# The Neurodevelopmental Effects of PFAS Exposure through Drinking Water

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Duke Global Health Institute
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2022

ABSTRACT

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# Abstract

Per- and polyfluoroalkyl substances (PFAS) are persistent organic pollutants that have become globally ubiquitous in the environment and in humans. One local population facing disproportionate PFAS exposure risk through their drinking water was Pittsboro, NC residents. *In utero* PFAS exposure is associated with an array of long-term health effects; however, the mechanism of toxicity is poorly understood. The aim of this study is to determine the causal relationship between *in utero* PFAS mixture exposure and cognitive deficits, emotional dysfunction, and behavioral dysregulation in rats. Using animal models, this study addresses the neurodevelopmental effects of gestational exposure to clearly defined PFAS concentrations seen in Pittsboro's drinking water and a 5,000-fold concentration as the positive control. To quantitatively assess toxicity, animal subjects exposed to PFAS-laden drinking water during fetal development underwent a battery of assessments from an established behavioral testing framework. Dams exposed to the high-dose mixture yielded smaller litters on average. Offspring in the low-dose group of environmental relevance demonstrated significantly smaller weights ($p<0.05$) and smaller anogenital distances on average just prior to weaning (PND 21). In the behavioral battery, low and high-dose-exposed rats made fewer attempts to explore different arms of the elevated plus maze, indicating a heightened anxiety response. In the figure-8 maze, males in the high-dose group displayed hyperactivity compared to the other groups. These findings suggest that maternal PFAS exposure may be able to cause

diminished fertility, small pup size, increased anxiety, and hyperactivity in rats. However, continued investigation is necessary to obtain sufficient statistical power.

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# 1. Introduction

Between 23 and 40% of all global premature deaths have been attributed to environmental factors, particularly exposures to polluted air, water, and soil.[1,2] Per- and polyfluoroalkyl substances (PFAS) are a prime example of a pollutant class associated with morbidities and premature mortality. PFAS are synthetic organic compounds developed in the 1930s and used throughout industry and commerce since the 1940s.[3,4] The exceptionally strong chemical bond between the carbon and fluorine atoms enables PFAS to resist degradation and persist in the environment, which gives rise to their nickname "forever chemicals."[4] In addition to their stability, they act as surfactants to repel oil and water, have low surface energy, and exhibit low friction properties.[4,5] The signature chemical behaviors of PFAS have been utilized in essential products from nonstick cookware to firefighting foams, leading them to become ubiquitous in the environment.[4,5] Global biomonitoring data have revealed diverse human exposure sources, including contaminated drinking water, food and food packaging, outdoor air, household dust, and soil.[6–8] Consequently, the serum of over 95% of Americans is estimated to contain detectable quantities of PFAS, but certain populations face disproportionate exposure risks.[9–11]

## *1.1 Drinking Water: The Primary PFAS Exposure Route*

Ingestion of contaminated drinking water and food is considered the primary PFAS exposure route for the general population.[8] According to national monitoring efforts by the United States Environmental Protection Agency (EPA) and the United

States Geological Survey (USGS), the average sum PFAS content of drinking water in the United States is estimated to be 19.5 ng/L.[12] As of 2016, the EPA set a non-enforceable lifetime health advisory level for two common PFAS compounds—perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA)—in drinking water at 70 ng/L.[13,14] A synthesis of the USGS and EPA database, along with additional public water testing data, demonstrated that the drinking water of over 200 million US residents includes detectable levels of PFAS.[15] Further, approximately 900,000 US residents are exposed to PFOS and PFOA concentrations at or above the advisory level through private wells or municipal water.[15] However, most Americans are said to be exposed to concentrations between 2.5 ng/L and 10 ng/L.[15]

A similar analysis of municipal drinking water commissioned by the Environmental Working Group corroborated the prevalence and variability in known levels of PFAS contamination. In a study encompassing 31 US states, concentrations in only 8% of tap water samples fell below a detection limit of 1 ng/L, 51% contained 10 ng/L or higher, and concentrations over 100 ng/L were discovered in four public drinking water supplies.[16] Despite the majority of national drinking water exposures falling below the EPA health advisory level, deleterious health consequences of chronic low-level exposure cannot be ruled out, particularly in vulnerable populations.

## *1.2 PFAS and Environmental Justice*

Environmental health disparities are caused by a combination of social and environmental factors. According to the environmental justice movement, certain disadvantaged groups are more susceptible to toxic environmental exposures and their

associated health consequences.[17] Vulnerable populations—such as communities of color, low-income, and rural communities—will often be exposed to pollution at a higher frequency than their neighbors and face a larger disease burden.

Marked environmental health disparities have been demonstrated in the case of PFAS. For example, a Northwestern Environmental Health Research Institute analysis found that 39,000 low-income households in the US fall within a five-mile radius of sites with known PFAS contamination—a figure 15% higher than that expected from census data.[18] A lack of financial resources and political influence compared to multibillion-dollar industries contributes to the disproportionate pollution exposures in disadvantaged communities. Further, environmental health disparities will be perpetuated by a lack of willingness to disseminate knowledge surrounding PFAS use and effects.[19] However, expanding the scientific knowledge base on the dangers of PFAS pollution to communities will create exigence among global stakeholders to act on the issue.

## *1.3 Pittsboro, NC: a Local Case Study*

People living in Pittsboro, North Carolina, are experiencing disparate exposures to PFAS pollution in their water supply. This small city with a median household income of approximately $45,000—which is $24,000 below the US national average—is facing the consequences of industrial activity.[20,21] Chemours, a well-established chemical manufacturing company, has been implicated in the contamination of the Pittsboro drinking water source [22]. Despite citizen outrage and lawsuits, effluent from the Chemours Fayetteville Works Plant allegedly continues to add persistent organic pollutants to local water bodies.[22,23]

Exceptionally high PFAS levels were discovered in the Cape Fear River (CFR) and its tributaries, which run through Pittsboro, due to industrial effluent releases dating back to 1980.[24,25] Preliminary monitoring data from Duke University found elevated ambient PFAS levels in the CFR basin and the communities which depend on it as a water source. In Pittsboro, sum PFAS concentrations up to 1,076 ng/L and 270.8 ng/L were measured at drinking water intakes and finished household drinking water, respectively.[26] The readings, far exceeding the non-enforceable EPA advisory level, are over 30 times higher than the neighboring city of Durham and are strikingly higher than surrounding cities.[26] Residential exposure via contaminated drinking water would explain why early data show the blood levels of PFAS to be between two and four times higher in Pittsboro than in the general US population.[27] Within this context, a primary goal of this study is to better understand any health implications related to this elevated exposure.

## *1.4 PFAS Health Effects*

Concern surrounding PFAS has risen among affected North Carolina residents due to the potential deleterious human health effects associated with exposure. Laboratory and epidemiologic data have linked PFAS exposure to increased cancer risk, immune system dysfunction, poor antibody response to vaccines, endocrine disruption, nephrotoxicity, thyroid disorders, high cholesterol, pregnancy-induced hypertension, asthma, and diminished fertility.[28] Fetuses are particularly susceptible to long-term adverse health effects due to PFAS transfer via the placenta and breastmilk.[29–31] Thus, this study is centered around the effects of prenatal and perinatal exposure.

