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Developmental exposure to the Fox River PCB mixture modulates behavior in juvenile mice

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ABSTRACT

Developmental exposures to PCBs are implicated in the etiology of neurodevelopmental disorders (NDDs). This observation is concerning given the continued presence of PCBs in the human environment and the increasing incidence of NDDs. Previous studies reported that developmental exposure to legacy commercial PCB mixtures (Aroclors) or single PCB congeners found in Aroclors caused NDD-relevant behavioral phenotypes in animal models. However, the PCB congener profile in contemporary human samples is dissimilar to that of the legacy Aroclors, raising the question of whether human-relevant PCB mixtures similarly interfere with normal brain development. To address this question, we assessed the developmental neurotoxicity of the Fox River Mixture (FRM), which was designed to mimic the congener profile identified in fish from the PCB-contaminated Fox River that constitute a primary protein source in the diet of surrounding communities. Adult female C57BL/6 J mouse dams (8–10 weeks old) were exposed to vehicle (peanut oil) or FRM at 0.1, 1.0, or 6.0 mg/kg/d in their diet throughout gestation and lactation, and neurodevelopmental outcomes were assessed in their pups. Ultrasonic vocalizations (USVs) and measures of general development were quantified at postnatal day (P) 7, while performance in the spontaneous alternation task and the 3-chambered social approach/social novelty task was assessed on P35. Triiodothyronine (T3) and thyroxine (T4) were quantified in serum collected from the dams when pups were weaned and from pups on P28 and P35. Developmental exposure to FRM did not alter pup weight or body temperature on P7, but USVs were significantly decreased in litters exposed to FRM at 0.1 or 6.0 mg/kg/d in the maternal diet. FRM also impaired male and female pups' performance in the social novelty task. Compared to sex-matched vehicles, significantly decreased social novelty was observed in male and female pups in the 0.1 and 6.0 mg/kg/d dose groups. FRM did not alter performance in the spontaneous alternation or social approach tasks. FRM increased serum T3 levels but decreased serum T4 levels in P28 male pups in the 1.0 and 6.0 mg/kg/d dose groups. In P35 female pups and dams, serum T3 levels decreased in the 6.0 mg/kg/d dose group while T4 levels were not altered. Collectively, these findings suggest that FRM interferes with the development of social communication and social novelty, but not memory, supporting the hypothesis that contemporary PCB exposures pose a risk to the developing brain. FRM had sex, age, and dose-dependent effects on serum thyroid hormone levels that overlapped but did not perfectly align with the FRM effects on behavioral outcomes. These observations suggest that changes in thyroid hormone levels are not likely the major factor underlying the behavioral deficits observed in FRM-exposed animals.

1. Introduction

Polychlorinated biphenyls (PCBs) are pervasive environmental pollutants that have been the focus of neurotoxicological studies for decades. This research has identified the developing brain as a particularly vulnerable target of PCBs (Pessah et al., 2019). Epidemiological

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studies have linked early-life PCB exposures to adverse neurodevelopmental outcomes across domains related to cognition (e.g., IQ, language, memory, learning), and attention and executive function (Schantz et al., 2003; Sprowles et al., 2022; Boucher et al., 2009; Berghuis et al., 2018). These associations have been corroborated in experimental animal models (Schantz et al., 1996, 1995; Johansen et al., 2011). More recently, emerging epidemiological evidence has raised concerns about the potential for PCBs to increase the risk of neurodevelopmental disorders (NDDs), particularly attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) (Mendola et al., 2002; Bullert et al., 2021; Xu et al., 2023; Mehri et al., 2021). The few experimental animal studies that have examined the effects of developmental PCB exposures on NDD-relevant behavioral domains support these concerns (Johansen et al., 2014; Sethi et al., 2021; Jolous-Jamshidi et al., 2010).

With a few exceptions (Sethi et al., 2021; Granillo et al., 2019), most of the published human and animal literature describing PCB effects on NDD-relevant behaviors have focused on the Aroclors and other legacy commercial PCB mixtures or individual PCB congeners found in them (Hens and Hens, 2017; Klocke et al., 2020). However, the PCB congener profile in contemporary human samples is increasingly dissimilar from that of the legacy Aroclors (Li et al., 2022; Marek et al., 2014; Koh et al., 2016). This raises critical questions of whether the developmental neurotoxicity of the legacy commercial mixtures or single PCB congeners predict neurotoxic outcomes following exposure of the developing brain to PCB mixtures more representative of contemporary human exposures. This concern is heightened by the observation that the Fox River mixture (FRM), an experimental mixture based on the PCB congener profile found in fish from the Fox River, is more potent than Aroclor 1254 with respect to sensitization of the ryanodine receptor (Kostyniak et al., 2005), a molecular initiating event in PCB developmental neurotoxicity (Bal-Price et al., 2015).

The Fox River is a major tributary of Lake Michigan that flows across central and eastern Wisconsin and provides habitat for large populations of game fish that comprise the primary protein source for several communities in surrounding areas (Christensen et al., 2016; Steenport et al., 2000). Between 1954 and 1971, numerous factories and industries that discharged effluent into the lower Fox River used PCBs to manufacture carbonless copy paper (Imamoglu and Christensen, 2002). Despite attempts to decrease PCB contamination in the Fox River via biological degradation or dechlorination of PCBs in water and sediment (Imamoglu et al., 2004; Kaya et al., 2018), high concentrations of PCBs persist in the water column of the Fox River (Guo et al., 2017). Consumption of fish from the Fox River has been positively correlated with elevated serum levels of PCBs (Schantz et al., 2010) and impaired cognitive functioning (Sprowles et al., 2022) in adolescent humans. Comparable deficits in cognitive behavior have been documented in adult rodents exposed developmentally to FRM (Sable et al., 2006). However, to date, the effects of FRM on NDD-relevant behaviors in early life have not been explored.

To address this data gap, we assessed NDD-relevant behaviors in weanling mice exposed to FRM in the maternal diet throughout gestation and lactation. Since many PCBs have been shown to reduce circulating levels of thyroid hormone (Little et al., 2022), and reduced thyroid hormone levels can negatively impact brain development (O'Shaughnessy et al., 2018; Bernal, 2000), we also quantified the effect of developmental exposure to FRM on circulating levels of triiodothyronine (T3) and thyroxine (T4) in dams and postnatal pups.

2. Materials and methods

The ARRIVE guidelines were followed in reporting experimental materials and methods.

2.1. Materials

The Fox River PCB Mixture (FRM) is a synthetic PCB mixture containing 35 % Aroclor 1242, 35 % Aroclor 1248, 15 % Aroclor 1254, and 15 % Aroclor 1260 by weight (Kostyniak et al., 2005). All Aroclors for this study, including Aroclor 1242 (catalog #: C-242 N, lot #: 0114-A), Aroclor 1248 (catalog #: C-248 N, lot #: 1063–24B), Aroclor 1254 (catalog #: C-254 N, lot #: 124–191-B) and Aroclor 1260 (catalog #: C-260 N, lot #: 021–020–1 A) were purchased from AccuStandard, Inc. (New Haven, CT, USA). The FRM was prepared by adding 525 mg of Arclor1242, 525 mg of Aroclor 1248, and 225 mg of Aroclor 1254 to a vial containing 225 mg of Aroclor 1260. The mixture was kept at 30 °C for 5 min and homogenized by shaking. Three individual aliquots of the FRM used in this study were authenticated by gas chromatograph-tandem mass spectrometry as described (Li et al., 2022). The FRM was highly similar to the original FRM (Kostyniak et al., 2005), with a similarity coefficient cos Θ of 0.986 and a relative percent difference (RPD) of 22 % (Fig. 1). The FRM used in the studies reported here was also similar to the FRM used in our previous study (Li et al., 2022), with a cos Θ of 0.979 and a RPD of 40 %. A dataset reporting the authentication of the FRM and the comparison with earlier FRM preparations is available on Iowa Research Online (Li et al., 2023).

The FRM was dissolved in peanut oil at a stock concentration of 20 mg/ml and stored in amber glass vials at −20 °C. A 12.5 μL or 125 μL aliquot of 20 mg/ml peanut oil/FRM stock was mixed into 10 g of peanut butter with a metal spatula for 5 min daily before use. Organic peanut butter (Trader Joe's, Monrovia, CA) and organic peanut oil (Spectrum Organic Products, LLC, Melville, NY) were purchased from Trader Joe's (Seattle, WA).

2.2. Animals

All procedures involving animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Washington, Seattle Institutional Animal Care and Use Committee.

C57Bl/6 J WT mice were purchased from Jackson Labs (Sacramento, CA, United States). All animals were housed in clear plastic shoebox cages containing corn cob bedding and maintained on a 12 h light and dark cycle at 22 ± 2°C with 40–50 % humidity. Feed (Diet 5010 before mating, Diet 5021 during and following mating, LabDiet, Saint Louis, MO, United States) and water were available *ad libitum*.

