or 15 weeks have elapsed, at which point males will be removed from the study. In instances of incompatibility and aggression between cage mates that cannot be remedied, animals may be separated and housed individually. If there are insufficient males or females to ensure 1:1 matings, an extra animal may be individually housed.

Also unlike traditional rodent species, the white-footed mouse has small litters of between one and five pups, therefore, litter sizes will not be adjusted. As such, it is anticipated that overcrowding will not be an issue.

Will animals bred on this protocol be transferred and used on any other protocols?

- ☐ Yes
☒ No

15.13 Will the project employ the use of behavioral testing?

- ☐ Yes
☒ No

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15.17 Will the project employ other procedures not yet described in this protocol?

☒ Yes

☐ No

Describe other procedures:

Oral gavage: Animals will be gently restrained or "scruffed" by pinching the skin at the base of the neck, behind the ears, between the thumb and fingers. Oral gavage will be performed with an appropriately sized oral gavage needle or feeding tube for the species (e.g., 18 gauge needle, 1 inch), per The Guide (2011). The gavage needle is 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 is removed and the animal is briefly released before the procedure is attempted again. Once the material has been dispensed, the animal is briefly observed for any signs of aspiration. When dosing is complete, Study Staff will observe each animal for any signs of aspiration. 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.

IP injections: 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.

15.18 Will post-mortem tissues be collected in support of study objectives?

☒ Yes

☐ No

List tissues collected:

This tissue list may be altered at the discretion of the Pathologist and SD/PI based on observations at the time of necropsy.

Uterus (with oviducts and cervix, if possible)
Ovaries
Testes
Epididymides (total and cauda for the samples used for sperm counts)
Prostate (if possible)
Brain
Liver
Kidneys
Heart
Spleen
Thymus
Pituitary
Thyroid (trimmed and weighed post-fixation)
Adrenal glands

In addition to the organs listed above, samples of peripheral nerve, muscle, spinal cord, eye plus optic nerve, gastrointestinal tract, urinary bladder, lung, trachea (with thyroid and parathyroid attached), bone marrow, vas deferens, mammary gland (males and females), and vagina will be collected.

15.19 Will unused tissues from this protocol be shared with other investigators?

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☒ Yes

☐ No

List what tissues will be shared, with whom, and for what purpose. Describe any pathogen testing that will be conducted.

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

16.0 Study Endpoint

16.1 Endpoint Criteria:

State the projected study endpoint for animals, including a plan for disposition of surviving animals:

P generation males will be euthanized after 1) pups are born (and at least 10 weeks of exposure), or 2) after 15 weeks of 1:1 male:female matings. Timing of necropsy of P generation males is not critical and may be spread out as facility/personnel demands necessitate. P generation females will be euthanized at weaning (approximately PND 21-28). F1 generation animals will be euthanized at puberty (approximately PND60). In the event of gross abnormalities or malformations at birth that interfere with normal behaviors (e.g., eating, drinking, proper postural adjustments), F1 generation animals will be euthanized immediately.

16.2 Early Endpoints:

Describe early endpoints for animals. Examples include weight loss, loss of locomotion, significant lowering of body temperature, decreased food or water consumption, and decreased activity. If weight loss is used as a criterion for early removal from the study, frequency of weighing must be described.

Intervention euthanasia will be conducted on moribund animals, but P generation animals are not expected to become ill on this study, based on the literature and previous range finding studies. 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 (20% body weight loss compared to controls); emaciation; 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 Attending Veterinarian (AV) may be consulted to evaluate potentially moribund animals, unless the SD/PI plans to immediately euthanize the animal. Intervention euthanasia will be conducted on animals determined to be moribund.

16.3 Will death be used as an endpoint for any animals on this protocol?

☐ Yes

☒ No

17.0 Euthanasia

17.1 Will the project involve euthanasia of animals as part of the planned studies?

☒ Yes

☐ No

Euthanasia

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Species	White-footed mouse
Strain, if applicable	
Euthanasia method	Decapitation- Refs. QSARC 800 & QSARC 801
Route	
Dose	
Volume	
Method of ensuring death	n/a

Add another entry:

+

-

If applicable, for conditional methods of euthanasia, describe how those conditions will be met. For those methods NOT within the current AVMA guidelines, provide a description and scientific justification.

Adult animals will be rendered deeply unconscious with CO₂ prior to being decapitated. Adult animals will be non-responsive to strong toe-pinch and to palpebral stimulation. Decapitation and exsanguination must be performed because the blood volume required for analysis could not be collected via the originally proposed method (i.e., cardiac puncture) in previous range finding studies.

If warranted, neonatal pups (<7 days old) requiring euthanasia will be euthanized via decapitation without being rendered unconscious under CO₂ exposure. Pups older than 7 days will be exposed to CO₂ for an extended period of time to be rendered unconscious prior to decapitation. See references QSARC 800 (APHC 2017b) and QSARC 801 (APHC 2017c).

18.0 Animal Husbandry

18.1 Special Husbandry and Procedures:

Are special husbandry practices required which are outside institute standard practice?

Examples of special husbandry practices may include change from standard food or water, withholding food or water, change from standard bedding, change from standard cage change frequency, temporary withholding of enrichment, use of metabolic cages.

- ☒ Yes
☐ No

Describe special husbandry practices below:

Food consumption for all study animals will be monitored based on weight of the food hopper. Therefore, food for all study animals will be provided by study staff in exception of QSARC SOP 707 (APHC, 2017d). Food enrichment may not be used due to food consumption monitoring.

Enrichment devices made from plastic or nylon (e.g., Nylabones, rodent retreats, rodent tubes) are unacceptable,

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given the compounds under investigation.

18.2 Will animal studies take place outside the vivarium?

- ☐ Yes
☒ No

18.4 Will other than standard housing methods be required?

- ☐ Yes
☒ No

18.5 Will exceptions to or deviations from IACUC-approved environmental enrichment practices, including single housing, be required?

- ☒ Yes
☐ No

Describe enrichment strategy and provide scientific justification if environmental enrichment is not provided. Animal monitoring should include observations for abnormal behaviors (such as self-injurious behavior or stereotypies). Include expected duration of exceptions or deviations.

Enrichment devices made from plastic or nylon (e.g., Nylabones, rodent retreats, rodent tubes) are unacceptable, given the compounds under investigation.

Every attempt will be made to socially house all animals. In instances of incompatibility and aggression between cage mates that cannot be remedied, animals may be separated and housed individually. If there are insufficient males or females to ensure 1:1 matings, an extra animal may be individually housed.

18.6 Do you need to house more than the allowable number of animals in a single enclosure?

- ☐ Yes
☒ No

19.0 Veterinary Medical Care

19.1 Describe any veterinary care that is NOT in accordance with the Institute's program of veterinary medical care.

N/A

20.0 Exceptions/Justifications

20.1 Check all applicable:

- ☐ Use of Paralytics
☐ Restraint Devices
☐ Prolonged Restraint (e.g.; >12 hrs in nonhuman primates)

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IACUC number (03 -) 18-08-01

IACUC approval date 13 August 2018

IACUC expiration date 13 August 2021

- ☐ Multiple Survival Surgery
- ☐ Multiple Major Survival Surgery
- ☒ Use of Non-Pharmaceutical Grade Chemicals
- ☐ Use of Complete Freund's Adjuvant
- ☐ Death as an Endpoint
- ☐ Food/Water Restriction
- ☒ Restriction of Environmental Enrichment, including Single Housing
- ☐ Other (Describe and justify below)

Deviations/exceptions MUST be justified by the P.I. If not listed elsewhere, all items that require scientific justification must be listed here.

See 15.2.

21.0 Biohazard/Safety

21.1 Will biohazardous material be used as part of this protocol?

- ☒ Yes
- ☐ No

List biohazardous agents and describe safety precautions, programs designed to protect personnel, and surveillance procedures in place to monitor potential exposures. Examples include viral/bacterial agents, toxins, radioisotopes, oncogenic viruses, chemical carcinogens, recombinant DNA.

Risks associated with this study include bites/scratches/needle sticks, transmission or zoonotic diseases, the development of animal allergies, and the potential exposure to PFAS.

21.2 List other safety considerations specific to this protocol, such as waste anesthetic gases and/or equipment that poses a potential threat (e.g.; lasers, imaging).

N/A

21.3 Other Considerations:

Describe any other considerations not addressed previously.

N/A

22.0 Attachments

22.1 Would you like to add attachments to this document?

Examples of attachments include pain and distress score sheets, flow charts, wildlife permits, or other documents associated with this protocol.

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Version 2.0, 10/2017

IACUC number (03 -) 18-08-01

IACUC approval date 13 August 2018

IACUC expiration date 13 August 2021

☒ Yes

☐ No

22.2 If yes, Please attach files.

Attach Files

23.0 Assurances

The law specifically requires several written assurances from the P.I. It states that "research facilities will be held responsible if it is subsequently determined that an experiment is unnecessarily duplicative, and that a good faith review of available sources would have indicated as much." No response is required under the title paragraph of this section.

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.

B. Duplication of Effort: I have made a reasonable, good faith 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 statistician to evaluate the statistical design or strategy of this proposal, and that the minimum number of animals needed for scientific validity are used.

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

E. Training: I verify that the personnel performing the animal procedures/manipulations 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 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" which the DoD has embraced, namely, "Responsibility" 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.