PFAS as a maternal stressor has been associated with developmental, neurocognitive, and behavioral impacts in population-level studies. In early childhood studies, associations were uncovered between PFAS exposure and visual-motor deficits,[32] lower IQ test scores, [33] externalizing behavior difficulties,[34–36] worsened executive function,[37] neuropsychological developmental issues,[38] and attention deficit hyperactivity disorder (ADHD) and related symptoms.[36,39,40] In a Taiwanese birth cohort study, PFOS exposure was found to affect gross motor development in two-year-old children.[41] An Ohio cohort study found that PFOA exposure may also adversely affect psychomotor development and externalizing scores, while PFOS exposure may impede executive function.[42–44] Conversely, null associations between PFAS exposure and changes in neurological outcomes were observed in cohort studies out of Denmark, Norway, and West Virginia.[45–47] The conflicting results of unique exposure scenarios necessitate further investigation of the neurodevelopmental consequences of PFAS exposure.

## *1.5 Study Aims and Hypothesis*

While many cohort studies have demonstrated associations between environmental PFAS contamination and human health complications, they are unable to show causation or mechanism. Experimental studies using animal models are needed to investigate the causal relationship between *in utero* PFAS exposure and the neurobehavioral effects seen in population studies. In addition, humans are exposed to a mixture of PFAS compounds, rather than single compounds in isolation. Most previous studies have focused on exposure to individual PFAS compounds. This study aims to address the neurodevelopmental toxicity of a clearly defined PFAS mixture mimicking

drinking water in Pittsboro, NC, to better inform health effects and intervention strategies. We hypothesize that *in utero* exposure to this PFAS mixture will cause cognitive deficits and behavioral dysregulation in rat offspring by disrupting placental-fetal brain hormonal signaling.

# 2. Methods

## *2.1 Setting & Subjects*

All experimentation and data collection took place at the Neurobehavioral Research Lab in the Psychiatry Department of Duke University Medical Center (Durham, NC). Sprague-Dawley strain rats (Charles Rivers Labs, Raleigh, NC, USA) and their offspring comprised the subjects of this study. Rats are an ideal candidate for neurobehavioral experiments due to their outgoing nature, low aggression, display of behaviors relevant to humans, similarities to basic structures and functions of the human brain, and high intelligence compared to other animal models such as mice.[48,49] The Sprague-Dawley rat strain in particular is widely used in environmental toxicology when assessing offspring effects of parental toxicant exposure.[50,51]

Rats were raised in a reversed day-night light schedule (12:12 hrs), and all behavioral assays were conducted during their active period under low-ambient light conditions. Unless fasting was required for food-motivated behavioral tests (see below), all animals were given *ad libitum* access to food and water. Animals were housed in colony rooms where temperature was maintained between 20 and 25°C and humidity did not exceed 65%. Animal welfare checks, temperature, and humidity were logged daily. Cages were changed once per week at minimum. Animal husbandry, behavioral testing, and all other laboratory duties were performed by Melissa Marchese (MSc-GH candidate), Tianyi Zhu (MSc-GH candidate), Katherine Wang (undergraduate), and Caroline Palmer (undergraduate).

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Duke University under protocol A214-20-11. During the study, the facilities and protocol underwent semi-annual Institutional Animal Care and Use Committee inspections and a tri-annual inspection by the American Association for the Accreditation of Laboratory Animal Care.

## *2.3 Procedures*

The experimental timeline is presented in **Table 3** and **Figure 1**. Adolescent female rats were delivered to the laboratory weighing between 200 and 250 grams. Upon arrival, female rats were singly housed and given at least seven days to acclimate before beginning the prenatal exposure. Each female rat was randomly assigned a cage number and an exposure group. Female rats received either deionized water (control), a low-dose drinking water mixture to mimic concentrations in Pittsboro, or a high-dose positive control mixture (5,000-fold higher than the low-dose) (**Table 1**). The low-dose mixture reflects PFAS concentrations measured in a 2019 sampling effort of North Carolina drinking water by the Stapleton Lab (Duke University, Durham, NC). The high-dose PFAS mixture concentrations were selected based on a review of previous animal toxicity studies where significant exposure-attributable changes were observed (**Table 2**). The first cohort, a pilot study, was used to ensure the doses were appropriate (i.e., acceptable general health, no significant signs of stress, no major reproductive harm such as miscarriage, no significant weight loss).

After a seven-day minimum acclimation period, female rats were given *ad libitum* to their respective drinking water mixtures. Drinking water mixtures were supplied

through one-pint glass bottles with rubber stoppers. Water consumption was measured by weight every three days and water was replenished. Bottles were washed on a weekly basis. After seven days of exposure to the mixture, female rats entered a five-day mating period where a male was placed into their home cage. After five days, males were removed. Drinking water exposure continued throughout pregnancy until birth.

On post-natal day (PND) 1, litters were culled to a uniform size—eight pups—with the goal of an even offspring sex distribution—four males and four females. An even sex distribution allows for the observation of sex-specific responses to the exposures. Pups were weaned when the youngest litter was 20 days old. From each litter, one male and one female were randomly selected for behavioral testing. The remaining six pups were raised separately for pathological analysis.

**Table 1: PFAS Exposure Dosing [ng/L]**

| PFAS Compound | Low-dose Concentration | High-dose Concentration |
|---|---|---|
| PFBS | 7.9 | 39500 |
| PFHxS | 9.6 | 48000 |
| PFOS | 15.4 | 77000 |
| PFBA | 96.1 | 480500 |
| PFPA | 158.4 | 792000 |
| PFHxA | 261.4 | 1307000 |
| PFHpA | 144.7 | 723500 |
| PFOA | 48.2 | 241000 |
| PFNA | 7.9 | 39500 |
| PFDA | 9.0 | 45000 |
| **Total** | **758.6** | **3793000** |

**Table 2: Review of Reproductive & Developmental Outcomes from Previous Studies**

| PFAS Compound | Dose (mg/kg/day) | Dosing method & timeline | Observed effects | Source |
|---|---|---|---|---|
| **Neurodevelopmental Studies** | | | | |
| **PFOS** | 0.1 / 0.3 / 1.0 | Oral dose<br><br>Pregnant females: GD 0 – PND 20 | 1.0 mg/kg/day: Low maternal body weight compared to control, increased motor activity and reduced habituation on PND 17 for male offspring only<br><br>Maternal NOAEL: 0.3 mg/kg/day (males); >1.0 mg/kg/day (females) | [52] |
| **PFOS** | 0.1 / 0.4 / 1.6/ 3.2 | Gavage<br><br>Males: 6 weeks prior to mating-mating<br>Females: 6 weeks prior to mating-lactation, across two generations | 0.4 mg/kg/day: reductions in body weight gain and feed consumption (F0); delayed eye opening (F1 pups)<br><br>1.6 mg/kg (critical dose): 50% mortality among prenatally exposed rat pups within 4 days after delivery; delayed eye opening, air righting, surface righting, and pinna unfolding in F1 pups<br><br>3.2 mg/kg/day: decreased length of gestation, number of implantation sites, and increased numbers of dams with stillborn pups | [53] |
| **PFHxS** | 0.3 / 1 / 10 | Gavage<br><br>14 days prior to mating through sacrifice (21 days of lactation for females, treatment day 42 for males) | No significant effects on functional observational battery | [54] |
| **PFOS** | 3 | Gavage<br><br>GD 2 to 21 | No effects observed in T-maze (spatial learning and memory) | [55] |
| **PFOS - mice** | 6 | Gavage<br><br>GD 12–18 | Reduced body weight on PND 4 and 8, diminished resistance during tail pull & vertical screen climb ability, diminished grip strength, temporarily decreased | [56] |