2.3. Exposure paradigm

A schematic of the experimental paradigm is shown in Fig. 2. Adult female nulliparous C57BL/6J mice (8–10 weeks old) were randomly divided into one of four experimental groups using a random number generator (GraphPad Prism 8 software- San Diego, CA, RRID: SCR_002798): vehicle (peanut oil in peanut butter); FRM at 0.1 mg/kg/d; FRM at 1 mg/kg/d; or FRM at 6.0 mg/kg/d. All animals were weighed daily before feeding and the amount of peanut butter was adjusted accordingly to account for weight changes throughout pregnancy and lactation. Two weeks prior to mating, dams were housed two per cage, and PCB dosing was initiated. Individual animals were placed in a clean cage containing a sterile weigh boat holding the FRM-peanut butter mixture. Animals were monitored to ensure the dose was fully consumed before they were placed back in the home cage. An age-matched male was placed in the cage for 7 d during mating. During this period, females were checked for the presence of a copulatory plug, which was considered gestational day 0. After mating, dams were housed singly and with their pups after parturition. At postnatal day 2 (P2), pups were culled or cross-fostered within dose groups to ensure all litters consisted of 6–8 pups. One male and one female pup from each litter were euthanized on P4 to collect brains for caspase-3 analyses (these data will be reported in a companion paper). After weaning at P28, pups were group-housed

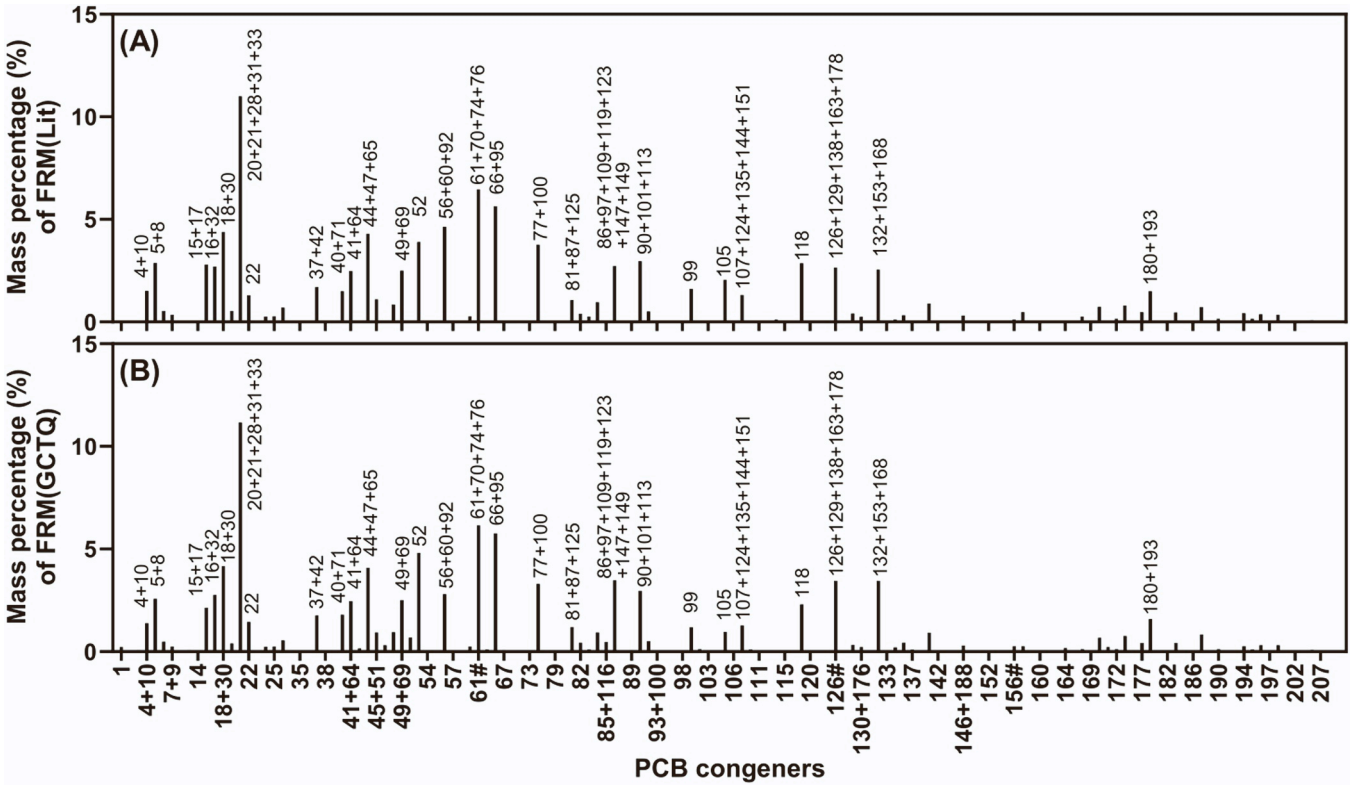


Fig. 1. Comparison of PCB congener profiles of the (A) original FRM (Kostyniak et al., 2005) and (B) the FRM prepared for this study. The original FRM was analyzed by gas chromatography-electron capture detection, whereas the FRM for this project was analyzed by gas chromatograph-tandem mass spectrometry. The PCB profiles were collapsed to give the same list of single or co-eluting PCB congeners and allow a comparison of the PCB profiles. Only PCB congeners with a mass percentage of > 1 % are shown.

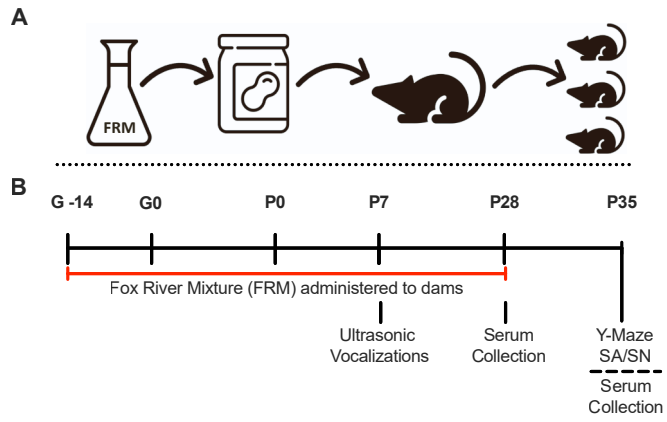


Fig. 2. Schematic of experimental design. (A) Dams were exposed to the Fox River PCB Mixture (FRM) throughout gestation and lactation via ingestion of peanut butter containing the FRM at varying doses. (B) Dams were exposed to FRM starting 2 weeks before mating and exposure continued through gestation (G) and lactation until pups were weaned on postnatal day (P) 28. Ultrasonic vocalizations were assessed at P7, and the spontaneous alternation task and social approach/novelty (SA/SN) task were both conducted at P35. Serum was collected from dams on P28 and from pups on P28 and P35 for thyroid hormone measurements.

with same-sex littermates. One male and one female pup from each litter and their respective dams were euthanized on P28; on P35, one male and one female pup from each litter were euthanized to collect brain tissues for immunohistological analyses of neuroinflammation and neurogenesis (these data will be reported in a companion paper) and serum for thyroid hormone measurement. The pups euthanized on P35 were tested in several behavioral paradigms before euthanasia (Fig. 2B).

2.4. Behavioral Tests

Ultrasonic vocalizations were recorded using an Avisoft UltraSoundGate microphone and Avisoft Recorder USGH software (version 4.2, Avisoft Bioacoustics, Glienicke, Germany). Spectrograms were analyzed using Avisoft SASLab Pro software (version 5.2, Avisoft Bioacoustics) as previously described (Brielmaier et al., 2012; Berg et al., 2018). Since ultrasonic vocalizations can vary within a litter (Rieger and Dougherty, 2016), all pups in the litter (n=6–8 pups per litter) were analyzed. Briefly, pups at P7 were removed from their home cage, and placed singly in a separate container with corn cob bedding within a sound-attenuating chamber equipped with an Avisoft UltraSoundGate microphone. Ultrasonic vocalizations were recorded for a total of 3 min. After each recording period, body mass and temperature were recorded, after which pups were returned to their home cage. The temperature of the room was maintained at 22 ± 2°C. An experienced individual blinded to exposure group manually quantified ultrasonic calls. The total number of calls per pup over the 3 min recording period was averaged across all pups within the litter. Each litter was assigned a single total number of ultrasonic vocalizations (e.g., the average USV value of each litter was the statistical unit of measure). One-way ANOVA was used to detect differences in calls with FRM dose as the variable ($p \leq 0.05$). Multiple comparisons were assessed using the Tukey *post-hoc* test ($p \leq 0.05$).

Spatial memory was assessed at P35 using the spontaneous alternation task (y-maze) performed as previously described (Adhikari et al., 2022; Kraeuter et al., 2019). The Y-shaped apparatus was made of non-reflective matte white finished acrylic ¼" thick (P95 White, Tap Plastics, Sacramento, CA, USA). The 3 arms (33.5 cm length x 6 cm width x 23 cm height) were separated by 120° between each other.

Subjects were placed at the end of the designated start arm facing the center of the Y, and the sequence of entries into each arm during the 8 min test period was recorded by a camera mounted above the apparatus. An investigator blinded to exposure group scored transitions between the three arms. The percentage of spontaneous alternations was calculated as the number of triads (entries into each of the three different arms of the maze in a sequence of three without returning to a previously visited arm) relative to the number of entries. Two-way ANOVA was used to detect differences in alternation with dose and sex as the variables ($p \leq 0.05$). Multiple comparisons were assessed using the Tukey *post-hoc* test ($p \leq 0.05$).