Principal Investigator signature

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.08.15 08:42:19 -04'00'

Date Aug 15, 2018

Painful Procedures:

I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals that WILL be relieved with the use of anesthetics, analgesics, and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

For IACUC use only

IACUC number (03 -) 18-08-01

DOD E-IACUC protocol format
Version 2.0, 10/2017

IACUC approval date 13 August 2018

IACUC expiration date 13 August 2021

Principal Investigator signature

JACKOVITZ.ALLISON.MARIE.1367161236 Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.08.15 08:43:11 -04'00'

For IACUC use only

DOD E-IACUC protocol format
Version 2.0, 10/2017

IACUC number (03 -) 18-08-01

IACUC approval date 13 August 2018

IACUC expiration date 13 August 2021

ADDENDUM 1: References

- Laboratory Animal Data: Quick Reference Guide, 2nd edition (2014). American Association for Laboratory Animal Science.
- APHC (2017a). QSARC 804. Individual Animal Identification (Aberdeen Proving Ground, MD).
- APHC (2018). QSARC 812. Use of Top-Loading Balances for Animal Weighing (Aberdeen Proving Ground, MD).
- APHC (2017b). QSARC 800. Animal Euthanasia (Aberdeen Proving Ground, MD).
- APHC (2017c). QSARC 801. Use and Maintenance of Decapitation Equipment (Aberdeen Proving Ground, MD).
- APHC (2017d). QSARC 707. Animal Health Technician Animal Husbandry and Vivarium Duties (Aberdeen Proving Ground, MD).
- 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.
- Biser JA, Vogel LA, Berger J, Hjelle B, Loew SS (2004). Effects of heavy metals on immunocompetence of white-footed mice (*Peromyscus leucopus*). *Journal of Wildlife Disease*; 40(2): 173-184.
- Butenhoff JL, Ehresman DJ, Chang S, Parker GA, Stump DG (2009). Gestational and lactational exposure to potassium perfluorooctanesulfonate in rats: Developmental neurotoxicity. *Reproductive Toxicology*; 27: 319-330.
- Chang S, Butenhoff JL, Parker GA, Coder PS, Zitzoq JD, Krisko RM, Bjork JA, Wallace KB, Seed JG (2018). Reproductive and developmental toxicity of potassium perfluorohexanesulfonate in CD-1 mice. *Reproductive Toxicology*; 78: 150-168.
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- Fenton SE, Hamm JT, Birnbaum LS, and Youngblood GL (2002). Persistent abnormalities in the rat mammary gland following gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Toxicological Sciences*, 67: 63-74.

Fuentes S, Colomina MT, Rodriguez J, Vicens P, Domingo JL (2006). Interactions in developmental toxicology: Concurrent exposure to perfluorooctane sulfonate (PFOS) and stress in pregnant mice. *Toxicology Letters*; 164: 81-89.

Godin AJ. (1977). *Wild Mammals of New England*. (Johns Hopkins University Press).

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Johnson MS, Ferguson JW, Holladay SW (2000). Immune effects of oral 2,4,6-dinitrotoluene (TNT) exposures to the white-footed mouse, *Peromyscus leucopus*. *International Journal of Toxicology*, 19: 5-11.

Lieder PH, Chang S, York RG, Butenhoff JL (2009). Toxicological evaluation of potassium perfluorobutanesulfonate in a 90-day oral gavage study with Sprague-Dawley rats. *Toxicology*; 255: 45-52.

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Organisation for Economic Co-Operation and Development (OECD) (2011). Test No. 443: Extended One-Generation Reproductive Toxicity Test, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris.

Organisation for Economic Co-Operation and Development (OECD) (2016). Test No. 421: Reproduction/Developmental Toxicity Screening Test, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris.

Organisation for Economic Co-Operation and Development (OECD) (2008). Environment, Health, and Safety Publications Series on Testing and Assessment No. 42: Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment.

Rattner BA and Hoffman DJ (1984). Comparative toxicity of acephate in laboratory mice, white-footed mice, and meadow voles. *Archives of Environmental Contamination and Toxicology* 13: 483-491.

Reddy G, Reddy TV, Choudhury H, Daniel FB, Leach GJ (1997). Assessment of environmental hazards of 1,3,5-trinitrobenzene (TNB). *Journal of Toxicology and Environmental Health*, 52: 101-114.

Song P, Li D, Wang W, Zhong X (2018). Effects of perfluorooctanoic acid exposure during pregnancy on the reproduction and development of male offspring. *Andrologia*.

Tice RR, Onniston BG, Boucher R, Luke CA, Paquette DE (1987). Environmental Biomonitoring with Feral Rodent Species. In: *Short-Term Bioassays in the Analysis of Complex Environmental Mixtures*. V. Sandu, D.M. DeMarini, M.J. Mass, M.M. Moore, J.S. Mumford, eds. (Plenuni Press, New York, NY).

ADDENDUM 2: Good Laboratory Practices Protocol Requirements

PROJECT SPONSOR:

Dr. Andrea Leeson
Deputy Director & Environmental Restoration Program Manager of SERDP
4800 Mark Center Drive, Suite 17D08
Alexandria, VA 22350-3605

TESTING FACILITY:

Army Public Health Center
Aberdeen Proving Ground, MD 21010-5403

PROPOSED START DATE: September 2018

PROPOSED END DATE: July 2021

IDENTIFICATION OF THE TEST SYSTEM: Test system will be identified as follows:

Specimens shall be identified by test system, study, nature, and date of collection. This information shall be located on the specimen container or shall accompany the specimen in a manner that precludes error in the recording and storage of data.

SOURCE/VENDOR:

Peromyscus Genetic Stock Center, University of South Carolina, Columbia, SC 29208;
USDA license number 56-R-0003

HISTOPATHOLOGY:

F0 GENERATION: At the time of termination or premature death, all animals will be necropsied and examined macroscopically for any structural abnormalities or pathological changes, paying special attention to the organs of reproductive development, where appropriate. For all females, uteri will be examined for the presence and number of implantation sites and ovaries will be examined for the number or corpora lutea, as possible. Wet weights of the organs listed below (tissue list may be altered at the discretion of the pathologist and SD/PI based on observations at the time of necropsy) from all F0 generation animals will be determined as soon as possible after dissection to avoid drying. If organs cannot be weighed immediately, they may be placed in a weigh-boat and a moist paper towel used to cover the weigh-boat, but the organs should not be allowed to come into contact with water. A single testis and epididymis from each animal (either left or right, but the same side from all animals) 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. All other organs will be placed in 10% buffered formalin for at least 24 hours for fixation.

Sperm parameters may be measured in all F0 generation males. After wet weight of the epididymides is determined as described above, at least one epididymis (either left or right, but the same side from all animals) will be reserved for histopathological examination. The remaining epididymis will be used for enumeration of cauda epididymis sperm reserves, sperm motility, and morphology.

F1 GENERATION: At the time of termination or premature death, all animals will be necropsied and examined macroscopically for any structural abnormalities or pathological changes, paying special attention to the organs of reproductive development, where appropriate. Wet weights of organs listed below (tissue list may be altered at the discretion of the pathologist and SD/PI based on observations at the time of necropsy) from all F0 generation animals will be determined as soon as possible after dissection to avoid drying. If organs cannot be weighed immediately, they may be placed in a weigh-boat and a moist paper towel used to cover the weigh-boat, but the organs should not be allowed to come into contact with water. A single testis and epididymis from each animal (either left or right, but the same side from all animals) 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. All other organs will be placed in 10% buffered formalin for at least 24 hours for fixation.

Sperm parameters may be measured in all F1 generation males. After wet weight of the epididymides is determined as described above, at least one epididymis (either left or right, but the same side from all animals) will be reserved for histopathological examination. The remaining epididymis will be used for enumeration of cauda epididymis sperm reserves, sperm motility, and morphology.

Records of gross findings for a specimen from postmortem observations shall be available to a pathologist when examining that specimen histopathologically.

STUDY CONDUCT AND RECORD KEEPING:

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

Animal use protocol: Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

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.

All data generated during the conduct of a study, except those that are generated by automated data collection systems, shall be recorded directly, promptly, and legibly in ink. All data entries shall be dated on the day of entry and signed or initialed by the person entering the data. Any change in entries shall be made so as not to obscure the original entry, shall indicate the reason for such change, and shall be dated and signed or identified at the time of the change. In automated data collection systems, the individual responsible for direct data input shall be identified at the time of data input. Any change in automated data entries shall be made so as not to obscure the original entry, shall indicate the reason for change, shall be dated, and the responsible individual shall be identified.

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

APHC IACUC Protocol Modification Review/Approval

Study Director/ Principal Investigator: Allison Jackovitz

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 1

Submission Date: Oct 16, 2018

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.10.16 12:42:25 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 16 Oct 18

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.10.16 14:28:22 -04'00'

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

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2018.10.16 14:51:37 -04'00'

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.10.24 13:32:42 -04'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☐ YES ☒ NO ☐

Oct 16, 2018

Date

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2018.10.16 14:50:16 -04'00'

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Oct 24, 2018

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.10.24 13:33:18 -04'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Oct 24, 2018

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 1

Submission Date: 16 October 2018

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

PFAS are compounds manufactured for use in paints, cleaning agents, non-stick cookware and food containers, and water-impermeable products. Concerns about PFAS stem from their ubiquitous presence in the environment, widespread reports of general toxicity, and the resistance of these compounds to degradation. Data from this study will be used to derive Toxicity Reference Values (TRVs). TRVs are critical components of environmental risk assessments that help determine if the risk of environmental exposure is acceptable.

In this study, six PFAS will be administered daily to male and female white-footed mice (*Peromyscus leucopus*) in six separate experiments to evaluate the effects of combined pre- and postnatal exposure to PFAS on development and reproduction. Parental generation animals will be dosed with the test compounds by oral gavage for 10-12 weeks (based on sex). Fetuses and offspring will only be exposed to the test compounds through pregnancy and maternal transfer (lactation) and will be maintained on the study through puberty. All animals will be monitored for body weight changes and signs of toxicity. Immune function will be evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood will be collected from anesthetized animals via decapitation and trunk blood collection.

Blood will be analyzed for chemical concentration, hormones, etc., as available. Selected tissues will be weighed and processed for histopathology. Immunological organs (thymus and spleen) may also be processed and evaluated for immune effects. This study will provide information about the effects of PFAS on the adult male and female reproductive system. Examination of physical and functional development following combined pre- and postnatal exposure is expected to identify specific target organs in the offspring and may reveal effects not seen with more abbreviated exposures.

This modification seeks to add Rebecca Kilby and Caroline Procell as personnel qualified to deliver compound via oral gavage. Additionally, this modification seeks to transfer as many as 20 *peromyscus* from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species.

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	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)]	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: _____	
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
N/A	N/A	N/A	N/A

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,208 **B:** 0 **C:** 3,720 **D:** 1,488 **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):

See 5.G.

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]*).