| | | | | |
|---|---|---|---|---|
| | | | number of falls in the rotarod in male offspring | |
| **Other Developmental Studies** | | | | |
| **Perfluoroundecanoic acid (PFUA)** | .1 / .3 / 1 | Gavage<br><br>Females: 41-46 days (14 days before mating to day 4 of lactation)<br><br>Males: 42 days (14 days before mating) | Low birth weight/pup weight gain (1mg/kg only), increased blood urea nitrogen (1mg/kg), increased liver weight (.3 mg/kg males; 1 mg/kg females), centrilobular hypertrophy of hepatocytes (.3 mg.kg), focal necrosis (1 mg/kg) | [57] |
| **PFOA - MICE** | 2 / 10 / 25 | Gavage<br><br>Pregnant Females: GD 11–16 | 2 mg/kg: decreased placental weight, decreased ratio of fetal weight/placental weight, increased No. of resorptions and dead fetuses<br><br>10 & 25 mg/kg: Dose-dependent effects on above measurements, fetal weight, necrotic changes in placenta | [58] |
| **PFOS** | 5 / 20 | Pregnant Females: GD 12-18 | reduced fetal body weight and placental weight (20 mg/kg/day), increased fetal serum corticosterone (5 & 20 mg/kg/day), significant downregulation of growth factors, hormones, and cell structure genes (20mg/kg/day) | [59] |
| **PFHxS** | 0.05 / 5 / 25 | Pregnant Females: GD 7 – PND 22 | 25 mg/kg/day:<br>Slightly increased liver weight in male pups<br><br>Decreased T4 levels in dams and pups (5 & 25 mg/kg/day) | [60] |

**Table 3: Experimental Timeline**

| Experimental Day | Sample Collection and Procedures |
|---|---|
| Day -7 – -5 | Begin acclimation and take baseline weight measurements |
| Day 0 | Exposure begins |
| Day 7 – 12 | Mating begins |
| Day 27 – Day 44 | Births |
| Day 57-62 (week 4) | Begin Behavioral Testing: elevated plus maze |
| Day 64-69; 85-90 (week 5 & week 8) | Behavioral testing: Figure-8 locomotor activity apparatus |
| Day 71-76 (week 6) | Behavioral testing: Novelty suppressed feeding |
| Day 78-83 (week 7) | Behavioral testing: Novel object recognition |
| Day 92-97 (week 9) | Forced swim test |

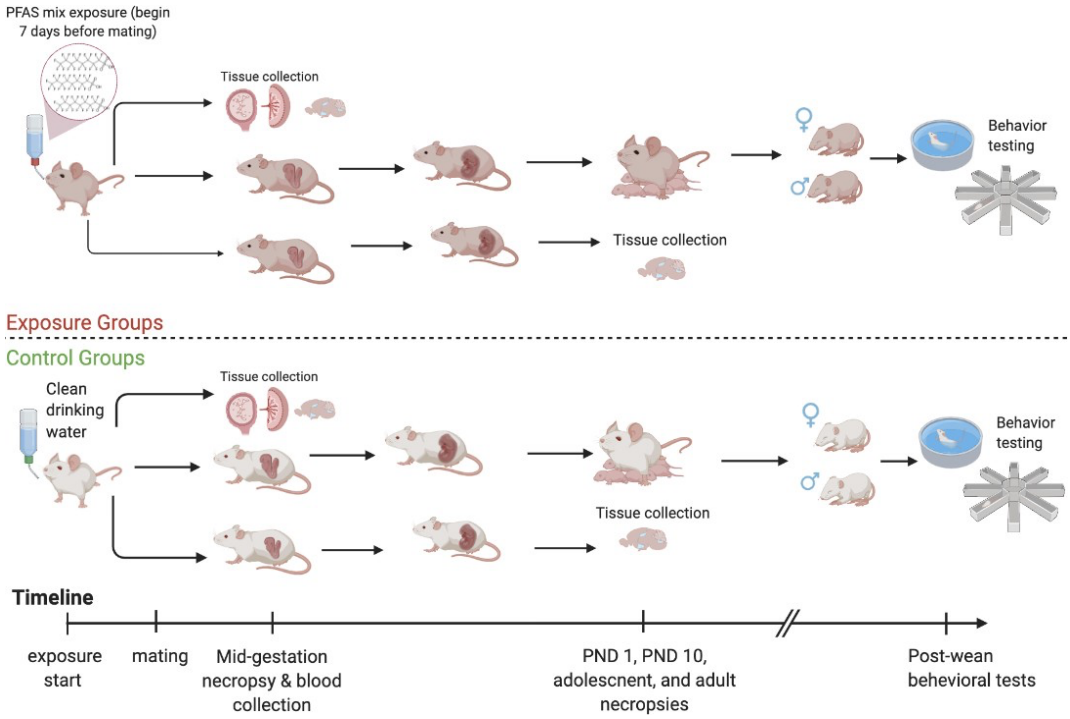


**Figure 1: Graphical representation of experimental timeline**

## *2.4 Measures*

### 2.4.1 Pregnancy Outcomes

Maternal weight was individually tracked on a weekly basis beginning with the prenatal exposure and ending at weaning. Measured pregnancy outcomes include pregnancy success after a five-day mating window, litter size, and pup survival rate.

### 2.4.2 Growth and Reflex Measurements

Neonatal development was tracked by measurements of weight and anogenital distance. Weights and anogenital distances were measured for individual pups on PND 1, 2, 4, 7, 10, 14, 17, and 21. Anogenital distance is measured using a digital caliper and is defined as the distance from the anal to the urethra opening. Two neonatal reflex tests were conducted in parallel.

*Righting Reflex*

Righting reflex ontogeny was measured on PND 2, 4, and 6. Pups are individually placed on their back in a supine position on a clean surface. The time it takes for the pup to return to the prone position is recorded. The testing session was terminated after a maximum of 30 s if pups were unable to right themselves.

*Negative Geotaxis*

The negative geotaxis reflex was evaluated on PND 7, 9, 11, and 13. The testing apparatus consists of a 45-degree slope made of a metal mesh for pups to grip onto. Pups are individually placed with their head facing down the incline and due to vestibular cues of gravity, the pups will turn to face up the slope. The time it takes for the pup to turn itself around and face upward is recorded. If the rat cannot successfully demonstrate the

reflex after a maximum of three 60 s trials, the session is concluded and considered a failure.