Sociability and social novelty were measured using the social approach and social novelty test at P35. Mice were given 3 h rest between the spontaneous alternation task and the social approach/social novelty task to reduce the potential effects of stress on the latter task. For this test, we used a three-chambered social box constructed from white plastic with removable doors as described previously (Silverman et al., 2010; Keil et al., 2019). Mice were moved into the behavioral testing room (lux level 110) in their home cage and allowed at least 30 min to acclimate prior to testing. The sociability assay consisted of one 5 min period and three 10-min periods for a total of 35 min. First, animals were placed in the center of the three-chambered arena for a 5 min acclimation period. Second, in the habituation phase, the doors were removed, and mice were allowed free exploration of the entire arena for 10 min. If an animal failed to explore during this time, they were removed from the study. Third, animals were allowed to interact for 10 min with either an empty, upside-down wire pencil cup (object) or an identical cup containing a novel age- and sex-matched "stranger mouse". The two cups were placed on opposite sides of the arena and the middle chamber was left empty. Placement of the empty cup and the cup with the mouse was counterbalanced between the left and right sides of the apparatus to eliminate side bias. Finally, the empty cup was replaced with an identical cup containing a novel age- and sex- matched WT "stranger mouse" and the test subject was given 10 min to interact with the novel and familiar stranger mice. The placement of the novel and familiar mouse was counterbalanced between the left and right sides. Both the area and the cups were cleaned with 70 % ethanol and allowed to dry completely between each subject to eliminate scent cues. Animals used as stranger mice were habituated to sitting under the wire cup for 30 min every day for 3 days prior to the testing phase to avoid erratic behavior during the testing phase of the assay. Behavior was recorded using Noldus EthoVision XT (version 11.0), an automated tracking and analysis software (Noldus Information Technology Inc., Leesburg, VA) and analyzed by an investigator blind to exposure group. Paired t-tests were used to detect within-group differences in sniff time, number of interactions, and time in chamber ($p \leq 0.05$). One-way ANOVA was used to detect between-group differences in the time spent in the middle chamber ($p \leq 0.05$). Two-way ANOVA was used to detect between-group differences in the number of entries with dose and sex as the variables ($p \leq 0.05$). Multiple comparisons were assessed using the Tukey *post-hoc* test ($p \leq 0.05$).

2.5. Thyroid hormone assay

Levels of triiodothyronine (T3) and thyroxine (T4) were measured in maternal serum collected from dams (which were 4 months old at the time of collection) at the conclusion of the FRM dosing period, and in serum from P28 and P35 pups. Blood was collected during the second half of the animals' 12-hour light cycle. Animals were anesthetized with 4 % isoflurane in medical grade oxygen and once determined to be properly anesthetized as determined by lack of response to toe pinch and tail pinch, blood was collected via cardiac stick using a 5 ml luer lock syringe (Becton Dickinson, San Jose, CA) fitted with a 30 gauge BD PrecisionGlide Needle (Becton Dickinson, San Jose, CA). Blood was transferred to a BD vacutainer blood collection tube (4 ml, Becton Dickinson, San Jose, CA) and 30 min after collection, centrifuged at 2000 *x* g for 10 min at 4°C. The supernatant (serum), was collected and placed into 2 ml cryogenic vials (ThermoFisher) and frozen at −80°C. T3 (Invitrogen, Carlsbad, CA, USA; Cat# EIAT3C) and T4 levels (Invitrogen; Cat# EIAT4C) were determined using enzyme-linked immunosorbent assays (ELISA) per the manufacturer's instructions. Concentrations of T3 and T4 hormones in serum were measured at 450 nm absorbance on a spectrophotometer microplate reader (BioTek Instruments, Winooski, VT). Samples were run in duplicates, and values were normalized to blank controls within each plate. Two-way ANOVA was used to detect differences in T3 or T4 levels with dose and sex as the variables. Multiple comparisons were assessed using the Tukey *post-hoc test* ($p \leq 0.05$).

3. Results

This study was part of a larger study designed to assess the effects of developmental exposure to the FRM on multiple endpoints, including molecular and cellular indices of neurodevelopment, the gut microbiome and microbial metabolites, and intestinal pathology. Here, we report the effects of developmental FRM exposure on behavioral endpoints in pups and circulating levels of thyroid hormones in the dams and pups.

3.1. Developmental exposure to FRM in the maternal diet altered ultrasonic vocalizations

To assess whether gestational and lactational exposure to FRM caused general maternal toxicity or interfered with overall pup development, we measured the weight of dams and pups. There was no effect of FRM on the weight of the dams measured on the final day of FRM exposure when pups were P28 (Fig. 3A). Similarly, FRM had no effect on pup weight at P7 (Fig. 3B), P28 (Fig. 3C) or P35 (Fig. 3D).

Ultrasonic vocalizations are commonly measured to assess early social communication (Sethi et al., 2021; Scattoni et al., 2009). An important component of communication between the dam and pup, pup ultrasonic vocalizations are whistle-like sounds at frequencies between 30 and 90 kHz (Grimsley et al., 2011; Branchi et al., 2001) emitted between P2 and P12 to orient maternal responsiveness to the pup for approach and/or retrieval (Cohen-Salmon et al., 1985; D'Amato et al., 2005; Yin et al., 2016). We tested the effect of gestational and lactational exposure to the FRM on the number of ultrasonic vocalizations at P7 in individual pups isolated from the dam immediately prior to testing (Fig. 4A). Male and female pups did not exhibit significantly different responses as measured by a two-way ANOVA with sex and dose group as variables ($F=1.195$, $p=0.2769$), therefore, male and female data were pooled and the average value of the entire litter was used as the statistical unit. Exposure to FRM at 6.0 mg/kg/d significantly decreased the number of vocalizations compared to vehicle controls (n=11–19 litters per dose group; $F=3.069$; $p=0.0362$). There was a trend towards decreased vocalizations in the 0.1 mg/kg/d dose group compared to vehicle control but this did not reach statistical significance ($p=0.1$). Pup weight and temperature can have a significant effect on the number of vocalizations therefore Pearson's correlation coefficient was computed to assess the linear relationship between the number of vocalizations and temperature (Fig. 3C) as well as the number of vocalizations and pup weight at P7 (Fig. 3D). Vocalizations and temperature have a weak positive association ($r=0.3292$, 95 %CI 0.06–0.55, $p = 0.0151$) whereas vocalizations and weight have a biologically negligible association ($r=0.09$, 95 %CI −0.18–0.35, $p=0.51$). Additionally, we found no significant effects of FRM on these endpoints (Figs. 3B and 4B) ($F=0.5671$, $p=0.6391$ and $F=1.088$, $p=0.3681$, respectfully), supporting the interpretation that FRM effects on vocalizations are likely not due to FRM effects on pup body temperature or weight.

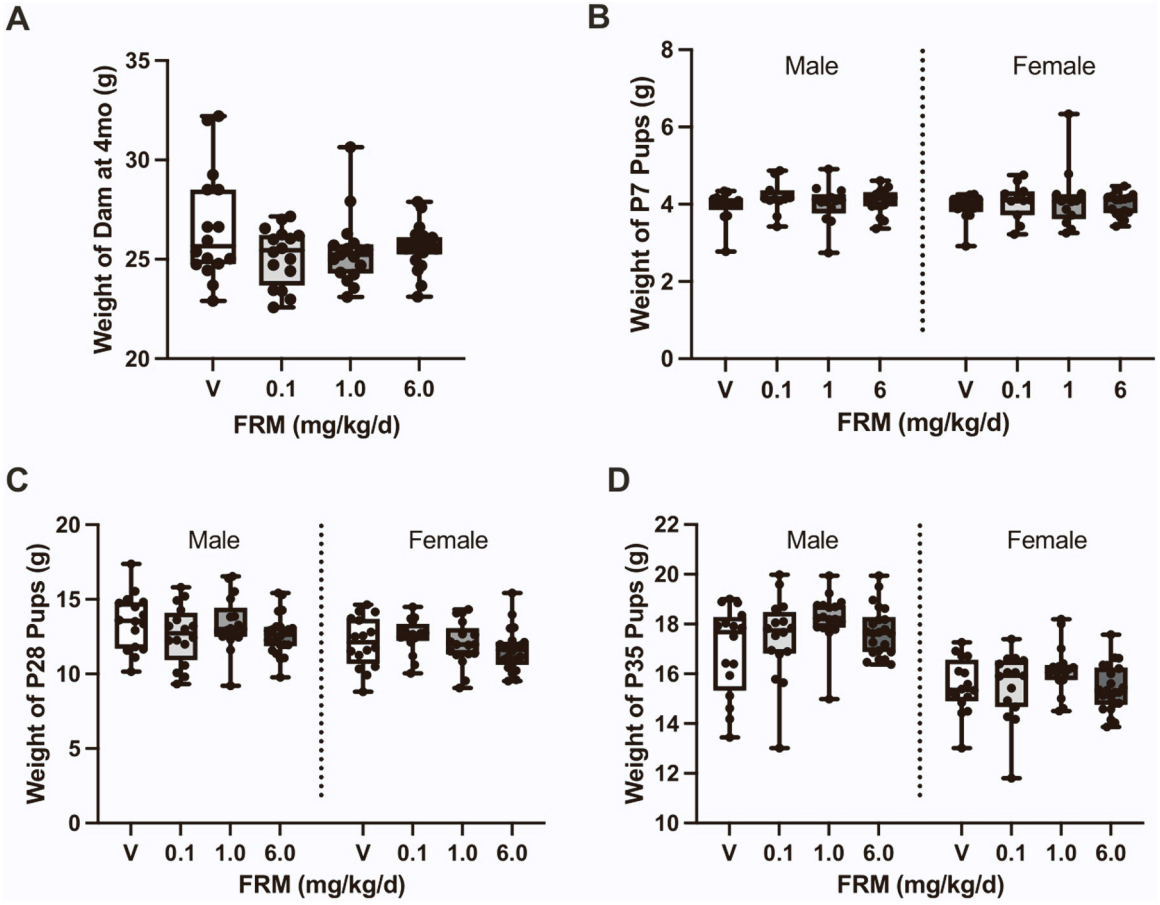


Fig. 3. Exposure to the Fox River PCB mixture (FRM) had no significant effect on maternal or pup weights. (A) Dam weight was measured on P28, which corresponded to 4 months of age. Weight of the pups at (B) P7, the day of ultrasonic vocalization testing, (C) P28, the final day of PCB exposure and (B) P35 at the conclusion of behavioral testing. Data are presented as box and whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=16–22 per dose group for pups, which included males and females; n= 16–22 per dose group for dams). There were no signfiicant differences from vehicle at $p < 0.05$ as determined using one-way ANOVA and post hoc Tukey's test.