One of the primary effects in laboratory animals exposed to PFAS is altered immune function. By piloting the foreign red blood cell challenge, we can evaluate feasibility, time involvement, and adverse events, and improve upon the study design (if necessary) prior to evaluating immune function in peromyscus exposed to PFAS. To do so, as many as 20 peromyscus may be transferred from 18-04-01 to 18-08-01. The immune challenge will be performed as described in 9.1. Animals will receive an intraperitoneal (IP) injection as described in 15.1 and 15.17. Animals will be anesthetized with CO2 and euthanized via decapitation as described in 11.5 and 17.1, respectively. Tissues will be collected as described in 15.18.

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.)*

New qualified personnel: Rebecca Kilby

Activities to be performed on this protocol: Oral gavage

Formal oral gavage training with lab mice: 7 September 2018 ; 2 October 2018

Formal oral gavage training with peromyscus: 10, 11 September 2018, 2 October 2018

Qualifications and Experience: ALAT, RVT, B.S., Organizational Management, 25+ years animal research experience

New qualified personnel: Caroline Procell

Activities to be performed on this protocol: Oral gavage

Formal oral gavage training with lab mice: 20, 21, 22 August 2018; 5, 6, 7 September 2018; 2 October 2018

Formal oral gavage training with peromyscus: 10, 11 September 2018, 2 October 2018

Qualifications and Experience: B.S., Biology, 3+ years animal experience

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: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 2

Submission Date: Nov 2, 2018

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.11.02 12:14:08 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 2 Nov 18

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by
SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.11.05 10:06:45 -05'00'

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

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

Date

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2018.11.07 14:34:25 -05'00'

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Nov 7, 2018

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2018.11.07 21:05:37 -05'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Nov 7, 2018

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 2

Submission Date: 2 November 2018

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

PFAS are compounds manufactured for use in paints, cleaning agents, non-stick cookware and food containers, and water-impermeable products. Concerns about PFAS stem from their ubiquitous presence in the environment, widespread reports of general toxicity, and the resistance of these compounds to degradation. Data from this study will be used to derive Toxicity Reference Values (TRVs). TRVs are critical components of environmental risk assessments that help determine if the risk of environmental exposure is acceptable.

In this study, six PFAS will be administered daily to male and female white-footed mice (*Peromyscus leucopus*) in six separate experiments to evaluate the effects of combined pre- and postnatal exposure to PFAS on development and reproduction. Parental (P) generation animals will be dosed with the test compounds by oral gavage. Fetuses and offspring will only be exposed to the test compounds through pregnancy and maternal transfer (lactation) and will be maintained on the study through puberty. All animals will be monitored for body weight changes and signs of toxicity. Immune function will be evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood will be collected from anesthetized animals via decapitation and trunk blood collection.

Blood will be analyzed for chemical concentration, hormones, etc., as available. Selected tissues will be weighed and processed for histopathology. Immunological organs (thymus and spleen) may also be processed and evaluated for immune effects. This study will provide information about the effects of PFAS on the adult male and female reproductive system. Examination of physical and functional development following combined pre- and postnatal exposure is expected to identify specific target organs in the offspring and may reveal effects not seen with more abbreviated exposures.

This modification seeks to adjust the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning. Thus far in the study, all sires and dams appear to be co-parenting pups without signs of aggression. Rather than potentially destabilizing a co-parenting system and adding stress, this modification seeks to maintain sires in the same cage as the dams and pups through weaning. As such, 1:1

matings will be maintained (e.g., animals will be co-housed) through weaning or until 15 weeks of mating has elapsed where no pups have been produced and where there is no evidence of pregnancy.

This modification also seeks to adjust how long P generation males are exposed to PFAS or vehicle control. In the currently approved protocol, P generation males will be exposed to PFAS or vehicle control for at least 10 weeks: four weeks prior to mating, the variable time to mating and conception (up to 12 weeks), the duration of pregnancy (about three weeks), and after the mating period (as needed to ensure at least 10 weeks of dosing). Since this modification seeks to maintain sires with dams and pups through weaning, sires will also be dosed during this period to prevent "recovery." As such, P generation animals of both sexes will be exposed to PFAS or vehicle control for at least 10 weeks (four weeks prior to mating, three weeks of gestation, and three weeks through weaning). The maximum duration of dosing will depend on how quickly (and if) pups are generated. The protocol states that if there are insufficient males, for example due to male death before mating, then male(s) which have already mated may be paired (1:1) with a second female from the same dose group such that all females are paired. This will also be applied if a male from a pair dies before pups are produced. Therefore, P generation animals could be exposed to PFAS or vehicle control for as many as 40 weeks if they are producing multiple litters. Given the potential for a quite lengthy period of exposure, all animals will be closely monitored for signs of toxicity. At the conclusion of exposure, P generation animals will be removed from the study.

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)	X
	Other: _____	
MAJO	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	Transfer of animals from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species. Addition of two qualified personnel to deliver compound via oral gavage.	20 Peromyscus leucopus	24 October 2018

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,208 **B:** 0 **C:** 3,740 **D:** 1,488 **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).**

9.0 Materials and Methods

9.1 Experimental Design and General Procedures:

B. EXPOSURE DURATION: As a modification to the currently approved protocol, P generation animals will be dosed daily with the test article for four weeks prior to mating, the variable time to mating and conception (up to 12 weeks), the duration of pregnancy (about three weeks), and through weaning (approximately PND 21-28). Since this modification also seeks to maintain sires with dams and pups through weaning, sires will be dosed during this period to prevent "recovery." After 15 weeks of 1:1 male:female matings have elapsed where no pups have been produced and where there is no evidence of pregnancy, P generation animals will be removed from the study. As stated in the currently approved protocol, fetuses and pups will be exposed via maternal transfer. At weaning, dams and sires will be removed from the study. First (F1) generation animals will be maintained through puberty (approximately PND60).

C. CONTROLS: As a modification to the currently approved protocol, P generation animals will be dosed daily with the vehicle control for four weeks prior to mating, the variable time to mating and conception (up to 12 weeks), the duration of pregnancy (about three weeks), and through weaning (approximately PND 21-28). Since this modification also seeks to maintain sires with dams and pups through weaning, sires will be dosed during this period to prevent "recovery." After 15 weeks of 1:1 male:female matings have elapsed where no pups have been produced and where there is no evidence of pregnancy, P generation animals will be removed from the study. As stated in the currently approved protocol, fetuses and pups will be exposed via maternal transfer. At weaning, dams and sires will be removed from the study. First (F1) generation animals will be maintained through puberty (approximately PND60).

BREEDING: As stated in the currently approved protocol, one male and one female mating (not between siblings) will be used in this study. All animals will be virgins. After four weeks of pre-mating dosing, one male will be placed in the home cage of one female from the same dose group. Unlike traditional rodent species, females cannot be examined for the presence of sperm or a vaginal plug in this species. Therefore, Day 0 of pregnancy cannot be confirmed in white-footed mouse. As a modification to the currently approved protocol, 1:1 matings will be maintained (e.g., animals will be co-housed) until pups are weaned or until 15 weeks have elapsed where no pups have been produced and where there is no evidence of pregnancy. Thus far in the study, all sires and dams appear to be co-parenting pups without signs of aggression. Rather than potentially destabilizing a co-parenting system and adding stress, this modification seeks to maintain sires in the same cage as the dams and pups through weaning. As stated in the currently approved protocol, if there are insufficient males, for example due to male death before mating, then male(s) that have already mated and co-parented pups through weaning may be paired (1:1) with a second female(s) such that all females are paired. If there are insufficient females, females will not be paired with a second male.

LITTERS: As stated in the currently approved protocol, each litter will be examined as soon as possible after delivery to establish the number of pups, stillbirths, live births, runts (pups that are significantly smaller than corresponding control pups), and the presence of gross

abnormalities. Live pups will be counted and litters weighed within 24 hours of parturition (day 0 or 1 post-partum) and at least on day 4 and 13 post-partum. Abnormal behavior in the offspring will be recorded. Classic developmental landmarks (e.g., ear opening, eye opening, nipple retention) will be observed, as possible. Unlike reproductive and/or developmental toxicity testing with traditional rodent species, the white-footed mouse has small litters of between one and five pups, therefore, litter sizes will not be adjusted. As a modification to the currently approved protocol, litters will be maintained with dams and sires (as possible) through weaning. Thus far in the study, all sires and dams appear to be co-parenting pups without signs of aggression. Rather than potentially destabilizing a co-parenting system and adding stress, this modification seeks to maintain sires in the same cage as the dams and pups through weaning.

15.0 Animal Manipulations

15.12 Breeding: As stated in the currently approved protocol, in order to produce the desired 20 litters, 31 pairs will be mated. The expected rate of mating with this species is 80%. The expected success rate of mating with this species is also 80%. One male and one female mating (not between siblings) will be used in this study. All animals will be virgins.

As stated in the currently approved protocol, after four weeks of pre-mating dosing, one male will be placed in the home cage of one female from the same dose group. Unlike traditional rodent species, females cannot be examined for the presence of sperm or a vaginal plug in this species. Therefore, Day 0 of pregnancy cannot be confirmed in white-footed mouse. As a modification to the currently approved protocol, 1:1 matings will be maintained (e.g., animals will be co-housed) until pups are weaned or until 15 weeks have elapsed where no pups have been produced and where there is no evidence of pregnancy. Thus far in the study, all sires and dams appear to be co-parenting pups without signs of aggression. Rather than potentially destabilizing a co-parenting system and adding stress, this modification seeks to maintain sires in the same cage as the dams and pups through weaning. As stated in the currently approved protocol, in instances of incompatibility and aggression between cage mates that cannot be remedied, animals may be separated and housed individually. If there are insufficient males or females to ensure 1:1 matings, an extra animal may be individually housed.

As stated in the original protocol, unlike traditional rodent species, the white-footed mouse has small litters of between one and five pups, therefore, litter sizes will not be adjusted. As such, it is anticipated that overcrowding will not be an issue.

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.**

16.0 Study Endpoint

16.1 Endpoint Criteria: The endpoint criteria in this study is not changing, rather the timing to the endpoint for P generation animals is being extended. As a modification to the currently approved protocol, P generation animals will be euthanized 1) after pups are weaned or 2) after 15 weeks of 1:1 male:female matings have elapsed where no pups have been produced and where there is no evidence of pregnancy. Therefore, P generation animals could be exposed to PFAS or vehicle control for as many as 40 weeks if they are producing multiple litters. Given the potential for a quite lengthy period of exposure, all animals will be closely monitored for signs of toxicity. As stated in the currently approved protocol, F1 generation animals will be euthanized at puberty (approximately PND60). In the event of gross abnormalities or malformations at birth that interfere with normal behaviors (e.g., eating, drinking, proper postural adjustments), F1 generation animals will be euthanized immediately.