### 2.4.3 Neurobehavioral Assays

After weaning, adolescent offspring began a battery of behavioral tests to evaluate motor function, emotional response, and cognitive development. The battery has been repeatedly validated to measure the behavioral effects of chronic maternal toxicant exposures.[61,62] In addition to broad measures of neurotoxicity and overall health, specific tests targeting depression, memory, attention, and anxiety were conducted. Rats were tested in random order, and scoring was blind for all tests.

*Elevated Plus Maze*

At four weeks of age, observations made in the elevated plus maze (Med Associates, St Albans, VT, USA) indicate anxiety-like behavior versus risk-taking behavior. The maze dimensions are 142 cm long x 104 cm wide x 76 cm tall. Two closed arms are enclosed by 15cm high walls, and two open arms have 2 cm railings. The assay begins when an individual subject is placed on an open arm of the maze. The rat can freely enter the two open and two closed arms for a total of 300 s. The number of center crossings and time spent in closed versus open arms is measured.

*Figure-8 Locomotor Activity Maze*

Observations are made in the figure-8 maze at five weeks of age and adulthood (week 8) to assess locomotor activity and habituation. The maze dimensions are 10 cm long x 10 cm wide with a total distance of 70 cm x 40 cm. Rats are individually placed in the figure-8-shaped maze and allowed to move freely for 1 hr. Activity is measured by

photobeams at eight evenly distributed points across the maze. The number of photobeam breaks is divided into 5 min intervals and analyzed by time block.

*Novelty Suppressed Feeding*

At six weeks of age, rats' feeding behaviors are assessed in a novel environment to measure fear responsivity. Rats are fasted 24 hrs prior to the test. During the test, rats are placed in a clean rectangular plastic cage that is different from their home cage. Testing occurs in a brightly lit testing room unique to their dark home colony room. Twelve food pellets are weighed and spread across the floor of the cage. Individual rats are given 600 s to consume as many pellets as possible. Eating consists of chewing and does not include sniffing food or holding it with paws or mouth. Latency to begin eating, number of eating bouts, total time spent eating, and post-test pellet weight are recorded. For quality control, rats are also assessed in their home cage environments within one week of testing. Fasted rats are allotted 600 s to consume a pre-measured quantity of food in their home cages. The weight of food consumed in the home cage is recorded.

*Forced Swim*

At eight weeks of age, animals are individually placed into a clear glass cylinder filled halfway with 25°C water for 300 s. Immobility of rats during the session is measured as an indicator of depression-like behavior. Immobility is defined as passive floating beyond what is necessary to keep the head above water (at least three paws are not moving). Subjects are removed from the water and dried before they are returned to their home cage.

## *2.5 Analysis*

A multivariate analysis of variance (ANOVA) was used to assess main effects of exposure on dependent variables. Any significant outcomes were followed by a two-tailed *post hoc* Dunnett's test for multiple comparisons. Litter was used as the unit of variance for main effects. A non-parametric Kendall's tau correlation coefficient was obtained to determine the relationship between weight and behavioral outcomes. Results for all statistical tests were considered significant if $p < 0.05$. Summary statistics are reported as means ± standard error. Creation of figures and statistical analyses were performed in R version 4.0.2 (Boston, MA, USA).

# 3. Results

## *3.1 Pregnancy Outcomes*

Pregnancy outcomes by exposure for each cohort are presented in **Table 4**. Across both cohorts, no significant associations were found between gestational PFAS exposure and number of pregnancies, average litter size, pup mortality, or day of delivery (out of five-day birth window). However, litter size was lower on average in the high-dose group (7.5 pups/litter) compared to the control (9.5 pups/litter) and the low-dose groups (13.4 pups/litter).

**Table 4: Pregnancy outcomes stratified by cohort and exposure (N= 19 litters).**

| | Number of Pregnancies | Average Litter Size (pups/litter) | Average Pup Mortality (%) | Average Day of Birth (out of 5-day window) |
|---|---|---|---|---|
| **Cohort 1** | | | | |
| Control | 3/4 | 8.75 | 2.8 | 1 |
| Low-dose | 4/4 | 10.50 | 2.4 | 3.8 |
| High-dose | 3/4 | 10.50 | 0 | 2.7 |
| **Cohort 2** | | | | |
| Control | 3/4 | 10.25 | 7.3 | 4 |
| Low-dose | 4/4 | 16.25 | 1.5 | 2.5 |
| High-dose | 2/4 | 4.50 | 0 | 3.5 |
| **Total** | | | | |
| Control | 6/8 | 9.5 | 5.3 | 2.5 |
| Low-dose | 8/8 | 13.4 | 1.9 | 3.1 |
| High-dose | 5/8 | 7.5 | 0 | 3 |

## *3.2 Growth and Development Outcomes*

Maternal exposure to PFAS-contaminated drinking water was not found to cause significant changes in the ontogeny of righting reflex or negative geotaxis for offspring

(data presented in **Appendix Figures A1 & A2**). However, significant age-specific changes in anogenital distance and weight were observed. Across both cohorts, we observed a significant interaction of exposure and age on weight, ($F(2, 324) = 7.60$, $p = 0.001$) (see **Figure 2**). This observation prompted further analyses of the effects of PFAS exposure on pup size during the pre-weaning period. A Dunnett's test of the simple main effects on each measurement day revealed significantly lower pup weight in the low-dose exposure group on PND 21 compared to both the high and control groups ($P = 0.0003$; 0.0234).

Additionally, we found a significant interaction of exposure and age on anogenital distance, ($F(2, 262) = 4.35$, $p = 0.0138$) (see **Figure 3**). On average, pups in the low-dose exposure group had shorter anogenital distances than those in the high and control exposure groups on PND 21. However, a *post hoc* Dunnett's test did not reveal significant main effects on any of the measurement days.

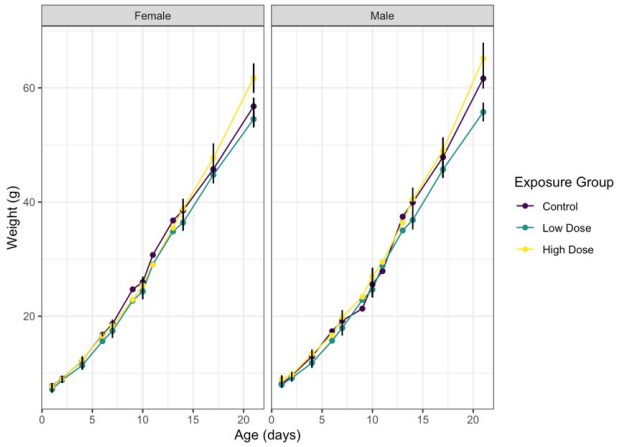


**Figure 2: Pup weight gain (PND 1 – 21) stratified by sex and maternal exposure group. N= 6 litters (control), 8 litters (low-dose), and 4 litters (high-dose).**

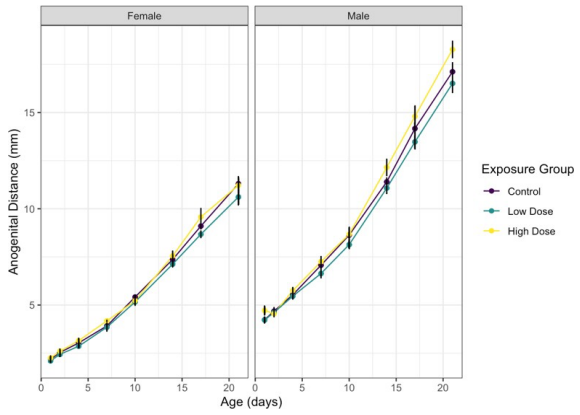


**Figure 3: Pup anogenital distance (PND 1 – 21) stratified by sex and maternal exposure group. N= 6 litters (control), 8 litters (low-dose), and 4 litters (high-dose).**

Finally, after weaning (age > 21 days), no significant relationship was observed between rat weight and exposure group.