3.2. Developmental exposure to FRM did not alter spontaneous alternation

The spontaneous alternation task assesses cognitive function through the lens of a spatial working and reference memory task (Kraeuter et al., 2019). Spatial navigation involves multiple cognitive functions, including but not limited to attention, memory, and decision-making (Berthoz and Viaud-Delmon, 1999; Brown and Chrastil, 2019), and deficits in these behaviors are associated with many NDDs (Faedda et al., 2022; Harper et al., 2023). Gestational and lactational exposure to FRM in the maternal diet did not significantly affect pup performance on the spontaneous alternation task (Fig. 5A) ($F_{(3125)}$=0.2169, p=0.8845) and no significant sex differences were identified ($F_{(1125)}$=2.367, p=0.1265). Similarly, the number of entries into the arms of the maze was not significantly altered by FRM exposure when compared to vehicle control using post hoc Tukey test (Fig. 5B)($F_{(3109)}$=1.163, p=0.3274), and there were no significant sex differences ($F_{(1109)}$=1.445, p=0.2319).

3.3. Developmental FRM exposure did not significantly affect social approach behavior

Deficits in social behavior are a core symptom of ASD and a component of many other NDDs (Association A.P. American psychiatric association. 2019; Lord et al., 2000). The three-chambered social approach task (Crawley, 2012; Nadler et al., 2004) was used to measure levels of sociability in FRM-exposed pups at P35. In this test, sociability is defined as the amount of time the test subject spends with an age- and sex-matched "stranger" mouse compared to an inanimate object, in this case, an empty inverted wire pencil cup (Silverman et al., 2012). Normal mice will spend significantly more time with the stranger mice than with the inanimate object. FRM-exposed groups performed similar to vehicle controls in all aspects of the social approach task. We found that FRM-exposed pups and vehicle controls of either sex spent significantly more time sniffing or interacting with the stranger mouse than with the empty cup (Fig. 6A, B). Additionally, in both FRM-exposed pups and sex-matched vehicle controls, the number of interactions (Fig. 6C, D) and time spent in the chamber (Fig. 6E, F) with the stranger mouse was significantly higher compared to the empty cup. The time spent in the middle chamber was analyzed using a one-way ANOVA and there were no significant differences in the males (F=1.508, p=0.2230) or females (F=1.153, p=0.3372), therefore, these were not included in further analysis. The total number of entries was quantified to rule out potential differences in overall activity between dose groups during the task. There were no significant differences between FRM-exposed pups and sex-matched vehicle controls ($F_{(3120)}$=0.3553, p=0.7854). (Fig. 6G). Statistical analyses are reported in Table 1.

3.4. Developmental exposure to FRM significantly altered social novelty and memory

The social novelty test was conducted to determine whether

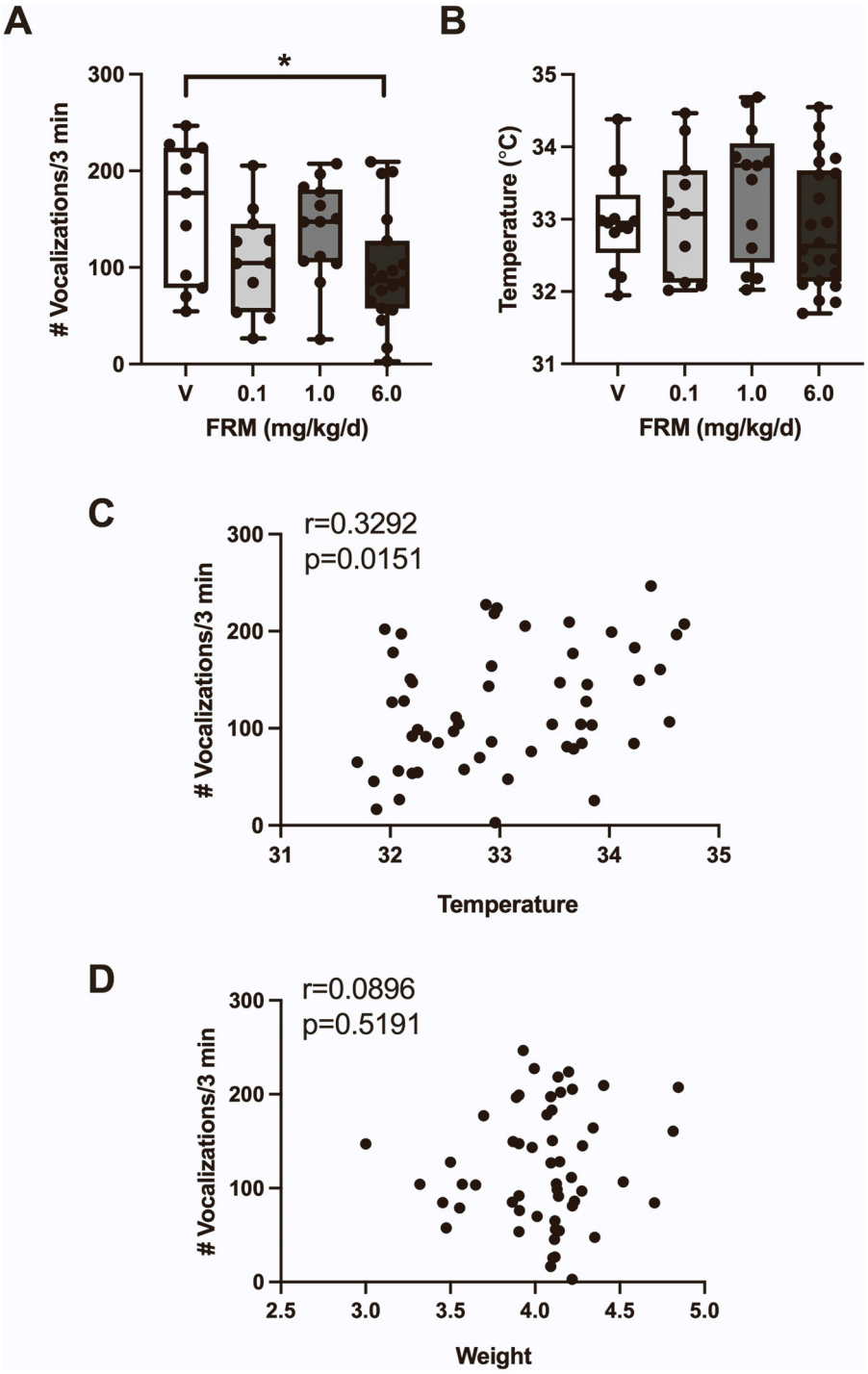


Fig. 4. Developmental exposure to the Fox River PCB mixture (FRM) altered social communication of P7 pups. (A) Each individual pup was placed into a soundproof chamber to record ultrasonic vocalizations for 3 min; the average of all pups in one litter was used as the statistical unit of measure. Data are presented as total number of vocalizations during the 3-min trial. (B) Temperature of pups measured immediately following isolation from the dam (n=11–19 litters per dose group, each point is one litter). Data are presented as box and whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=11–19 litters per dose group, statistical unit is litter). *Significantly different from the vehicle at $p < 0.05$ as determined using one-way ANOVA and *post hoc* Tukey's test. Pearson's correlation was used to determine if there were linear relationships between (C) vocalizations and temperature of pups at P7 (or (D) vocalizations and weight of pups (n=54 pairs). Data are presented as scatterplots with each dot representing the average value of an individual litter.

developmental exposure to FRM in the maternal diet altered social learning and preference for social novelty (Nadler et al., 2004; Moy et al., 2004; Yang et al). In this test, preference for social novelty and social memory is defined as the test subject spending significantly more time with a novel stranger mouse than the previously introduced, familiar stranger mouse (Silverman et al., 2010). Male (Fig. 7A) and female (Fig. 7B) pups in the 0.1 and 6.0 mg/kg/d FRM dose groups showed significantly decreased preference for social novelty compared to sex-matched vehicle controls as indicated by no significant differences in the time spent sniffing the novel *versus* familiar stranger mouse.