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: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 3

Submission Date: Nov 26, 2018

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.11.26 16:38:27 -05'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 27 Nov 18

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.11.27 11:26:51 -05'00'

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

GEHLING.ALICIA.MARIE.1364571689

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Date: 2018.11.27 12:14:49 -05'00'

NEWKIRK.KRISTIN.TORELL.1014786895

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Date: 2018.11.27 13:00:34 -05'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☐ YES ☒ NO ☐

GEHLING.ALICIA.MARIE.1364571689

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Date: 2018.11.27 12:15:01 -05'00'

Date

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Nov 27, 2018

NEWKIRK.KRISTIN.TORELL.1014786895

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Date: 2018.11.27 13:01:13 -05'00'

Date

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Nov 27, 2018

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 3

Submission Date: 26 November 2018

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

PFAS are compounds manufactured for use in paints, cleaning agents, non-stick cookware and food containers, and water-impermeable products. Concerns about PFAS stem from their ubiquitous presence in the environment, widespread reports of general toxicity, and the resistance of these compounds to degradation. Data from this study will be used to derive Toxicity Reference Values (TRVs). TRVs are critical components of environmental risk assessments that help determine if the risk of environmental exposure is acceptable.

In this study, six PFAS will be administered daily to male and female white-footed mice (*Peromyscus leucopus*) in six separate experiments to evaluate the effects of combined pre- and postnatal exposure to PFAS on development and reproduction. Parental (P) generation animals will be dosed with the test compounds by oral gavage. Fetuses and offspring will only be exposed to the test compounds through pregnancy and maternal transfer (lactation) and will be maintained on the study through puberty. All animals will be monitored for body weight changes and signs of toxicity. Immune function will be evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood will be collected from anesthetized animals via decapitation and trunk blood collection.

Blood will be analyzed for chemical concentration, hormones, etc., as available. Selected tissues will be weighed and processed for histopathology. Immunological organs (thymus and spleen) may also be processed and evaluated for immune effects. This study will provide information about the effects of PFAS on the adult male and female reproductive system. Examination of physical and functional development following combined pre- and postnatal exposure is expected to identify specific target organs in the offspring and may reveal effects not seen with more abbreviated exposures.

Currently, one experiment with one chemical is underway. After 8 weeks of 1:1 matings, only 18.5% of pairs have produced litters, which is fewer than anticipated. According to the breeder (the *Peromyscus* Genetic Stock Center), 80% of 1:1 matings should copulate and of those, 80% should produce litters. Therefore, the Primary Investigator (PI) investigated possible causes and found that unlike traditional rodent species, which are maintained on a 24 hour

light cycle with a photoperiod duration of 12 hours of illumination (QSARC SOP 707, Animal Health Technician Animal Husbandry and Vivarium Duties), *Peromyscus* should be maintained on a 24 hour light cycle with a photoperiod duration of 16 hours of illumination (Crossland 2006). In the currently approved protocol, special husbandry practices (e.g., practices in exception to QSARC SOP 707) are that food for all study animals will be provided by the study staff and that enrichment devices made from plastic or nylon are unacceptable given the compounds under investigation. This modification seeks to add an additional special husbandry practice: to adjust the light cycle to a 24 hour light cycle with a photoperiod duration of 16 hour of illumination, with lights on at 0400 and off at 2000 daily. This modification also includes a transition plan for increasing the illumination period over the course of four days.

It is well documented that photoperiods have significant effects on breeding success in rodents, birds, cattle, sheep, etc. (Nelson, Demas, and Klein, 1998; Dawson et al., 2001; Dahl, Buchanan, and Tucker 2000; Bittman, Karsch, and Hopkins 1983). Furthermore, a short day-length has been shown to trigger reproductive suppression in this species (Crossland et al., 2014). Given that lighting has a significant impact on breeding, that the light cycle currently in place is not best suited for the animal model in this protocol, that breeding is reduced compared to what was anticipated, and that the purpose of this study is to evaluate the effects of combined pre- and postnatal exposure to PFAS on development and reproduction, adjusting the light cycle immediately is critical to meeting the study objectives.

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	
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	X
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: _____	
MAJOR	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	Transfer of animals from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species. Addition of two qualified personnel to deliver compound via oral gavage.	20 Peromyscus leucopus	24 October 2018
2	Adjust the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning	N/A	08 November 2018

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,208 **B:** 0 **C:** 3,740 **D:** 1,488 **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:

Bittman EL, Karsh FJ, Hopkins JW. (1983). Role of the Pineal Gland in Ovine Photoperiodism: Regulation of Seasonal Breeding and Negative Feedback Effects of Estradiol upon Luteinizing Hormone Selection. *Endocrinology*; 113(1): 329-336.

Crossland JP. (2006). *Peromyscus* - A fascinating laboratory animal model. *Tech Talk*; 11, 1-2.

Crossland JP, Dewey MJ, Barlow SC, Vrana PB, Felder MR, and Szalai GJ. (2014). Caring for *Peromyscus* spp. in research environments. *Lab Animal*; 43(5): 162-166.

Dahl GE, Buchanan BA, Tucker HA. (2000). Photoperiodic effects on dairy cattle: A review. *Journal of Dairy Science*; 83(4): 885-893.

Dawson A, King VM, Bentley GE, Ball GF. (2001). Photoperiodic control of seasonability in birds. *Journal of Biological Rhythms*; 16(4): 365-380.

Nelson RJ, Demas GE, Klein SL. (1998). Photoperiodic mediation of seasonal breeding and immune function in rodents: A Multi-factorial approach. *American Zoology*; 38: 226-237.

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.)

18.0 Animal Husbandry

18.1 Special Husbandry and Procedures. The special husbandry practices in this study are not changing, rather one is being added. In the currently approved protocol, special husbandry practices are limited to 1) food for all study animals will be provided by the study staff in exception to QSARC SOP 707 and 2) enrichment devices made from plastic or nylon are unacceptable given the compounds under investigation. As a modification to the currently approved protocol, the light cycle will also be an exception to what is listed in QSARC SOP 707.

According to QSARC 707, "Rodent and most mammalian animal rooms will be set to a 24 hour light cycle with a photoperiod duration of 12 hours illumination, with lights on at 0600 at off at 1800 daily." However, *Peromyscus leucopus* should be maintained on a 24 hour light cycle with a photoperiod duration of 16 hours of illumination, which is especially important for breeding (Crossland 2006; Crossland et al., 2014). Given that the purpose of this study is to evaluate the effects of combined pre- and postnatal exposure to PFAS on development and reproduction, optimizing breeding conditions is critical to meeting the study objectives. Therefore, as a modification to the currently approved protocol, animals will be adjusted to a 24 hour light cycle with a photoperiod duration of 16 hour of illumination, with lights on at 0400 and off at 2000 daily.

To accomplish the transition from a 24 hour light cycle with a photoperiod duration of 12 hours illumination to a 24 hour light cycle with a photoperiod duration of 16 hours of illumination, animals will receive 30 additional minutes of illumination in the morning and the evening for four days in a row. On day one of the transition, the illumination period will be from 0530 to 1830. On day two of the transition, the illumination period will be from 0500 to 1900. On day three of the transition, the illumination period will be from 0430 to 1930. On day four of the transition (and going forward), the illumination period will be from 0400 to 2000.

Given that animals (in the first of six separate experiments of this study) have already received the test article for 12 weeks and have been in 1:1 matings for 8 of 15 possible weeks, the transition plan is less gradual/more aggressive than what may be recommended. However, this modification, including the transition plan, is the most appropriate way to get meaningful data from the animals. The exposure duration should not be extended to allow for animals to acclimate after the transition; animals are already being dosed for an extended period of time and by extending the exposure duration, more toxicity could occur. Furthermore, many animals on the study are already reaching their natural end of life and may not survive a longer exposure duration, which could reduce the sample size and thus compromise statistical power.

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: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 4

Submission Date: Dec 28, 2018

Study Director/ Principal
Investigator Signature:

JACKOVITZ.ALLISON.MARIE.1367161236

Digitally signed by JACKOVITZ.ALLISON.MARIE.1367161236
Date: 2018.12.28 13:45:03 -05'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 28 Dec 18

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2018.12.28 16:05:37 -05'00'

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

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.01.02 08:51:30 -05'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☐ YES ☒ NO ☐

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.01.02 08:51:45 -05'00'

Date

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Jan 2, 2019

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2019.01.02 11:09:58 -05'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Jan 2, 2019

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 4

Submission Date: 28 December 2018

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

In this study, PFAS is administered daily to male and female white-footed mice (*Peromyscus leucopus*) to evaluate the effects of combined pre- and postnatal exposure to PFAS on development and reproduction. Parental (P) generation animals are dosed with the test compounds by oral gavage. Fetuses and offspring are only exposed to the test compounds through pregnancy and maternal transfer (lactation) and then maintained on the study through puberty. All animals are monitored for body weight changes and signs of toxicity. Immune function is evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood is collected from anesthetized animals via decapitation and trunk blood collection.

This study will provide information about the effects of PFAS on the adult male and female reproductive system. Examination of physical and functional development following combined pre- and postnatal exposure is expected to identify specific target organs in the offspring and may reveal effects not seen with more abbreviated exposures.

Currently, one experiment with one chemical is underway. The remaining five compounds have not been started. The original protocol stated that sires would be removed from the home cage when pups were born, but thus far in the first experiment, all sires and dams are co-parenting pups without signs of aggression. Therefore, rather than potentially destabilizing a co-parenting system and adding stress, a previously approved modification adjusted the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning at approximately postnatal day 25 (PND25). However, rebreeding has occurred in the course of the first experiment and several P generation females have been pregnant at PND25 (i.e., when they were removed from the study, euthanized, and tissues were collected). Therefore, this modification clarifies how to handle the pups in those cases.