## *3.3 Behavioral Outcomes*

*Elevated Plus Maze*

Across both cohorts, maternal PFAS exposure was not associated with significant changes in percent open arm time (see **Appendix Figure A3**). However, promising trends associated with maternal exposure were observed with the number of center crossings (see **Figure 4**). Although no significant main effects were revealed through an ANOVA, subjects in the low and high-dose exposure groups did cross the center fewer times on average than those in the control. Additionally, males consistently crossed the center more often than females. Finally, there was a significant negative correlation between weight and time spent in closed arms ($\tau = -0.29$, $p = 0.0131$).

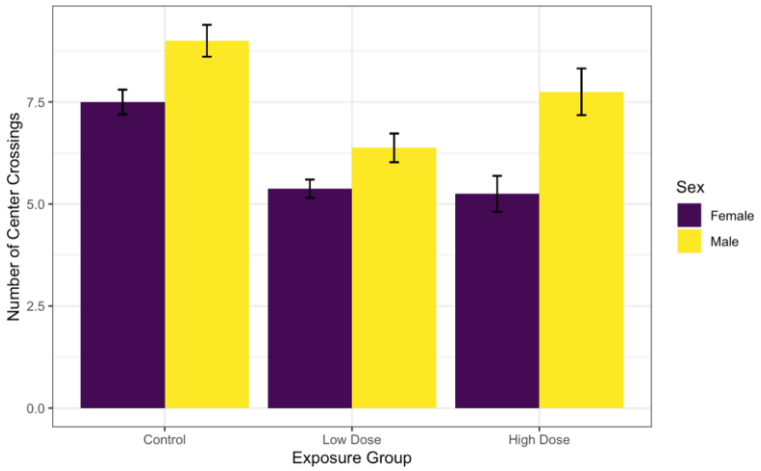


**Figure 4: Elevated plus maze (PND 28) number of center crossings (mean ± standard error). N= 6 litters (control), 8 litters (low-dose), and 4 litters (high-dose); 1 male and 1 female per litter.**

*Figure 8*

When adolescent locomotor activity was evaluated using a figure-8 maze, a main effect of maternal PFAS exposure was observed, ($F(2, 404) = 4.31$, $p = 0.0141$). Further analysis explored the exposure effects during the testing session course. According to a Dunnett's test, high-dose individuals displayed significant hyperactivity compared to low-dose individuals in time blocks 9 ($p = 0.0297$) and 10 ($p = 0.0356$).

When the data were stratified by sex, a stronger sex-specific main effect of exposure was observed, ($F(2, 404) = 6.66$, $p = 0.0015$). The one-hour habituation curve suggests slower habituation in females compared to males during early time blocks, and hyperactivity in high-dose males compared to control and low-dose males (see **Figure 5B**). Several simple main effects were found upon further examination of exposure group and time block. A Dunnett's test showed significantly reduced activity in low-dose females compared to control females in block 4 ($p= 0.0438$). Additionally, significant

hyperactivity was observed in high-dose males relative to control males in block 7 (p = 0.0286) and low-dose males in block 10 (p= 0.0459).

After rats were retested during adulthood (PND 56), a main effect of age was observed, (F(1, 812) = 13.39, p=0.0003). Ultimately, locomotor activity was higher during adulthood (see **Figure 5A**). However, no significant main effects related to maternal exposure were uncovered during adulthood. Habituation issues in adolescent high-dose males appeared to improve when they reached adulthood (see **Figures 5B** & **5C**). No significant correlations appeared between weight and activity at any age.

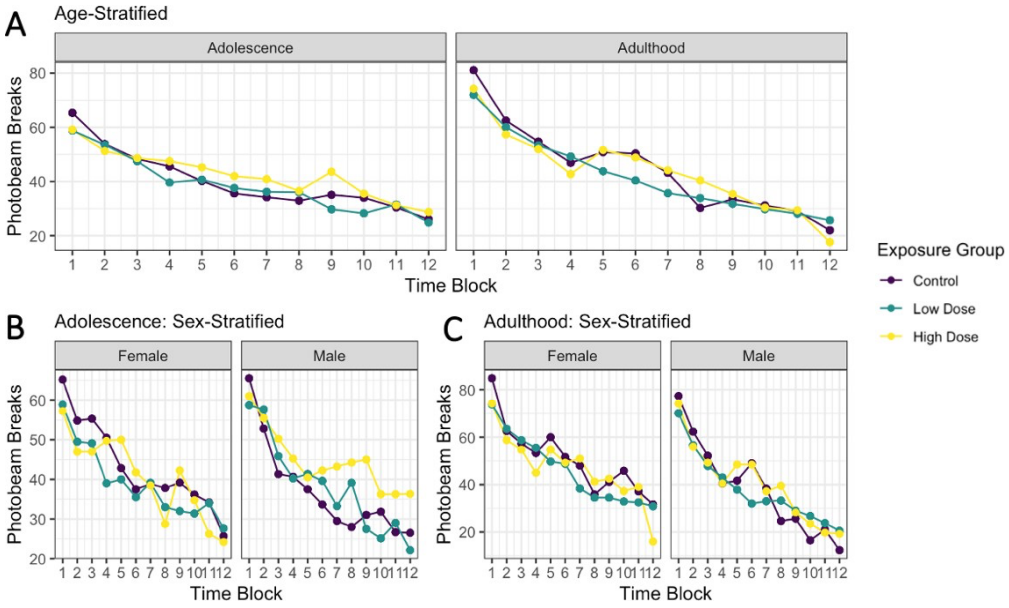


**Figure 5: (A) Adolescent versus adult figure-8 maze locomotor activity by time block (PND 35 & 56); (B) Adolescent figure-8 maze locomotor activity stratified by sex (PND 35); (C) Adult figure-8 maze locomotor activity stratified by sex (PND 56). N= 6 litters (control), 8 litters (low-dose), and 4 litters (high-dose); 1 male and 1 female per litter.**

*Novelty Suppressed Feeding*

Across both cohorts, PFAS exposure was not associated with significant changes in any measures of fear response (latency to eat, number of eating sessions, time spent eating, quantity of food consumed). Animals in the high-dose group demonstrated a longer average latency, and the effect was particularly pronounced among females (see **Figure 6A**). High-dose females also partook in the fewest number of eating sessions (see **Figure 6B**), spent the shortest amount of time eating (see **Figure 6C**), and ate the smallest quantities of food (see **Figure 6D**) compared to the low-dose and control animals. The same trend was not observed among high-dose males. No significant correlations were seen between weight and outcomes in this test.