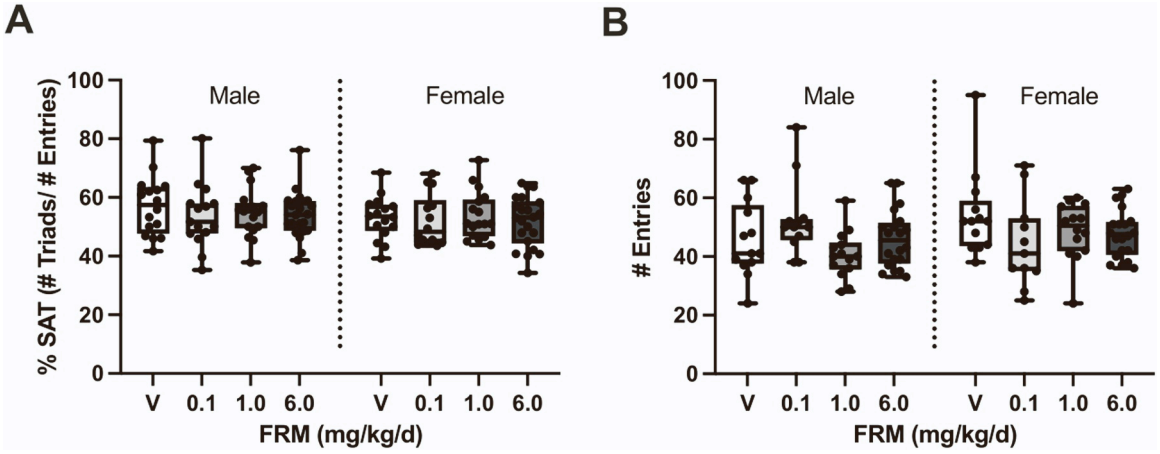


Fig. 5. Developmental exposure to FRM had no significant effect on performance of P35 pups in the spontaneous alternation (y-maze) task**.** Pups were placed into the y-maze apparatus to explore for 8 min. (A) The percentage of SAT (spontaneous alternating triads divided by the total number of entries). (B) Total number of entries between arms of the y-maze apparatus. Data are presented as box and whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=15–22 pups per sex per exposure group). *Significantly different from vehicle at $p<0.05$ as determined by two-way ANOVA with sex and dose as the variables followed by Tukey's *post hoc* analysis.

Measurement of the number of interactions with the novel versus familiar stranger mouse revealed significant differences between dose groups amongst female, but not male, pups (Figs. 7C and 7D). Specifically, female pups in the 0.1 and 6.0 mg/kg/d dose group showed a significantly decreased preference for social novelty (Fig. 7D). Relative to sex-matched controls, the amount of time spent in the chamber with the novel *versus* familiar stranger mouse was not significantly different in male pups exposed to FRM at 0.1 mg/kg/d in the maternal diet (Fig. 7E) or in female pups exposed to FRM at 0.1 or 6.0 mg/kg/d in the maternal diet (Fig. 7F). The time spent in the middle chamber was analyzed using a one-way ANOVA and there were no significant differences in the males (F=2.401, p=0.0808) or females (F=0.7021, p=0.555), therefore, these were not included in further analysis. There were no significant differences in the number of total entries made by FRM-exposed pups of either sex compared to sex-matched vehicle controls (Fig. 7G)($F_{(3106)}$=1.274, p=0.2872). Statistical analyses are reported in Table 1.

3.5. *Dose, sex, and age-dependent effects of developmental FRM exposure on circulating levels of thyroid hormones*

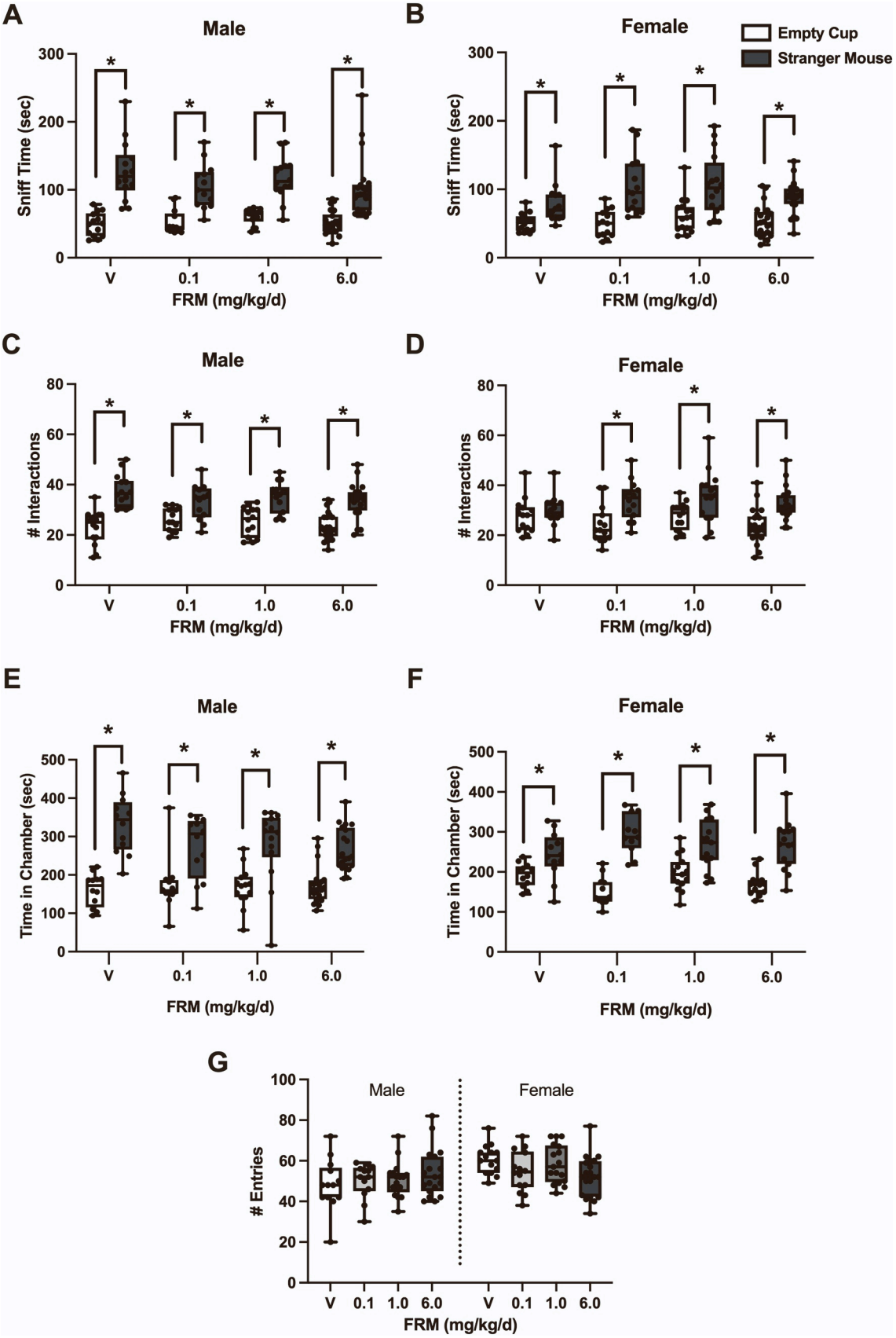
In P28 males, serum T3 levels (Fig. 8A) were significantly increased while serum T4 levels (Fig. 8B) were significantly decreased in the 1.0 and 6.0 mg/kg/d dose groups (F=2.796, p=0.0432, F=2.825, p=0.0412). In contrast, there were no significant effects of FRM on T3 or T4 levels in P28 females (Fig. 8A, B). At P35, FRM had no significant effect on serum T3 levels in males, but significantly decreased serum T3 levels in females in the 6.0 mg/kg/d dose groups (Fig. 8C) (F=6.401, p=0.0012). The FRM did not significantly alter serum T4 levels at P35 in either males or females (Fig. 8D) (F=0.4866, p=0.6935). Measurement of thyroid hormone levels in serum collected from dams at weaning (when pups were P28), revealed a significant decrease in T3 levels in the 6.0 mg/kg/d dose group (Fig. 8E) (F=3.686, p=0.0314), but no significant effects of FRM on T4 levels (Fig. 8F) (F=1.959, p=0.1584).

Pearson correlation was used to assess whether there was a linear relationship between the serum T3 and T4 levels of P35 pups and the behavioral endpoints measured in this study. Ultrasonic vocalizations were not correlated with T3 levels (Fig. 9A) (r=0.1658, 95 %CI −0.1536–0.4539, p=0.3066) or T4 levels (Fig. 9B) (r=0.2579, 95 % CI −0.0582–0.5271, p=0.1081). There were also no significant correlations between T3 levels and spontaneous alternation performance (Fig. 9C) (r=0.0763, 95 % CI −0.2410–0.3788, p=0.64), social approach behavior (Fig. 9E) (r=-0.1959, 95 % CI −0.4783–0.1231, p=0.2256), or social novelty behavior (Fig. 9G) (r=0.1669, 95 % CI −0.1710–0.4697, p=0.3307). Similarly, T4 levels also did not correlate with the spontaneous alternation (Fig. 9D) (r=-0.1187, 95 % CI −0.4148–0.2003, p=0.4658), social approach (Fig. 9F) (r=0.0549, 95 % CI −0.2611–0.3602, p=0.7365), or social novelty behavioral tasks (Fig. 9H) (r=0.05191, 95 % CI −0.2814–0.3741, p=0.7637).

4. Discussion

It has previously been reported that consumption of PCB-contaminated fish from the Fox River is associated with cognitive deficits in adolescent humans (Sprowles et al., 2022) and in adult mice exposed developmentally to FRM (Sable et al., 2006). Our findings from this studies extend these observations by demonstrating effects of developmental exposure to the FRM, a human-relevant PCB mixture, altered early communication and social novelty tasks in weanling mice. Additionally, we found that exposure to the FRM altered serum thyroid hormone levels in pups and dams, but these changes were inconsistent and exhibited dose-response relationships that varied from those of the FRM-associated behavioral effects. These observations provide further support for the hypothesis that PCBs are putative risk factors for NDDs, and in particular, ADHD and ASD (Keil-Stietz and Lein, 2023; Panesar et al., 2020).