Thus far, second litters from 1:1 mating have not been produced; however, if a second litter is produced, this modification also clarifies that pups will be anesthetized with CO₂ and euthanized via decapitation within 24 hours of being found if they are born before the dam is removed from the study. Because first litters are generally smaller than subsequent litters in this model species, data from first and second litters must be analyzed separately. As such,

there is not an added benefit to maintaining animals from second litters on study or to allowing pregnant females scheduled for necropsy to give birth to second litters.

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	
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	
	Changes in dosing procedures (e.g., dose, volume, or timing)	
	Other: <u>Clarification as to how a second litter (inadvertently generated by dams and sires co-parenting a first litter) will be handled.</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	Transfer of animals from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species. Addition of two qualified personnel to deliver compound via oral gavage.	20 Peromyscus leucopus	24 October 2018
2	Adjust the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning	N/A	08 November 2018
3	Adjust the 24 hour light cycle to photoperiod with a 16 hour duration of illumination	N/A	27 November 2018

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,208 **B:** 0 **C:** 3,740 **D:** 1,488 **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:

AVMA Guidelines for Euthanasia of Animals: 2013 Edition

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

15.0 Animal Manipulations

15.12 Breeding: According to a previously approved modification, 1:1 matings will be maintained (e.g., animals will be co-housed) until pups are weaned or until 15 weeks have elapsed where no pups have been produced and where there is no evidence of pregnancy.

The intent of this modification was to allow dams and sires to co-parent litters through weaning rather than potentially destabilizing a co-parenting system and adding stress by removing the sire. However, rebreeding has occurred several times in the course of the first experiment. Thus far, second litters from 1:1 mating have not been produced, however; in the event that second litters are produced, pups will be removed from the study within 24 hours of being found.

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.**

16.0 Study Endpoint

16.1 Endpoint Criteria: The endpoint criteria in this study is not changing, rather it is being clarified. As stated in the currently approved protocol, P generation animals will be euthanized 1) after pups are weaned or 2) after 15 weeks of 1:1 male:female matings have elapsed where no pups have been produced and where there is no evidence of pregnancy. As a clarification to the currently approved protocol, if P generation females are pregnant with a second litter when they are euthanized (at approximately PND25), fully developed unborn pups will be handled as described in 17.0. As a further clarification, if dams and sires that are maintained together to co-parent a first litter generate a second litter, then the second litter will be removed from the study, as described in 17.0, within 24 hours of being found.

17.0 Euthanasia

The form of euthanasia in this study is not changing, rather it is being clarified. If P generation females are discovered to be pregnant after being euthanized and during post-mortem tissue collection, then fully developed unborn pups will be immediately decapitated. If dams and sires that are maintained together to co-parent a first litter generate a second litter, then the second litter will be anesthetized with CO₂ and euthanized via decapitation, as described in QSARC 800 and 801. Adequate exposure time to CO₂ should be provided such that a neonate is unresponsive to painful stimuli prior to decapitation (AVMA, 2013).

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: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 5

Submission Date: Mar 12, 2019

Study Director/ Principal
Investigator Signature:

NARIZZANO.ALLISON.MARIE.1367161236

Digitally signed by NARIZZANO.ALLISON.MARIE.1367161236
Date: 2019.03.12 08:06:23 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: 12 Mar 19

AURO receipt:

SEYFERT.ALLISON.MARIE.1473772210

Digitally signed by SEYFERT.ALLISON.MARIE.1473772210
Date: 2019.03.12 15:36:19 -04'00'

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

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.03.19 12:24:12 -04'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☐ YES ☐ NO ☐

Mar 19, 2019

Date

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.03.19 12:24:01 -04'00'

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Mar 29, 2019

Date

NEWKIRK.KRISTIN.TORELL.1014786895

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Date: 2019.03.29 11:00:42 -04'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Mar 29, 2019

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 5

Submission Date: 12 March 2019

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

This study includes six separate experiments with six separate test compounds. The six test compounds are PFOS, PFOA, PFHxS, PFBS, PFNA, and 6:2 FTS. The test compounds will be administered daily to Parental (P) generation male and female white-footed mice (*Peromyscus leucopus*) via oral gavage. Fetuses and offspring are only exposed to the test compounds through pregnancy and maternal transfer (lactation). All animals are monitored for body weight changes and signs of toxicity. Immune function is evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood is collected from anesthetized animals via decapitation and trunk blood collection. Data from this study will ultimately be used to derive Toxicity Reference Values (TRVs). Statistical analysis of data from the first experiment (with PFOS) is underway. Experiments with the remaining five test compounds have not been initiated, but based on the results of the PFOS experiment and suggestions from the Sponsor, this modification seeks to refine several aspects of the study design.

According to the originally approved protocol, 1:1, male: female matings between non-siblings will be maintained (e.g., animals will be co-housed) until pups are born or 15 weeks have elapsed, at which point males will be removed from the study. However, upon observing dams and sires co-parenting newborn litters early in the PFOS experiment, Modification 2 was submitted. Per Modification 2, rather than potentially destabilizing a co-parenting system and adding stress, dams and sires will be maintained (e.g., animals will be co-housed) through weaning or until 15 weeks of mating has elapsed where no pups have been produced and where there is no evidence of pregnancy. As such, Modification 2 also adjusted how long P generation animals were exposed to the test compound or vehicle control; this, specifically, was because the maximum duration of dosing depended on how quickly (and if) pups were generated. Therefore, in the PFOS experiment, the dosing duration for P generation animals ranged from 11 to 23 weeks, which made for complicated data analysis. Some successful pairs also produced second litters in this time period, which also complicated data analysis. Taking both factors experienced with the PFOS experiment into consideration, proposed changes for the five future experiments include 1) removing the sire when a litter is produced (to prevent rebreeding of dams and inadvertent production of a second litter) and 2) dosing P generation animals for a finite period (i.e., 16 weeks).

Additionally, this modification seeks to adjust how long F1 generation animals are maintained on study. Per the original protocol, F1 generation animals will be maintained on study through puberty. However, data from the PFOS experiment suggests this effort is not worthwhile (e.g., the number of animals generated was too small to ensure sufficient statistical power for the intended endpoints). Therefore, for the five future experiments, F1 generation animals will be removed from the study at PND4 rather than at puberty (approximately PND60). The primary endpoints to be evaluated will be male and female (P generation) reproductive performance, mating behavior, conception, development of conceptus, parturition, and litter survivability.

Given these changes, an additional modification is how P generation animals are allocated to treatment groups. At the Sponsor's request, instead of having 31 pairs (1:1, male: female), four groups (control, low, medium, and high), and a total of 248 P generation animals per experiment, this modification seeks to have 25 pairs (1:1, male: female), five groups (control, low, medium, medium-high, and high), and a total of 250 P generation animals per experiment). As such, an additional 10 animals are being requested (two per experiment for the five remaining experiments).

This modification also seeks to improve conditions for study animals, and thus data generation, by incorporating additional enrichment into home cages and changes to the macroenvironment. In the PFOS experiment, enrichment was limited to certified plastic-free forms of enrichment (e.g., Nestlets). Based on experimentation with *Peromyscus* on Animal Use Protocol 18-04-01 and other forms of plastic-free enrichment, this modification seeks to include additional enrichment for the five future experiments, such as uncoated cardboard huts and Manzanita wood gnawing sticks. Additionally, macroenvironment refinements will be put in place from the time animals are received in house, to include having animals on the appropriate 24 hour light cycle with a photoperiod duration of 16 hours of illumination (as changed with Modification 3), covering windows on the animal rooms doors with co-extruded polycarbonate film, and placing cage racks on anti-vibration pads. Finally, food enrichment may be used following routine handling/procedures during the prolonged acclimation period and the experimental period as a form of reward.

This modification also seeks to adjust how animals are received from the vendor. Per the original protocol, all animals intended for Animal Use Protocol 18-08-01 will be received on Animal Use Protocol Number 18-04-01 when they arrive from the vendor and after an extended acclimation period, animals will be transferred to this protocol. This modification seeks to receive animals directly onto this protocol.

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

	Type Of Change	X
ADMINISTRATIVE I AURO/Q AU/SEM	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., 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	X
	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	X
	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	Transfer of animals from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species. Addition of two qualified personnel to deliver compound via oral gavage.	20 Peromyscus leucopus	24 October 2018
2	Adjust the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning.	N/A	08 November 2018
3	Adjust the 24 hour light cycle to photoperiod with a 16 hour duration of illumination.	N/A	27 November 2018
4	Clarification about how to handle second litters inadvertently generated by sires and dams co-parenting pups from a first litter.	N/A	02 January 2019

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,208 **B:** 0 **C:** 3,740 **D:** 1,488 **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:

Organisation for Economic Cooperation and Development (OECD) Environment, Health and Safety Publications Series on Testing and Assessment No. 43 (2008). Guidance document on mammalian reproductive toxicity testing and assessment.

APHC (2017e). QSARC 809. Animal Procurement, Receipt, and Transfers (Aberdeen Proving Ground, MD).

Characterization of Train-Induced Vibration and its Effect on Fecal Corticosterone Metabolites in Mice (2015). Journal of the American Association for Laboratory Animal Science; 54(6): 737-744.

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

9.0 Materials and Methods

9.1 Experimental Design and General Procedures:

B. EXPOSURE DURATION and C. CONTROLS: P generation animals will be dosed daily with the test compound or vehicle control for a finite period (i.e., 16 weeks). F1 generation animals will be removed from the study at PND4 rather than at puberty (approximately PND60). By removing F1 generation animals from the study at PND4, F1 generation animals will have 21 fewer days of exposure to the test compound or vehicle control.

D. STUDY DESIGN:

BREEDING: 1:1 male: female matings will be maintained (e.g., animals will be co-housed) until pups are born or until 12 weeks have elapsed where no pups have been produced. If pups are born, the sire will be removed from the home cage to prevent rebreeding. Sires removed from the home cage of a dam and litter will be housed as described below (5.D.15.12). If there are insufficient males, for example due to male death before mating, animals will not be repaired. In the PFOS experiment, repairing P generation animals was fairly unsuccessful and complicated data analysis.