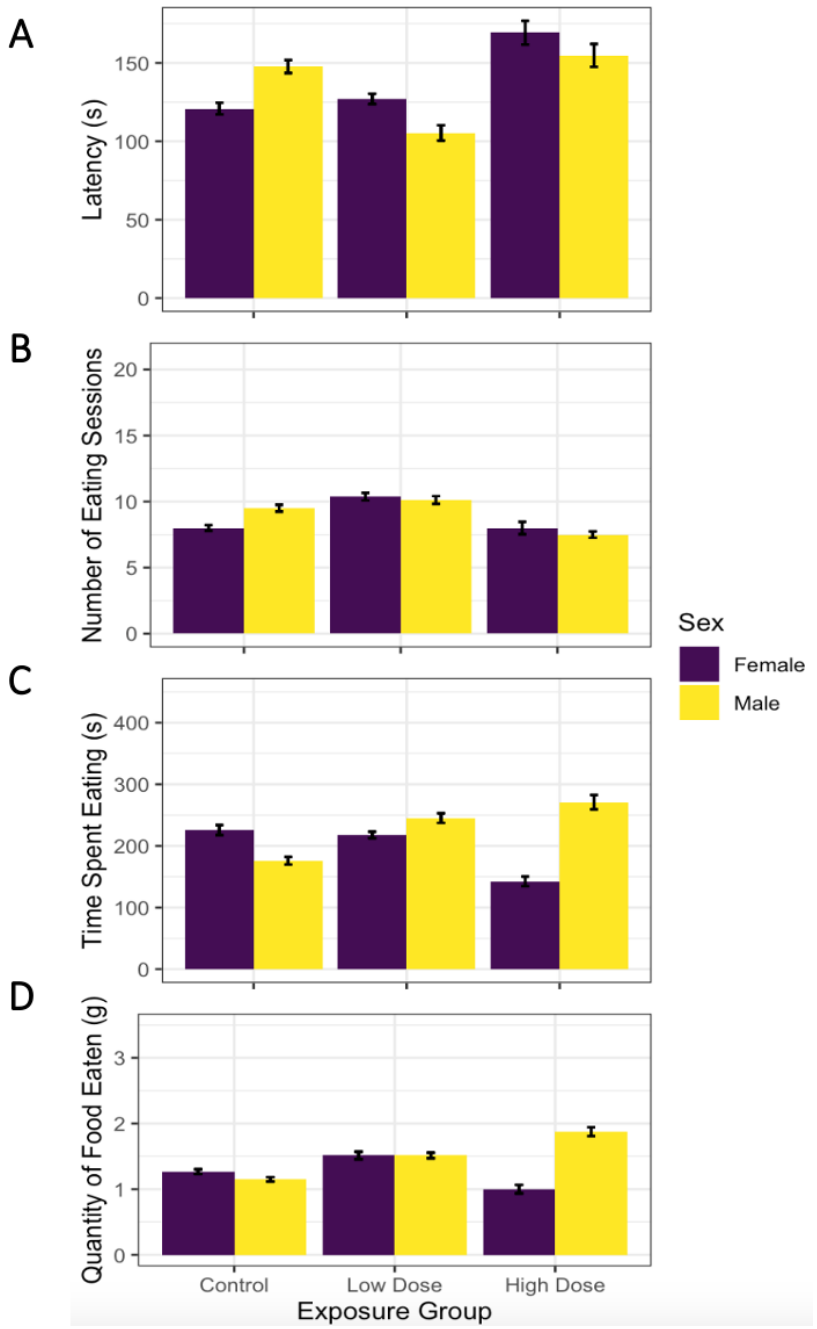


**Figure 6: (A) Latency to eat during novelty suppressed feeding (PND 42); (B) Number of eating sessions; (C) Time spent eating; (D) Quantity of food eaten. N= 6 litters (control), 8 litters (low-dose), and 4 litters (high-dose); 1 male and 1 female per litter.**

*Forced Swim*

No significant effects of maternal PFAS exposure were seen in the forced swim test for cohorts 1 and 2. On average, low-dose males spent more time immobile than control and high-dose males, but the difference was not significant (see **Figure 7**). The opposite trend was seen in females, where both PFAS-exposed groups spent less time immobile than the control group. Further, there was a significant positive correlation between weight and time spent immobile ($\tau = 0.30$, $p = 0.0108$).

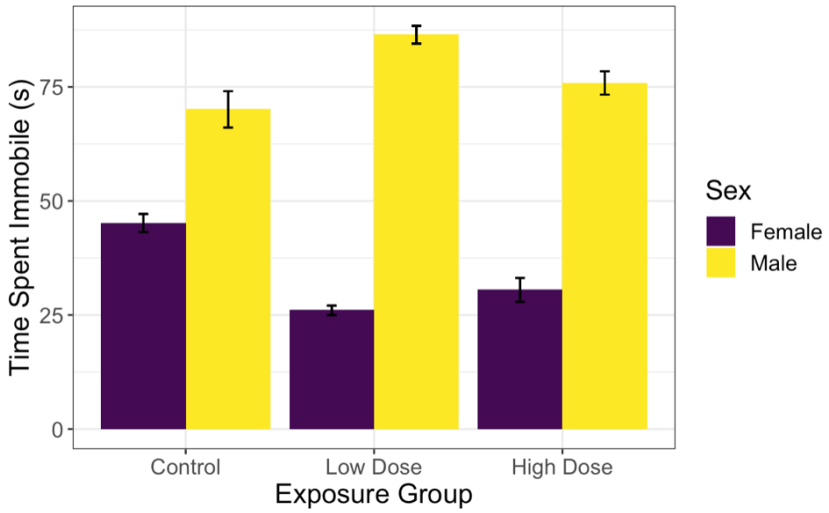


**Figure 7: Average time spent immobile (out of 300s) during forced swim test (PND 63). N= 6 litters (control), 8 litters (low-dose), and 4 litters (high-dose); 1 male and 1 female per litter.**

# 4. Discussion

In this pilot study, maternal exposures to PFAS in drinking water at environmentally relevant levels and 5,000-fold the environmental concentration were associated with developmental changes in the F1 generation. Additionally, maternal PFAS exposure demonstrated effects on fear response, habituation, and locomotor activity in the F1 generation. However, due to small sample size and the use of litter (rather than individual animal) as the unit of variance, further testing is necessary to draw definitive conclusions with sufficient statistical power.