Social behaviors like communication, sociability, and social memory are critical cognitive abilities that are impaired in humans diagnosed with NDDs (Shultz et al., 2018; Khalil et al., 2018; Harpin et al., 2016) and in rodent models of NDDs (Moy et al., 2004; Ricceri et al., 2007; Kight et al., 2021; Silverman and Ellegood, 2018). Our data demonstrate that exposure to the FRM in the maternal diet during gestation and lactation caused deficits in human-relevant social behaviors in weanling mice. Communication is a complex social behavior mediated by multiple brain regions, including the medial prefrontal cortex, amygdala, and anterior cingulate cortex (Lovinger, 2008; Gan-Or and London, 2023; Frith, 2007). In early life, communication aids in maternal and paternal care; in later life, communication is key to developing and maintaining social relationships (Portfors, 2007; Motomura et al., 2002). To assess early-life communication, we measured the ultrasonic vocalizations of P7 pups following maternal isolation and found that developmental exposure to FRM caused non-monotonic dose-dependent deficits. The number of USVs can be negatively impacted by decreased pup weight or temperature, which are reflective of general development and maternal care, respectively (Hahn and Lavooy, 2005). These endpoints did not differ between exposure groups, indicating that the decreased USVs



(caption on next page)

Fig. 6. Developmental exposure to FRM in the maternal diet had no significant effect on social approach of P35 pups. During the social approach trial, pups were placed in a 3-chambered apparatus and following a period of habituation, were given 10 min to interact with an empty cup and a cup with an age- and sex-matched "stranger" mouse placed inside. Social approach data are presented as a comparison of the time spent sniffing the stranger mouse versus the empty cup in (A) males and (B) females (n=14–20 pups per sex per exposure group). The total number of interactions were quantified in (C) males and (D) females. The time spent in the chambers corresponding with the empty cup and stranger mouse were quantified in (E) males and (F) females. (G) Total number of entries into any of the chambers. Data are presented as box and whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values. (A-F) *Significantly different at $p < 0.05$ as determined using paired t-test. (G) *Significantly different at $p < 0.05$ from vehicle control as determined using two-way ANOVA and post hoc Tukey's test.

Table 1
Statistical analysis conducted on Social Approach and Social Novelty behavioral tests. Paired t-tests were used to compare time with the stranger mouse versus the empty cup for the social approach task and the novel mouse versus familiar mouse for the social novelty task.

Task	Sex	Exposure Group	Sniff Time			# Interactions			Chamber Time		
			t-value	DF	p-value	t-value	DF	p-value	t-value	DF	p-value
Social Approach	**Male**	**Vehicle**	5.59	14	<0.0001	5.38	13	0.0001	5.17	11	0.0003
		0.1 mg/kg/d	5.51	13	0.0001	3.66	12	0.0033	4.227	11	0.0014
		1.0 mg/kg/d	7.35	14	<0.0001	5.65	13	<0.0001	5.24	13	0.0002
		6.0 mg/kg/d	6.51	22	<0.0001	6.08	21	<0.0001	4.97	19	<0.0001
	Female	**Vehicle**	4.16	13	0.0012	**1.19**	**13**	**0.2547**	2.21	11	0.0492
		0.1 mg/kg/d	4.34	13	0.0008	3.15	13	0.0076	6.78	10	<0.0001
		1.0 mg/kg/d	4.19	16	0.0008	2.86	14	0.0126	2.95	14	0.0105
		6.0 mg/kg/d	5.89	20	<0.0001	6.54	21	<0.0001	5.44	14	<0.0001
Social Novelty	**Male**	**Vehicle**	4.17	12	0.0013	4.31	12	0.001	3.26	11	0.0076
		0.1 mg/kg/d	**1.76**	**13**	**0.1023**	2.767	13	0.016	**1.04**	**11**	**0.3208**
		1.0 mg/kg/d	3.74	8	0.0057	2.879	8	0.0205	2.35	9	0.0434
		6.0 mg/kg/d	**1.26**	**14**	**0.2292**	3.076	14	0.0082	2.56	14	0.0224
	Female	**Vehicle**	3.79	14	0.002	3.05	14	0.0086	4.25	11	0.0014
		0.1 mg/kg/d	**1.46**	**13**	**0.1685**	**1.71**	**13**	**0.1126**	**0.88**	**10**	**0.4011**
		1.0 mg/kg/d	3.41	15	0.0039	4.162	15	0.0008	4.05	14	0.0012
		6.0 mg/kg/d	**0.08**	**20**	**0.94**	**0.62**	**20**	**0.5421**	**1.12**	**18**	**0.2764**

observed in FRM-exposed pups are likely due to the direct effects of PCBs on neural circuits that regulate this form of communication. These findings are consistent with previous reports that ultrasonic vocalizations were significantly decreased in P7 mice developmentally exposed to the MARBLES PCB mixture (Sethi et al., 2021), which replicates the congener profile of the 12 most abundant PCBs detected in the serum of Northern California women at increased risk for having a child diagnosed with an NDD (Sethi et al., 2019). Ultrasonic vocalizations were also decreased in P10 rat pups following developmental exposure to a mixture of PCB 47 and PCB 77 at 25 ppm, but not 12.5 ppm (Krishnan et al., 2014).

Sociability and social memory are two other important measures of social behavior that are often assessed in neurotoxicological studies (Grandjean and Landrigan, 2014; Miodovnik et al., 2011). Using the three-chambered social approach task, we found no effects of developmental exposure to FRM on general sociability in the mice at P35 (Silverman et al., 2010). This observation is consistent with a previous study of Sprague Dawley rats that reported exposure to Aroclor 1221 in the maternal diet during gestation and lactation did not alter social approach in adult animals (Reilly et al., 2015). In contrast, in mice exposed to the MARBLES mix in the maternal diet during gestation and lactation, sociability was decreased in male, but not female, weanling mice exposed to this PCB mixture at 0.1, but not 1.0 or 6.0 mg/kg/d (Sethi et al., 2021). These observations suggest that PCB mixtures with differing congener profiles may differentially alter sociability.

In contrast to social approach, the social novelty task measures preference for social novelty as a type of working social memory. We found that developmental exposure to FRM significantly decreased social novelty in male and female pups in the 0.1 and 6.0 mg/kg/d dose groups. When the social novelty data were analyzed as the number of interactions or the time spent in each of the chambers, males had no significant group differences, but amongst females, these measures were significantly decreased in the same dose groups that showed decreased social novelty. These findings suggest that developmental exposure to FRM had stronger effects on social novelty in females than males. Importantly, we observed no effects of FRM exposure on the number of entries and number of interactions, indicating that dose group differences in the social novelty test were not due to effects of FRM on activity or interest levels. While studies assessing the effects of developmental PCB exposure on working social memory are limited, similar deficits in social novelty have been reported in adult rats exposed developmentally to Aroclor 1221 (Reilly et al., 2015).

Spatial memory and navigation are highly dependent on executive function skills, including attention, visual discrimination, perception, and sensory discrimination (Faedda et al., 2022). These skills are often disrupted following exposures to PCBs (Bastien et al., 2022). However, we found that developmental exposure to the FRM did not cause deficits in performance in the spontaneous alternation task, which is a test of spatial working memory. The lack of effect of the FRM on this behavior was not due to possible FRM effects on activity level since we observed no significant differences in the number of entries between exposure groups. This finding further confirms that FRM effects on social novelty are not due to alterations in the level of exploration. To our knowledge, there are no published studies of PCB effects on performance in the y-maze, but studies that employed alternative tests of spatial memory, including the radial arm maze or Morris Water maze, have similarly reported no or minimal effects of developmental PCB exposure. For example, Sprague-Dawley rats exposed throughout gestation and lactation to PCB 28, PCB 118, or PCB 153 exhibited no significant differences in latency to complete the 8-arm radial maze, with the exception of males exposed to PCB 153 at a high dose of 64 mg/kg/d, which were impaired relative to vehicle controls (Schantz et al., 1995). In a later study, this same group assessed coplanar PCBs and found small deficits in spatial memory using the radial arm maze (Schantz et al., 1996). Varying impacts on performance in the Morris water maze have been reported by three different labs that studied Long-Evans rats exposed throughout gestation and lactation to Aroclor 1254 in the maternal diet. Two studies observed no impairment in Morris water maze performance in 3-month-old animals exposed to Aroclor 1254 at 6.0 mg/kg/d (Gilbert et al., 2000) or 8 mg/kg Aroclor 1254 (Zahalka et al., 2001). However, a third independent study found that weanling rats exposed to Aroclor 1254 in the maternal diet at 1, but not 6,