LITTERS AND F1 GENERATION ANIMALS: Litters will be removed from the study at PND4, as described below (5.C. Changes in sample size evaluation, data analysis plan, archiving of data). Because F1 generation animals will be removed from the study at PND4, they will not be evaluated for immune challenge. As such, F1 generation animals will not receive a 0.1 mL IP injection of foreign red blood cells (RBCs) suspended in sterile saline.

15.0 Animal Manipulations

15.12 Breeding: 1:1 male: female matings will be maintained (e.g., animals will be co-housed) until pups are born or until 12 weeks have elapsed where no pups have been produced (i.e., the conclusion of the study). If pups are born, the sire will be removed from the home cage to prevent rebreeding. Every attempt will be made to co-house sires of the same dose group when they are removed from the home cage of a dam and litter; however, if sires of the same dose group are unavailable, an animal may be individually housed. Similarly, every attempt will be made to co-house dams of the same dose group when litters are removed at PND4; however, if dams of the same dose group are unavailable, an animal may be individually housed. Finally, if there are insufficient males, for example due to male death before mating, animals will not be repaired. In the PFOS experiment, repairing P generation animals was fairly unsuccessful and complicated data analysis.

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

According to Organisation for Economic Cooperation and Development guidelines (OECD 2008), the test compound should be administered to groups of animals such that 20 litters per dose level are generated in order to determine whether the test compound affects the offspring and to establish a dose-response relationship. In the PFOS experiment, in order to produce the desired 20 litters, 31 pairs were mated; this number of pairs was selected based on the expected rate of mating (80%) and mating success (80%) with this species under optimized

breeding colony conditions. However, data from the PFOS experiment suggests this effort is not worthwhile (e.g., the number of animals generated was too small to ensure sufficient statistical power for the intended endpoints). For example, in the PFOS experiment, 13 of 30 control animals (43%), 12 of 31 low-dose animals (39%), 17 of 31 medium-dose animals (55%), and 6 of 30 high-dose animals (20%) generated litters, which is insufficient to establish a dose-response relationship for the intended endpoints (e.g., developmental landmarks, malformations, etc.). The overall litter success rate in the PFOS experiment was 39%.

That being said, OECD guidelines also suggest that the test compound should be administered to groups of animals such that a minimum of eight pregnant females per dose level are generated to screen whether the test compound affects male and female reproductive performance, mating behavior, conception, development of conceptus, and parturition. Furthermore, there was a dose-response relationship in litter survivability in the PFOS experiment. Specifically, 100%, 91.7%, 52.9%, and 0% of litters born to P generation animals from the control, low, medium, and high dose groups, respectively, survived to at least PND1. Therefore, as a modification to the currently approved protocol, for future experiments with PFOA, PFHxS, PFBS, PFNA, and 6:2 FTS, the effects of a test compound on male and female reproductive performance, mating behavior, conception, development of conceptus, parturition, and litter survivability will be the primary endpoints to be evaluated. As such, F1 generation animals will be removed from the study at PND4 rather than at puberty (approximately PND60). PND4 was selected rather than PND1 to protect data integrity.

Given that fewer litters per dose group are necessary to evaluate the revised endpoints and to establish a dose-response relationship, it is a better allocation of animals, and ultimately better for deriving a TRV, to have slightly fewer animals per dose group and a fourth treatment group than to have more animals per dose group and three treatment groups.

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]).*

5.0 Animal(s) Used

5.1 List the animals used on this protocol

The P generation will be comprised of 25 pairs (1:1, male: female), five dose groups (control, low, medium, medium-high, and high), and a total of 250 P generation animals per experiment). As such, an additional 10 animals are being requested (two per experiment for the five remaining experiments). Given the 39% overall litter success rate in the PFOS experiment, 25 pairs per group is protective; if 39% of animals from each group produce a litter in future experiments, 10 litters will be generated per dose group. As mentioned above (5.C.), the test compound should be administered to groups of animals such that a minimum of eight pregnant females per dose level are generated to screen whether the test compound affects male and female reproductive performance, mating behavior, conception, development of conceptus, and parturition.

Special Considerations: Per QSARC 809 (Animal Procurement, Receipt, and Transfers), all animals will undergo a 5 day acclimation period upon receipt from the vendor. Per the original protocol, all animals intended for Animal Use Protocol 18-08-01 will be received on Animal Use Protocol Number 18-04-01 when they arrive from the vendor and after an extended acclimation

period, animals will be transferred to this protocol. This modification seeks to receive animals directly onto this protocol. It is more appropriate for animals to be received directly onto this protocol than onto 18-04-01 so that between the standard five day acclimation period and the initiation of the experiment, animals can acclimate to the Study Staff and routine handling (e.g., handling, weighing, “scruffing,” etc.). Acclimating animals to Study Staff and routine handling has historically decreased stress-related deaths during experiments with this species at this facility. The length of the extended acclimation period is at the discretion of the Primary Investigator/Study Director, but is anticipated to be between four and six weeks.

10.0 Refinement, Reduction, Replacement (3Rs)

10.1 Refinement: Based on experimentation with *Peromyscus* on Animal Use Protocol 18-04-01 and various forms of plastic-free enrichment, all animals will be provided with uncoated cardboard huts and Manzanita wood gnawing sticks.

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.**

16.0 Study Endpoint

16.1 Endpoint Criteria: The endpoint criteria in this study is not changing, rather the timing to the endpoint for P generation animals is being changed. P generation animals will be euthanized at the conclusion of a finite dosing period (i.e., 16 weeks of exposure to the test compound or vehicle control). F1 generation animals will be euthanized at PND4. Rationale for both of these changes are described above (5.B and 5.C.).

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.)*

18.0 Animal Husbandry

18.1 Special Husbandry and Procedures: Macroenvironment refinements will be put in place from the time animals are received in house, to include having animals on the appropriate 24 hour light cycle with a photoperiod duration of 16 hours of illumination (as changed with Modification 3), covering windows on the animal room doors with co-extruded polycarbonate film, and placing the housing racks on anti-vibration pads. Each of these changes may reduce stress on the animals and may alter research results. As described in Modification 3, it is well documented that photoperiods have significant effects on breeding success in rodents, birds, cattle, sheep, etc. Furthermore, a short day length has been shown to trigger reproductive suppression in this species. Given that lighting has a significant impact on breeding, having the animals on the proper light cycle from the time they are received from the vendor is imperative. Covering windows on the animal room doors with co-extruded polycarbonate film will serve dual purposes: to extrude residual light from the hallways and to reduce stress in the

animals from personnel passing by the doors throughout the day. Finally, excessive environmental vibrations can cause deleterious effects on animal health and environmental results (Atanasov et al., 2015). Placing the housing racks on anti-vibration pads may reduce stress on the animals and may alter research results.

Finally, food enrichment may be administered by Study Staff following routine handling/procedures (to include cage changes) during the prolonged acclimation period and the experimental period as a form of reward. It is well documented that animals can be conditioned to anticipate the receipt of a reward for undergoing a procedure and/or experimental treatment, and that this pairing can decrease the stress response associated with a procedure and/or experimental treatment.

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: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 6

Submission Date: Jul 26, 2019

Study Director/ Principal
Investigator Signature:

NARIZZANO.ALLISON.MARIE.1367161236

Digitally signed by NARIZZANO.ALLISON.MARIE.1367161236
Date: 2019.07.26 13:36:19 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: Jul 29, 2019

AURO receipt:

DAWSON.CHELSEA.LEE.1544
052116

Digitally signed by
DAWSON.CHELSEA.LEE.1544052116
Date: 2019.07.29 14:04:47 -04'00'

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

HOYT.NATHAN.ARTHUR.1293177410

Digitally signed by HOYT.NATHAN.ARTHUR.1293177410
Date: 2019.07.29 14:10:09 -04'00'

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2019.07.29 15:31:01 -04'00'

Attending/ Alternate Veterinarian Review Signature:

Approved via VVC: N/A ☐ YES ☒ NO ☐

Jul 29, 2019

Date

HOYT.NATHAN.ARTHUR.1293177410

Digitally signed by HOYT.NATHAN.ARTHUR.1293177410
Date: 2019.07.29 14:09:58 -04'00'

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Jul 30, 2019

Date

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2019.07.30 16:18:37 -04'00'

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Jul 30, 2019

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 6

Submission Date: 26 July 2019

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

This study includes six separate experiments with six separate test compounds. The six test compounds are PFOS, PFOA, PFHxS, PFBS, PFNA, and 6:2 FTS. The test compounds will be administered daily to Parental (P) generation male and female white-footed mice (*Peromyscus leucopus*) via oral gavage. Fetuses and offspring are only exposed to the test compounds through pregnancy and maternal transfer (lactation). All animals are monitored for body weight changes and signs of toxicity. Immune function is evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood is collected from anesthetized animals via decapitation and trunk blood collection. Data from this study will ultimately be used to derive Toxicity Reference Values (TRVs). Statistical analysis of data from the first experiment (with PFOS) and the in-life portion of the second experiment (with PFNA) are underway.

This modification seeks to adjust how long F1 generation animals are maintained on study. Per the Modification 5, F1 generation animals will be removed from the study at PND4 rather than at puberty (approximately PND60). However, upon re-review of the literature, one of the five remaining test compounds behaves slightly differently than the others. Specifically, the pattern of test article-induced neonatal death with PFNA is somewhat different from those elicited by PFOS or PFOA. According to Das et al., (2015), a majority of the PFNA-exposed pups survived a few days longer after birth than those exposed to PFOS or PFOA, which typically died within the first two days of postnatal life. Das et al. was identified during the literature search. It was oversight by the Study Director/Principal Investigator to not re-consult the literature after conducting the first experiment and before designing and submitting Modification 5. As such, F1 animals will be removed from the study at PND4-10, depending on the test compound being evaluated.

This modification also seeks to add five animals to the protocol to replace the animals compromised during shipment from the vendor (prior to initiating the second experiment).