Pups exposed to low doses of PFAS *in utero* were smaller than pups in control litters on average from birth to weaning. Pups exposed to the high dose drinking water mixture, however, were significantly larger than pups in both groups as they approached weaning age. This observation could be due to survivorship bias. The numbers of litters and pups in the high-dose groups were lower than those of the low-dose groups. The few successful pregnancies in the high-dose groups could have been more resistant to the effects of PFAS exposure, whereas the failed pregnancies were more susceptible. Further, the difference in weight on PND 21 diminished by PND 28 after offspring were weaned. Thus, weight deficits may not be due to an inability to gain weight by pups, but differences in dam milk production relative to offspring demand. This hypothesis is consistent with previous studies which observed morphological changes in mammary glands of PFAS-exposed rodents and lactational deficits in PFAS-exposed humans.[63,64]

After offspring were weaned, promising neurobehavioral trends were revealed in the domains of anxiety and locomotor activity. In the elevated plus maze assay, anxious

rats will gravitate towards dark, enclosed spaces to avoid potential threats. They will also spend less time venturing from closed to open spaces. In this study, individuals in the PFAS-exposed groups crossed through the center from one arm to another fewer times than those in the control. Hesitancy to explore different arms indicates a heightened fear response in the PFAS-exposed groups. Data from the novelty suppressed feeding test corroborated findings of increased anxiety in the highly exposed offspring, especially among females.

The figure-8 maze measures trends in locomotor activity and habituation, which can be extrapolated to human conditions such as ADHD and autism, respectively. In adolescents, females in the high-dose groups demonstrated higher activity than males during the early time blocks. The activity curve shows that high-dose females took longer to adjust to their environments. Further, males systematically demonstrated hyperactivity after habituation in the high-dose group. Adulthood figure-8 testing revealed increased hyperactivity over time, but the adolescent locomotor tendencies associated with exposure were not sustained.

Besides sex, several factors may influence behavioral outcomes such as weight, age, social isolation, handling frequency, and other individual differences.[65] To control for these potential confounders, animals were tested at the same age, were handled with equal frequency, and were doubly housed across cohorts. Weight could not be controlled because animals had *ad libitum* access to food. In the elevated plus maze, smaller animals spent more time in enclosed spaces, indicating they were more anxious. In the forced swim test, larger animals spent more time immobile, which could be due to mobility

issues in the enclosure or increased depression-like behaviors. In humans, low birth weight has been associated with increased risks for childhood behavioral issues.[66] However, there were no significant differences in birth weight among the exposure groups, so differences in birth weight are unlikely to be responsible for differences in behavioral outcomes as they relate to exposure.

Childhood overweight and obesity have also been associated with elevated PFAS exposure in human cohort studies.[67] In this study, no significant trends were observed between *in utero* PFAS exposure and post-weaning weights.

## *4.1 Study Considerations, Strengths, and Limitations*

The unique chemical properties of PFAS give rise to a dual lipophilicity and hydrophobicity, leading not only to persistence and bioaccumulation, but also accumulation in breastmilk and maternal transfer to offspring during nursing.[68,69] Although neonatal exposure during nursing is lower relative to body weight compared to maternal exposure, it is important to acknowledge that the results contain a perinatal component in addition to prenatal exposure.

The study design has several technical strengths. First, the dose range used for PFAS exposure was both appropriate for rats according to the cited rodent literature and environmentally relevant for affected populations. A high-dose positive control is strongly recommended by the EPA because it allows for more sensitive detection of results.[70] Further, the low-dose drinking water exposure directly mimics a primary human exposure pathway, aiding in animal to human extrapolation. Finally, the use of a mixture—which includes both legacy and emerging contaminants—represents realistic

human exposure scenarios in Pittsboro and other sites more effectively than single-chemical exposures.[71]

Despite the strong relevance to a contemporary human exposure scenario, limitations in the study design and timeline must be recognized. The data collection is ongoing, with four cohorts anticipated. To date, two cohorts have completed the full battery of behavioral testing and have undergone complete neonatal growth and reflex testing. Thus, sample size is currently a point of weakness, and the study is underpowered. Definitive causal relationships cannot be determined at this stage.

The lack of dose verification and toxicokinetic data also present limitations. Although drinking water mixtures were made with an analytical balance, the water samples have not been analyzed to verify PFAS concentrations. PFAS compounds are highly persistent and stable, but they have the potential to sorb to plastic and glass surfaces, so stock concentrations could change over time.[72,73] Future analysis plans include verification of PFAS concentrations in drinking water samples, offspring serum, and maternal serum from various timepoints during each cohort.

## *4.2 Implications for Further Research*

Although the preliminary results demonstrate a potential link between gestational PFAS exposure and offspring neurobehavioral changes, the toxicodynamic mode of action is still unclear. Endocrine disruption for pathways such as thyroid hormone may play a role in observed trends. In human studies, maternal thyroid hormone levels during pregnancy have been implicated in the onset of childhood

behavioral issues such as anxiety, depression, aggression, and externalizing problems.[74,75] Future study plans include concurrent measurement of maternal thyroid hormone levels in dams and offspring.

Further, the high-dose exposure groups demonstrated a lower pregnancy rate (62%) than the low-dose (100%) and control groups (75%). Coupled with a smaller average litter size (high = 7.5 pups/litter; low= 13.4 pups/litter; control= 9.5 pups/litter), the data suggest potential diminished fertility in the high-dose group. Factors underlying PFAS-associated fertility issues should be explored in future research.

## *4.3 Implications for Policy and Practice*

Enacting a substance ban or strictly limiting PFAS has sizable upfront economic costs. However, the legal, social, and health costs of inaction are far greater. Failing to reduce PFAS pollution will result in public health consequences that burden healthcare systems and adversely affect economic productivity on a national scale.

In the US, the estimated economic cost of hospitalizations and productivity losses due to PFOA-attributable low birth weight totaled 13.7 billion USD from 2003 to 2014.[76] Exposure to PFAS compounds through drinking water in the European Economic Area is associated with individual and social health costs estimated between 17 and 171 billion euros.[77] Once discovered, further costs of eliminating PFAS from the environment include water quality testing, human biomonitoring and health assessments, provision of temporary safe drinking water supplies, upgrading pipeline and water treatment plant infrastructure, improving treatment plant maintenance, and ecological restoration.[76,77]

Ultimately, governments and taxpayers may be liable for multimillion-dollar cost burdens to upgrade drinking water treatment facilities and multibillion-dollar burdens to remediate groundwater contamination.[78,79]

Global discussion surrounding the numerous consequences of PFAS pollution has prompted over 152 nations to sign onto the UNEP Stockholm Convention to restrict the production of persistent organic pollutants.[80] Although the US became a signatory in 2001, it has yet to accept the 2009 amendment, which restricts PFAS. Moreover, the Convention has not been ratified because the State Department "lack[s] the authority to implement all of its provisions."[80] The widespread inability to swiftly act on emerging contaminants can be partly ascribed to the current pace at which new chemicals are synthesized. Despite over 86,000 chemicals being reported to the EPA Toxic Substances Control Act Inventory for commercial use, only about 10,000 compounds in the database have undergone toxicity screening, and nine have been banned.[81] Chemical risk evaluations are federally required only for 20 "high priority" compounds and 20 "low-priority substances" at a time with a seven-year deadline due to the immense amount of resources required for each risk assessment.[82]

The current pollution regulation approach in the US is predominantly reactionary and assigns minimal responsibility for chemical risk assessment to chemical manufacturers. Implementation of the Precautionary Principle could reduce the enormous efforts expended by the EPA to independently assess chemical compounds and increase the proportion that is tested for toxicity. The Precautionary Principle, adopted by the European Union, serves to prevent potentially harmful environmental exposures by

requiring proof of a substance's safety before its use and release into the environment.[83] Preventative measures, rather than curative reactions, will play a significant role in alleviating the socioeconomic, ecological, and human health costs of PFAS pollution.