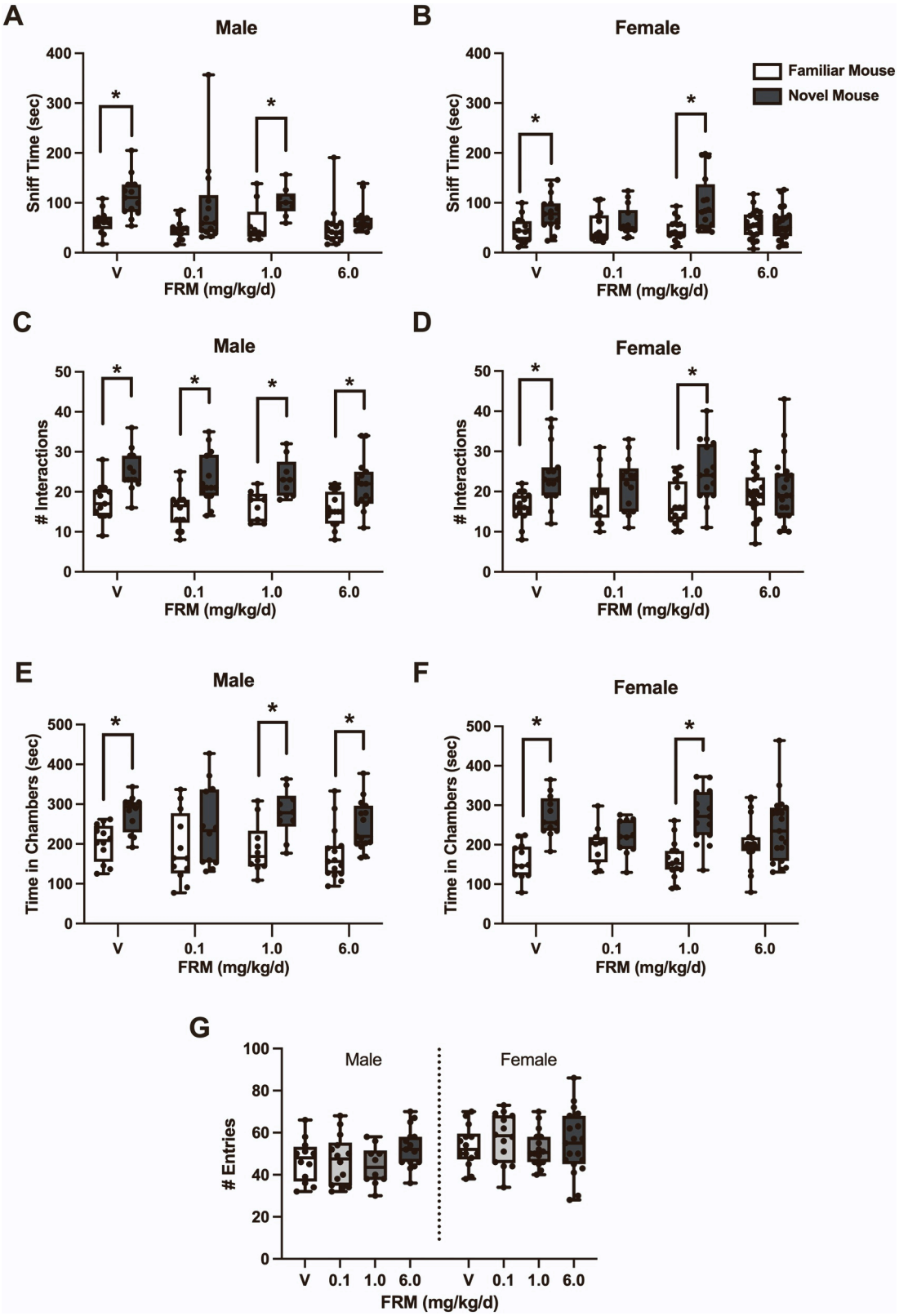


Fig. 7. Dose- and sex-dependent effects of developmental exposure to FRM on social novelty trial conducted at P35. The social novelty trial was conducted immediately following the conclusion of the social approach trial using the same 3-chambered apparatus. Animals were given 10 min to interact with the same stranger mouse used in the social approach trial and a cup with a novel stranger mouse. (A, B) The amount of time spent with the novel mouse compared to the familiar stranger mouse in males (A) and females (B). (C, D) The total number of interactions during the social novelty trial in males (C) and females (D). (E, F) The time spent in the chambers corresponding with the familiar stranger mouse and novel stranger mouse in males (E) and females (F). (G) Total number of entries into all chambers in males and females. Data are presented as box and whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=14–21 pups per sex per dose group). (A-F) *Significantly

different at $p < 0.05$ as determined using paired t-test. (G) *Significantly different at $p < 0.05$ from vehicle control as determined using two-way ANOVA and post hoc Tukey's test.

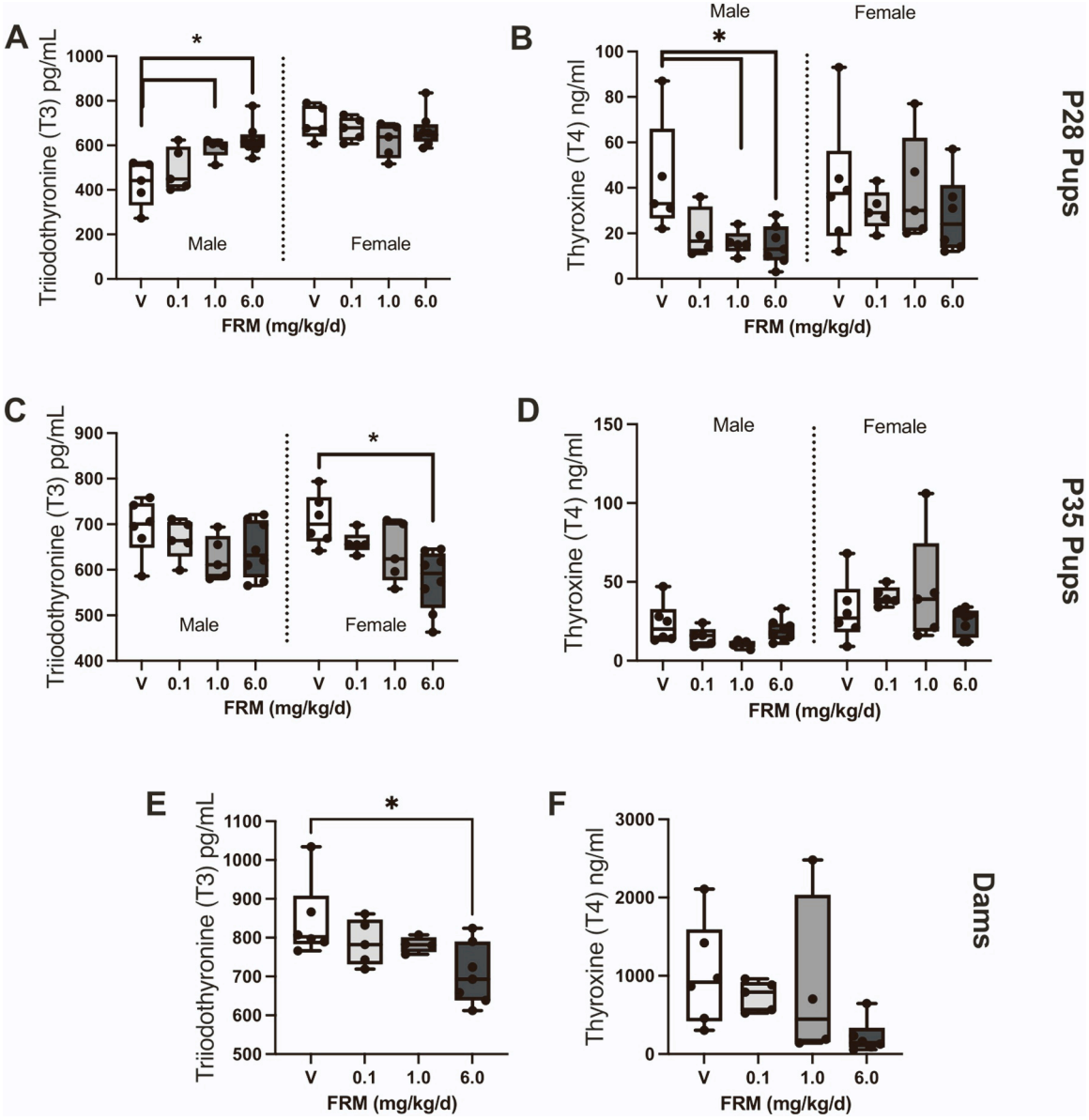


Fig. 8. Dose-, sex- and age-dependent effects of developmental exposure to FRM on serum thyroid hormone levels. Serum was collected from pups upon euthanasia at P28 (A, B), P35 (C, D) and from dams when pups were weaned at P28 (E, F). All serum samples were analyzed for levels of triiodothyronine (T3) (A, C, E) and thyroxine (T4) (G, B, F) at P35 was analyzed for (C) T3 and (D) T4 hormones. Dam serum was collected at the P28 tissue collection timepoint and was analyzed for (E) T3 and (F) T4 (n=4–7 per dose group). Data are presented as box and whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=5–8 pups per sex per dose group; n = 4–7 dams per dose group). *Significantly different from the vehicle at $p < 0.05$; pup data were analyzed using two-way ANOVA with sex and dose as variables, and *post hoc* Tukey's test; dam data were analyzed using one-way ANOVA and *post hoc* Tukey's test.

mg/kg/d were significantly impaired in the Morris water maze task (Yang et al., 2009). Collectively, these findings suggest the possibility that the y-maze is not sensitive enough to reveal spatial memory deficits and/or that PCB effects on spatial memory are congener-specific and exhibit a non-monotonic dose-dependent relationship.