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)]	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>Additional days of exposure to test article (via lactation), body weight measurements, and possible observation of mortality.</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	Transfer of animals from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species. Addition of two qualified personnel to deliver compound via oral gavage.	20 Peromyscus leucopus	24 October 2018
2	Adjust the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning.	N/A	08 November 2018
3	Adjust the 24 hour light cycle to photoperiod with a 16 hour duration of illumination.	N/A	27 November 2018
4	Clarification about how to handle second litters inadvertently generated by sires and dams co-parenting pups from a first litter.	N/A	02 January 2019
5	Remove sire when litter is produced and dose P generation animals for a finite period (i.e., 16 weeks). Adjust how long F1 generation animals are maintained (to PND4). Adjust how P generation animals are allocated to treatment groups (i.e., 5 groups of 25 animals rather than 4 groups of 31). Addition of 10 additional animals. Improvement of conditions for study animals, and thus data generation. Receipt of animals directly onto this protocol.	10 Peromyscus leucopus	29 March 2019

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,208 B: 0 C: 3,720 D: 1,498 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).

9.0 Materials and Methods

9.1 Experimental Design and General Procedures:

B. EXPOSURE DURATION and C. CONTROLS: F1 generation animals will be removed from the study at PND4-10, depending on the test compound being evaluated. According to Das et al., (2015), a majority of the PFNA-exposed pups survived a few days longer after birth than those exposed to PFOS or PFOA, which typically died within the first two days of postnatal life.

D. STUDY DESIGN:

LITTERS AND F1 GENERATION ANIMALS: Litters will be removed from the study at PND4-10.

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

This modification seeks to add five animals to the protocol to replace the animals compromised during shipment from the vendor (prior to initiating the second experiment).

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.**

16.0 Study Endpoint

16.1 Endpoint Criteria: The endpoint criteria in this study is not changing, rather the timing to the endpoint for F1 generation animals is being changed. F1 generation animals will be euthanized at PND4-10. Rationale for this change is described above (5.B).

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: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 7

Submission Date: Aug 19, 2019

Study Director/ Principal
Investigator Signature:

NARIZZANO.ALLISON.MARIE.1367161236

Digitally signed by NARIZZANO.ALLISON.MARIE.1367161236
Date: 2019.08.19 14:29:42 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: Aug 20, 2019

AURO receipt:

DAWSON.CHELSEA.LEE.1544
052116

Digitally signed by
DAWSON.CHELSEA.LEE.1544052116
Date: 2019.08.20 10:52:06 -04'00'

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

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.08.21 09:35:05 -04'00'

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2019.08.21 14:28:06 -04'00'

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

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.08.21 09:35:16 -04'00'

Date

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Aug 29, 2019

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2019.08.29 15:19:16 -04'00'

Date

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Aug 29, 2019

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 7

Submission Date: 19 August 2019

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

This study includes six separate experiments with six separate test compounds. The six test compounds are PFOS, PFOA, PFHxS, PFBS, PFNA, and 6:2 FTS. The test compounds will be administered daily to Parental (P) generation male and female white-footed mice (*Peromyscus leucopus*) via oral gavage. Fetuses and offspring are only exposed to the test compounds through pregnancy and maternal transfer (lactation). All animals are monitored for body weight changes and signs of toxicity. Immune function is evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood is collected from anesthetized animals via decapitation and trunk blood collection. Data from this study will ultimately be used to derive Toxicity Reference Values (TRVs). Statistical analysis of data from the first experiment (with PFOS) and the in-life portion of the second experiment (with PFNA) are underway.

Per Modification 5, every attempt will be made to co-house sires of the same dose group when they are removed from the home cage of a dam and litter. However, there was one instance of an animal developing abscesses from fight wounds, which necessitated euthanasia, several instances of animals found dead with trauma indicative of fighting, and several instances of animal found dead, likely from stress, even after being separated from a group of sires. Specifically, 12 total groups (of two or three) sires have been established and five were identified as incompatible. Of the five cages identified as incompatible and transitioned to individual housing, two cages had 100% survivability post-separation, two had 33% survivability and one had 50% survivability.

The Primary Investigator (PI) discussed several options for how to proceed with the Attending Veterinarian (AV), two Animal Health Technicians, and members of the Animal Use and Review Office. One suggestion was to give post-parturition sires a period of being individually housed prior to establishing groups of sires, the rationale was that a break may allow hormone levels to normalize. However, a similar practice was already in place. Per Modification 5, only sires of the same dose group are co-housed. As such, in all cases, there were several days of sires being individually housed post-parturition before establishing groups. Another option included continuing to establish groups of sires and increasing day-time observations in a collaborative effort between the Veterinary Support Office and Study Staff. However, since

mice are nocturnal, increasing day-time observations after establishing groups of sires is unlikely to result in early detection of incompatibility. Furthermore, in many cases, animals were found dead several days after being separated, suggesting that not identifying bullying early may cause undue stress, and in one case, abscesses from fight wounds developed post-separation, which necessitated euthanasia. Therefore, this modification seeks to adjust husbandry procedures so sires are individually housed after generating a litter. Sires that are currently, successfully, socially housed will remain socially housed unless evidence of incompatibility develops.

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:	
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	Transfer of animals from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species. Addition of two qualified personnel to deliver compound via oral gavage.	20 Peromyscus leucopus	24 Oct. 2018
2			
3	Adjust the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning.	N/A	08 Nov. 2018
4	Adjust the 24 hour light cycle to photoperiod with a 16 hour duration of illumination.	N/A	27 Nov. 2018
5	Clarification about how to handle second litters inadvertently generated by sires and dams co-parenting pups from a first litter.	N/A	02 Jan. 2019
	Remove sire when litter is produced and dose P generation animals for a finite period (i.e., 16 weeks). Adjust how long F1 generation animals are maintained (to PND4). Adjust how P generation animals are allocated to treatment groups (i.e., 5 groups of 25 animals rather than 4 groups of 31). Improvement of conditions for study animals, and thus data generation. Receipt of animals directly onto this protocol.	10 Peromyscus leucopus	29 March 2019
6	Adjust how long F1 generation animals are maintained on the study.	5 Peromyscus leucopus	30 July 2019

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,208 B: 0 C: 3,720 D: 1,508 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.)*

18.0 Animal Husbandry

18.5 Will exceptions to or deviations from IACUC-approved environmental enrichment practices, including single housing, be required?

Due to several instances of social incompatibility that resulted in the development of abscesses from fight wounds, which necessitated euthanasia, and other animals found dead

with trauma indicative of fighting, this modification seeks to adjust husbandry procedures so sires are individually housed after generating a litter. Sires that are currently, successfully, socially housed will remain socially housed unless evidence of incompatibility develops.

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: [REDACTED]

PROTOCOL #: 03-18-08-01

Protocol Approval Date: 13 August 2018

PROTOCOL
TITLE:

Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)

Modification Request #: 8

Submission Date: Sep 19, 2019

Study Director/ Principal
Investigator Signature:

NARIZZANO.ALLISON.MARIE.1367161236

Digitally signed by NARIZZANO.ALLISON.MARIE.1367161236
Date: 2019.09.18 15:52:44 -04'00'

For review use only:

Review Process: ADMIN ☐

FCR ☐

DMR ☒

VVC ☐

Received Date: Sep 19, 2019

AURO receipt:

DAWSON.CHELSEA.LEE.1544
052116

Digitally signed by
DAWSON.CHELSEA.LEE.1544052116
Date: 2019.09.19 12:13:09 -04'00'

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

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.09.26 10:46:59 -04'00'

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2019.09.27 14:44:34 -04'00'

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

GEHLING.ALICIA.MARIE.1364571689

Digitally signed by GEHLING.ALICIA.MARIE.1364571689
Date: 2019.09.26 10:47:15 -04'00'

Date

Safety Review Signature (if applicable):

Date

LTACO/GLP Review Signature (if applicable):

Date

IACUC Chair Review Signature:

Sep 27, 2019

NEWKIRK.KRISTIN.TORELL.1014786895

Digitally signed by NEWKIRK.KRISTIN.TORELL.1014786895
Date: 2019.09.27 14:45:03 -04'00'

Date

Modification IACUC APPROVED ☒

GLP REVIEWED ☐

Date: Sep 27, 2019

APHC IACUC Animal Use Protocol Modification

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

PROTOCOL #: **03-18-08-01**

Protocol Approval Date: 13 August
2018

PROTOCOL TITLE: **Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in the white-footed mouse (*Peromyscus leucopus*)**

Phone No.: 410-436-8772

FAX No.: n/a

Email: [REDACTED]

Modification Request #: 8

Submission Date: 18 September 2019

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

This study includes six separate experiments with six separate test compounds. The six test compounds are PFOS, PFOA, PFHxS, PFBS, PFNA, and 6:2 FTS. The test compounds will be administered daily to Parental (P) generation male and female white-footed mice (*Peromyscus leucopus*) via oral gavage. Fetuses and offspring are only exposed to the test compounds through pregnancy and maternal transfer (lactation). All animals are monitored for body weight changes and signs of toxicity. Immune function is evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood is collected from anesthetized animals via decapitation and trunk blood collection. Data from this study will ultimately be used to derive Toxicity Reference Values (TRVs). Statistical analysis of data from the first experiment (with PFOS) and the in-life portion of the second experiment (with PFNA) are underway.

As part of this protocol, Toxicology Directorate (TOX) purchases *Peromyscus* from the sole source, commercially available vendor. The vendor is unable to consistently meet APHC's demand for animals for each phase of the study, despite intervention from Contracting. Additionally, the vendor uses isoflurane gas for routine procedures with the animals. Isoflurane is a heavily fluorinated molecule, and repeated exposure may confound results in subsequent PFAS studies. Therefore, a colony of *Peromyscus maniculatus* (originally obtained from the same vendor) was obtained from Harvard University. Therefore, this modification seeks to add the model species *P. maniculatus* to this protocol. In doing so, the four remaining experiments on the protocol may be conducted with either *P. maniculatus* or *P. leucopus*. While it is not ideal for experiments to be conducted in two different species, doing so will not negatively impact the overall goal of this project, which is to derive TRVs for wild mammals.

This modification also seeks to add qualified personnel to various roles, to add Meredith Bohannon as the Co-Investigator, and to adjust Michael Quinn from the Co-Investigator to an Associate Investigator.