# 5. Conclusion

Exposure to PFAS compounds during pregnancy has the potential to detrimentally impact neurobehavioral and developmental metrics in a sex-specific manner. PFAS-exposed offspring demonstrated differential neonatal growth, a diminished fear response, slower habituation, and hyperactivity compared to unexposed offspring. The early results of this animal model study indicate that a vastly improved understanding of the long-term effects of PFAS exposure on developing babies is needed to justify their continued commercial and industrial use.

# Appendix

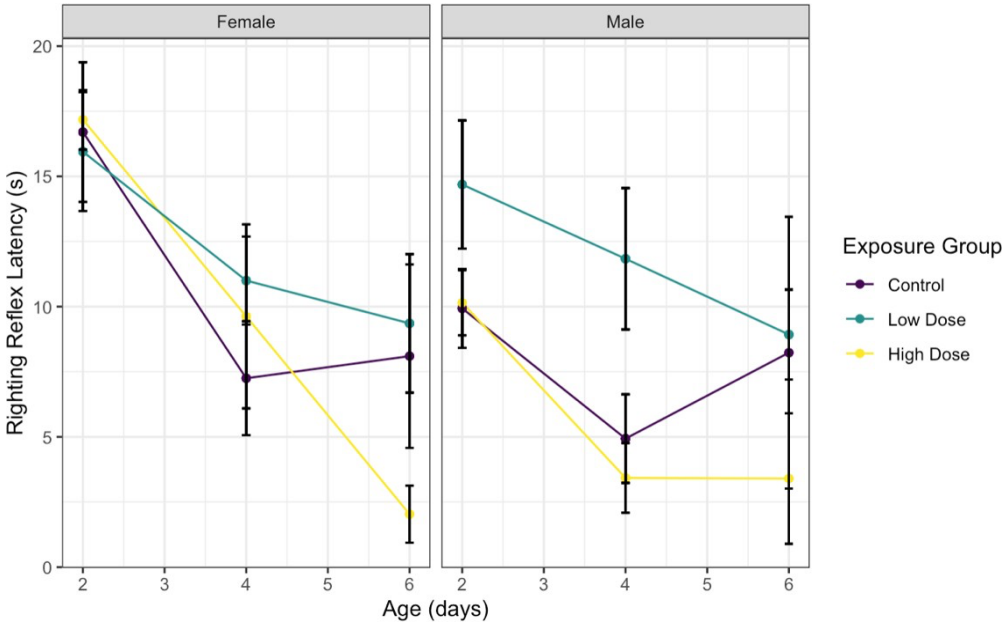


**Figure A1: Neonatal righting reflex ontogeny (PND 2, 4, 6) for cohorts 1 and 2.**

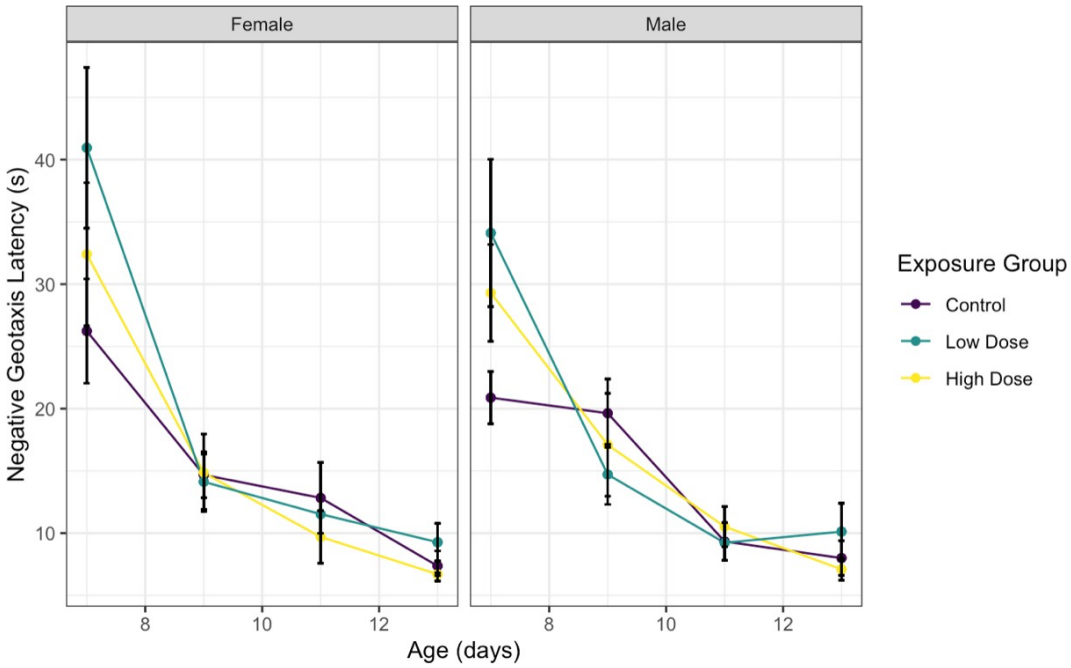


**Figure A2: Neonatal Negative Geotaxis (PND 7, 9, 11) for cohorts 1 and 2.**

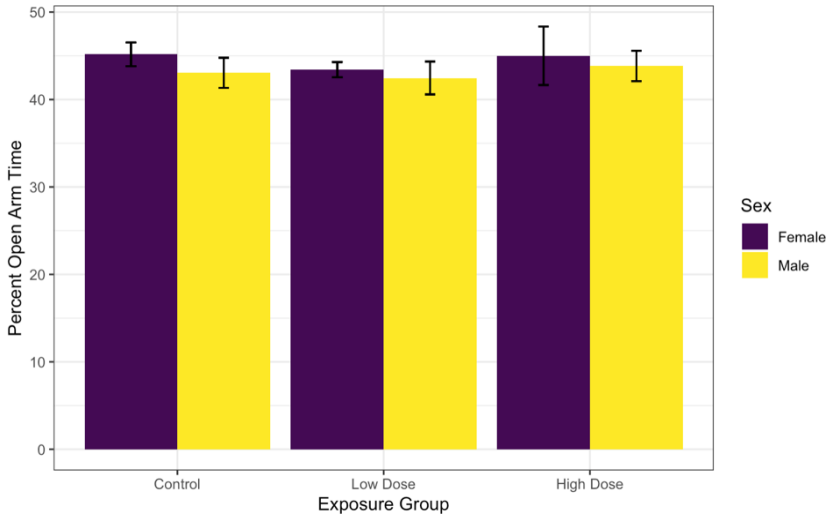


**Figure A3: Elevated plus maze (PND 28) percent open arm time (mean ± standard error). N= 6 litters (control), 8 litters (low-dose), and 4 litters (high-dose); 1 male and 1 female per litter.**

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