It is widely posited that PCBs interfere with normal neurodevelopment by decreasing circulating levels of thyroid hormone (Gauger et al., 2004). This premise originated from the well-documented importance of thyroid hormone in brain development (Bernal, 2000; Prezioso et al., 2018), as well as evidence that PCBs can decrease blood levels of thyroid hormones in humans (Curtis et al., 2019; Berlin et al., 2021) and experimental animal models (Sethi et al., 2021; Martin and Klaassen, 2010; Kato et al., 2003). However, there is also evidence that not all developmental PCB exposures decrease blood levels of thyroid hormones (Sethi et al., 2021; Yang et al., 2009). Here, we observed that gestational and lactational exposure to FRM resulted in significant sex, age–and dose-dependent changes in serum thyroid hormone levels in the dams and pups. Most of the significant changes were observed in the 1.0 and 6.0 mg/kg/d dose groups but the magnitude of the change did not increase with increasing dose. Male pups exhibited increased T3 levels

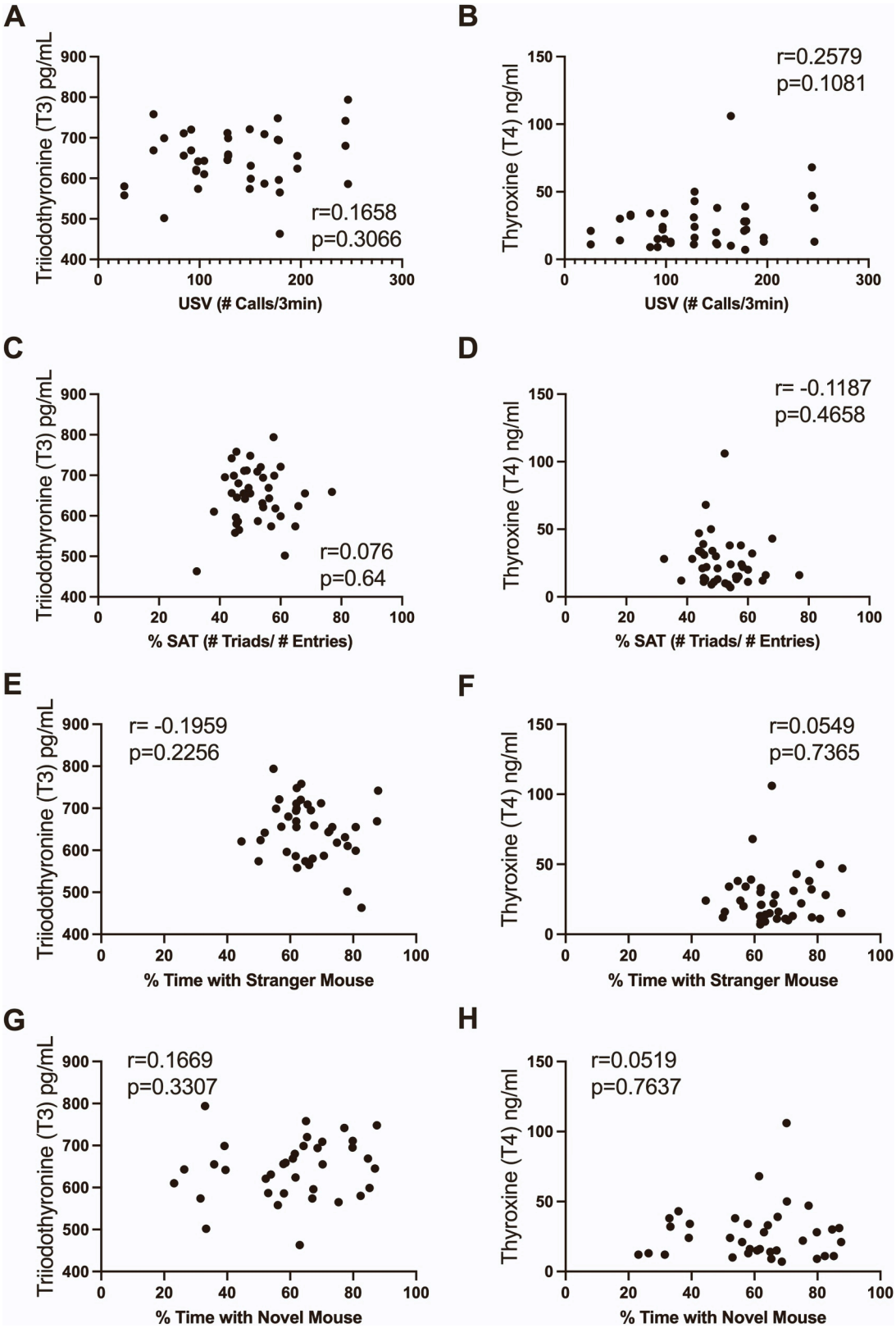


Fig. 9. FRM effects on thyroid hormone are not correlated with FRM effects on behavior. Pearson correlation analyses were used to assess linear relationships between serum triiodothyronine (T3) levels (A, C, E, G) or serum thyroxine (T4) levels (B, D, F, H) at P35 with ultrasonic vocalizations (A, B) measured as the number of calls during the 3 minute trial, spontaneous alternation (C, D) presented as the percent spontaneous alternation (spontaneous alternating triads divided by the total number of entries), social approach (E, F) presented as percent of total sniff time spent with the stranger mouse, or social novelty (G, H) presented as percent of total sniff time spent with the novel mouse. Data are presented as scatterplots (n=36–40 pairs).

and decreased T4 levels at P28, but no changes relative to vehicle controls at P35. Conversely, dams and female pups exhibited decreased T3 levels at P35. These sex-, age and dose-dependent patterns of FRM effects on thyroid hormone levels overlap but do not perfectly align with the FRM effects on behavioral outcomes. This was confirmed by correlation analysis that failed to identify any significant correlative relationships between FRM effects on thyroid hormone levels and FRM effects on behavioral outcomes. These analyses suggest that changes in thyroid hormone levels are not the major factor underlying the behavioral deficits observed in FRM-exposed animals.

The lack of a significant correlation between FRM effects on behavior and circulating levels of thyroid hormone are consistent with previous observations of animal models of developmental PCB exposure (Pessah et al., 2019). For example, developmental exposure to Aroclor 1254 caused spatial memory deficits in weanling rats independent of effects on serum thyroid hormone levels (Yang et al., 2009), and investigations of weanling mice exposed developmentally to the MARBLES PCB mixture revealed no correlation between altered thyroid hormone levels and behavioral deficits in USVs, repetitive behavior or sociability in MARBLES-exposed mice (Sethi et al., 2021). Moreover, neither the MARBLES PCB mixture nor any of the individual congeners in the MARBLES mixture exhibited agonistic or antagonistic activity on the thyroid hormone receptor (THR) as determined using a THR reporter cell line (Sethi et al., 2019). However, we cannot rule out the possibility that thyroid hormone-mediated mechanisms contribute to the behavioral deficits observed in mice developmentally exposed to FRM since individual PCB congeners found in the Aroclors used to make the FRM have been shown to alter THR-mediated signaling (Kostyniak et al., 2005). This possibility is further supported by reports that developmental exposure to Aroclor 1254 altered the expression of TH-responsive genes in the brain of weanling rats independent of significant changes in circulating levels of thyroid hormone in the dam or pups (Gauger et al., 2004). Further study is required to address this possibility in the mouse model of developmental FRM exposure.

Overall, our findings suggest that developmental exposure to FRM in the maternal diet may increase risk of deficits in NDD-relevant behaviors. Confidence in this conclusion is increased by the strong study design, including the inclusion of appropriate controls for each behavioral test to rule out non-specific effects of FRM that could have influenced behavioral outcomes, such as maternal toxicity, and FRM effects on activity and interest levels. However, it remains possible that PCBs altered motor capabilities (Roegge and Schantz, 2006; Gaum et al., 2021) or attention (Johansen et al., 2014; Lombardo et al., 2015), which could interfere with performance in the three-chambered task. Another limitation of this study is that we did not assess anxiety-like behavior or stress levels in these mice, which if elevated, can also alter social behavior (Bell et al., 2016; Orito et al., 2007). Finally, we conducted two behavior tests on the same day, which in spite of the 3-hour break between the tests to minimize effects of the first test on the second, could have produced additional stress to the animals (Lassarén et al., 2023).

Despite the fact that Aroclors were utilized to mimic the congener-profile of the human-relevant Fox River PCB mixture, the behavioral responses we've seen following developmental FRM exposure do not directly mirror the effects described in studies that assessed the developmental neurotoxicity of individual Aroclors. This suggests that risk assessments based on individual Aroclors may not fully capture the developmental neurotoxic potential of current human PCB exposures, and, therefore, further assessment of FRM and other human-relevant PCB mixtures in relevant animal models will be necessary to identify which PCBs are of most concern, and to understand how PCBs interact to modulate neurotoxic outcomes. Given the variable developmental neurotoxicity of PCB mixtures with different congener profiles, it is striking that the human epidemiological studies are largely consistent in reporting negative impacts of developmental PCB exposure on behavioral and cognitive deficits (Pessah et al., 2019).

CRediT authorship contribution statement

Ana Cristina Grodzki: Writing – review & editing, Methodology, Investigation. **Ilknur Dursun:** Writing – review & editing, Methodology, Investigation. **Felipe da Costa Souza:** Writing – review & editing, Methodology, Investigation. **Rebecca J. Wilson:** Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Youjun P. Suh:** Writing – review & editing, Methodology, Investigation. **Pamela J Lein:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Julia Y. Cui:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Hans-Joachim Lehmler:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Pamela J. Lein was hired as an expert witness by lawyers representing a group of plaintiffs alleging they were harmed by exposure to PCBs in school air. In that capacity, she testified as an expert witness on PCB neurotoxicity. The defendant was Pharmacia, a successor company to Monsanto.

Data Availability

Data will be made available on request.

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