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

	Type Of Change	X
ADMINISTRATIVE – AURO/QUAU/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>]	
	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	X
	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	Transfer of animals from protocol 18-04-01 to this protocol to pilot the foreign red blood cell challenge in this species. Addition of two qualified personnel to deliver compound via oral gavage.	20 Peromyscus leucopus	24 Oct. 2018
2	Adjust the co-housing procedure of P generation animals to maintain dams, sires, and pups in one cage (as possible) through weaning.	N/A	08 Nov. 2018
3	Adjust the 24 hour light cycle to photoperiod with a 16 hour duration of illumination.	N/A	27 Nov. 2018
4	Clarification about how to handle second litters inadvertently generated by sires and dams co-parenting pups from a first litter.	N/A	02 Jan. 2019
5	Remove sire when litter is produced and dose P generation animals for a finite period (i.e., 16 weeks). Adjust how long F1 generation animals are maintained (to PND4). Adjust how P generation animals are allocated to treatment groups (i.e., 5 groups of 25 animals rather than 4 groups of 31). Improvement of conditions for study animals, and thus data generation. Receipt of animals directly onto this protocol.	10 Peromyscus leucopus	29 March 2019
6	Adjust how long F1 generation animals are maintained on the study.	5 Peromyscus leucopus	30 July 2019
7	Adjust husbandry procedures so sires are individually housed after generating a litter.	N/A	29 August 2019

4. CURRENT APPROVED ANIMAL USAGE ON PROTOCOL:

Species/Total: Peromyscus leucopus/5,243 **B:** 0 **C:** 3,740 **D:** 1,503 **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):

Change in title: Reproductive and developmental toxicity testing of six per- and polyfluoroalkyl substances (PFAS) in *Peromyscus* spp.

Change in Co-Investigator: Meredith Bohannon.

Addition of Associate Investigator: Michael Quinn

Change in personnel: See 5.G.

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]).*

The number of animals on this protocol is not changing, merely another model species is being added. Since *P. maniculatus* and *P. leucopus* are from the genus *Peromyscus* and are wild-type, both are suitable for use on this protocol, being that the ultimate objective is to derive TRVs for wild mammals. As such, the four remaining experiments on the protocol (with 6:2 FTS, PFOA, PFHxS, and PFBS) may be conducted with either *P. maniculatus* or *P. leucopus*.

Besides being a suitable model species, *P. maniculatus* are readily available via an in-house colony. The colony was obtained from Harvard University when the animals were no longer needed by researchers, which is an overall reduction in animal use. *P. maniculatus* are also available via a vendor, and may be obtained via the vendor if the number produced by the colony is insufficient to complete the four remaining experiments on this protocol. Like *P. leucopus*, there is only one commercial vendor for *P. maniculatus*. According to the colony manager for the vendor, *P. maniculatus* are easier to breed and produce larger litters. As such, the historical delivery issues with obtaining animals from the vendor could be less severe by using *P. maniculatus* instead of (or in addition to) *P. leucopus*. At APHC, the number of animals produced per litter has been consistently larger in *P. maniculatus* (colony animals) than *P. leucopus* (used in Experiments 1 and 2), though the PI acknowledges that the colony animals are also manipulated less than the animals used in experiments. Overall, data generated from animals produced in-house may have more integrity than data generated from animals produced by the vendor. Besides issues with delivery, the vendor maintains poor

records of animal age, lineage, etc. In contrast, records for animals obtained from Harvard and produced in-house are quite thorough. Finally, animals produced in-house are not exposed to isoflurane gas, which the vendor uses for routine procedures. Isoflurane is a heavily fluorinated molecule and repeated exposure may confound results in subsequent PFAS studies.

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.)*

See attached table.

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

N/A

Person	Handling and Observations	IP injections	Oral gavage	CO2 anesthesia	Cervical dislocation	Decapitation and Exsanguination
Allison Jackovitz B.S. Biology, 9+ years animal research experience	6/4/09; 2/18/15; 3/12/15; 12/8/16; 12/20/16; 12/22/16; 1/24/17	2/18/15; 3/12/15; 12/20/16; 9/6/17; 9/13/17	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18	3/12/15; 7/6/17; 8/30/17; 9/6/17; 9/13/17	7/6/17; 9/6/17; 9/13/17	7/6/17; 8/30/17; 9/6/17; 9/13/17
Meredith Bohannon Ph.D., Environmental Sciences, 10+ years animal research experience	8/26/19; 8/28/19; 9/13/19; 9/16/19	9/4/19; 9/6/19	9/13/19; 9/16/19	9/17/19	9/17/19	9/17/19
Taryn Brown MLT ASCP, A.S., Business Administration, 1+ years of animal research experience	8/26/19; 8/28/19; 9/13/19	9/4/19; 9/6/19	9/10/19; 9/13/19	9/17/19	9/17/19	9/17/19
James Cox	Removed	Removed	Removed	Removed	Removed	Removed
Lee Crouse M.S., Environmental Sciences, 20+ years animal research experience	12/08/16; 8/7/19; 8/26/19; 8/28/19	12/20/16; 9/4/19	12/8/16; 8/26/19; 8/28/19	9/17/19	9/17/19	9/17/19
Blas Guigni Ph.D., Molecular Physiology, 5+ years animal research experience	9/12/19; 9/13/19; 9/16/19	9/12/19	9/12/19; 9/13/19; 9/16/19	9/17/19	9/17/19	9/17/19
Teresa Hanna ALAT, 17+ years animal research experience	6/30/11; 2/12/15; 2/18/15; 2/25/15; 3/12/15; 2/12/16; 12/20/16	9/4/19; 9/6/19	N/A	9/17/19	9/17/19	9/17/19

Person	Handling and Observations	IP injections	Oral gavage	CO2 anesthesia	Cervical dislocation	Decapitation and Exsanguination
Jarod Hanson DVM, Ph.D., Infectious Diseases/Virology, 25+ years animal experience	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18	N/A	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18	N/A	N/A	N/A
Lindsay Holden Ph.D., Biology, 10+ years animal research experience	8/26/19; 8/28/19	9/4/19; 9/6/19	N/A	T N/A	N/A	N/A
Rebecca Kilby ALAT, RVT, B.S., Organizational Management, 26+ years animal research experience	6/24/09; 10/14/08; 1/24/17; 8/14/19	10/14/08; 6/30/11	9/7/18; 9/10/18; 9/11/18; 10/2/18	05/08/09; 06/24/09; 12/05/17	5/12/2017	7/23/19
Emily Lent Ph.D., Natural Resources and Environmental Studies, M.S., Wildlife Biology, 20+ years animal research experience	10/12/07; 12/11/07; 1/23/08; 10/14/08; 2/12/15; 2/18/15; 12/12/16; 12/20/16	N/A	N/A	N/A	N/A	N/A
Caroline Procell B.S., Biology, 4+ years animal experience	12/20/16; 12/22/16; 1/24/17	12/20/16	8/20/18; 8/21/18; 8/22/18; 9/5/18; 9/6/18; 9/10/18; 9/11/18; 10/2/18	8/30/17; 9/13/17	8/30/17; 9/13/17	8/30/17
Michael Quinn Ph.D., Animal Science, 20+ years animal research experience	6/21/05; 10/14/08; 6/30/11; 2/25/15; 3/12/15; 12/20/16; 12/22/16; 1/24/17	6/30/11; 2/25/15; 3/12/15; 12/20/16; 9/6/17	12/20/16; 12/22/16; 3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18	7/6/17; 8/30/17; 9/6/17	7/6/17; 8/30/17; 9/6/17	7/6/17; 8/30/17; 9/6/17

Person	Handling and Observations	IP injections	Oral gavage	CO2 anesthesia	Cervical dislocation	Decapitation and Exsanguination
Thomas Sussan Ph.D., Biochemistry, Cellular and Molecular Biology, 19+ years animal research experience	9/6/17; 9/13/17	N/A	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18	9/6/17; 9/13/17	9/6/17; 9/13/17	9/6/17; 9/13/17
Lindsey Ward RLAT, A.S. Business Administration, 10+ years animal research experience	9/12/19	9/6/19	9/10/19; 9/12/19	9/17/19	9/17/19	9/17/19
Mark Way B.S., Biology, 19+ years animal research experience	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	2/12/15; 2/18/15; 2/25/15; 3/12/15; 12/20/16; 8/30/17; 9/6/17	3/5/18; 3/6/18; 3/7/18; 3/12/18; 3/13/18; 3/14/18	6/24/09; 2/12/15; 3/12/15; 7/6/17; 8/30/17; 9/6/17	9/6/17; 9/13/17	9/6/17; 9/13/17

Gray boxes indicate new qualified personnel.

GLOSSARY

AIC

Akaike Information Criterion

AFFF

Aqueous Film Forming Foam

ANOVA

Analysis of variance

ANCOVA

Analysis of covariance

APHC

Army Public Health Center

BMD

Benchmark dose

BMDL

Benchmark dose low

BMDS

Benchmark Dose Software

BMR

Benchmark response

CO₂

carbon dioxide

D_{mw}

phospholipid membrane-water distribution coefficient

DOD

Department of Defense

°C

Degrees Celsius

F₁ generation

First filial generation

g

grams

GD

Gestation day

IP

Intraperitoneal

L

liter

LC/MS/MS

Liquid chromatography-tandem mass spectrometry

LC-QTOF-MS

Liquid chromatography-quadrupole time-of-flight-mass spectrometry

LD50

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

LOAEL

Lowest observed adverse effect level

MANOVA

Multivariate analysis of variance

mg/kg-day

milligrams per kilogram per day

mg/mL

milligrams per milliliter

mL

milliliters

NOAEL

No observed adverse effect level

P generation

Parental generation

PAS

Periodic acid-Schiff

PBS

Phosphate buffered solution

PFAS

Per- and polyfluoroalkyl substances

PFAAs

Perfluoroalkyl acids

PFBS

Perfluorobutane sulfonate

PFC

Plaque forming cells

PFHxS

Perfluorohexane sulfonate

PFNA

Perfluorononanoic acid

PFOA

Perfluorooctanoic acid

PFOS

Perfluorooctane sulfonate

PGSC

Peromyscus Genetic Stock Center

PND

Postnatal day

POD

Point of Departure

RfD

Reference dose

St. dev.

Standard deviation

SEM

Standard error of mean

SERDP

Strategic Environmental Research and Development Program

SRBCs

Sheep red blood cells

T3

Triiodothyronine

T4

Thyroxine

μL

microliter

μm

micrometer

USEPA

United State Environmental Protection Agency

6:2 FTS

6:2 fluorotelomer sulfonate