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Effects of an environmentally relevant PFAS mixture on dopamine and steroid hormone levels in exposed mice

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ABSTRACT

In the present study, we investigated the dopaminergic and steroid hormone systems of A/J mice fed environmentally relevant concentrations of a perfluoroalkyl substance (PFAS) mixture over a period of 10 weeks. The PFAS mixture was chosen based on measured PFAS concentrations in earthworms at a Norwegian skiing area (Trondheim) and consisted of eight different PFAS. Dietary exposure to PFAS led to lower total brain dopamine (DA) concentrations in male mice, as compared to control. On the transcript level, brain tyrosine hydroxylase (*th*) of PFAS exposed males was reduced, compared to the control group. No significant differences were observed on the transcript levels of enzymes responsible for DA metabolism, namely – monoamine oxidase (*maoa* and *maob*) and catechol-O methyltransferase (*comt*). We detected increased transcript level for DA receptor 2 (*dr2)* in PFAS exposed females, while expression of DA receptor 1 (*dr1)*, DA transporter (*dat*) and vesicular monoamine transporter (*vmat*) were not affected by PFAS exposure. Regarding the steroid hormones, plasma and muscle testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E2) levels, as well as transcripts for estrogen receptors *(esr1* and *esr2*), gonadotropin releasing hormone (*gnrh)* and aromatase (*cyp19*) were unaltered by the PFAS treatment. These results indicate that exposure to PFAS doses, comparable to previous observation in earthworms at a Norwegian skiing area, may alter the dopaminergic system of mice with overt consequences for health, general physiology, cognitive behavior, reproduction and metabolism.

1. Introduction

Perfluoroalkyl substances (PFAS) are a group of synthetic, persistent chemicals with broad applications in many industrial and consumer products (Glüge et al., 2020). Due to the strong electronegativity and small atomic size of fluorine, PFAS have high surface activity, stability, and water- and oil-repellency (Fischer et al., 2016). These are properties that are desirable in a wide range of consumer products, including ski waxing products (Kotthoff et al., 2015). The global production of ski waxes is estimated to be several tons per year (Plassmann and Berger, 2010), and the chemical composition of these products is continuously evolving (Axell, 2010).

In a previous study, we showed that Bank voles (*Myodes glareolus*) inhabiting a skiing area in Trondheim (Norway) had significantly higher ∑PFAS concentration in their livers, compared to the voles from a reference area with no skiing activities (Grønnestad et al., 2019). We further reported that the composition of the various PFAS in the skiing area was similar with commercial ski wax, indicating that the concentrations measured in the Bank voles were most likely derived from ski waxing products (Grønnestad et al., 2019). In a follow-up study, we reported relationships between PFAS concentrations in the Bank voles from the skiing area and variables related to the dopaminergic and steroid hormone systems (Grønnestad et al., 2020). Specifically, the Bank voles from the skiing area had higher total brain dopamine (DA) concentrations and lower DA turnover (3,4-dihydroxyphenylacetic acid (DOPAC)/DA ratio), compared to the control. Furthermore, voles from

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the skiing area showed lower dopamine receptor 1 (*dr1)* and monoamine oxidase (*mao)* expression, and males showed lower cellular testosterone (T) concentrations, compared to the reference area. These findings indicated potential health concerns since the dopaminergic system and sex steroids are involved in a variety of physiological functions in mammals.

DA is the major catecholamine in the central nervous system and is derived from tyrosine (an amino acid), which is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (Th). L-DOPA is further metabolized to DA by DOPA decarboxylase (DDC). DA is eventually broken down into inactive metabolites by the enzymes Mao and catechol-O methyltransferase (Comt) (Eisenhofer et al., 2004). Although, different pathways exist, the main metabolites are DOPAC and homovanillic acid (HVA), where HVA is the main end product of the DA catabolism (Ashcroft, 1969). DA is involved in the regulation of a variety of functions, including locomotor activity, cognition, mood, fear, anxiety, as well as vascular and reproductive functions (Nakajima et al., 2013; Goschke and Bolte, 2014).

There are few studies on the effects of PFAS at environmentally relevant concentrations on the neuro-endocrine system in wildlife species. Laboratory studies on the effects on DA show diverging results. For example, Northern leopard frogs *(Lithobates pipiens)* exposed to perfluorooctane sulfonate (PFOS) and perfuorooctanoic acid (PFOA) (Foguth et al., 2019), and mice (*Mus musculus)* exposed to PFOS showed decreased DA levels (Long et al., 2013). On the other hand, wild Bank voles exposed to a mixture of PFAS (Grønnestad et al., 2020) and adult laboratory rats (*Rattus norvegicus*) exposed to PFOS (Salgado et al., 2016) and mice exposed to PFOA (Yu et al., 2016), showed elevated DA levels. These variations indicate that more studies are needed to fully elucidate the effects of individual PFAS and mixtures of PFAS on the brain dopaminergic system.

DA also plays a significant role in maintaining sex steroid homeostasis, that in turn control the reproductive system, as well as in other tissues not traditionally considered to be "steroid targets" (Evans, 1988). Androgens play a role in various physiological processes via pathways involving the androgen receptor (AR) (Mooradian et al., 1987). The AR pathway is essential for male physiology, reproduction and development of sexual characteristics (Davey and Grossmann, 2016). Estrogens regulate various physiological processes such as cell growth, reproduction, development and differentiation. Thus, changes in 17β-estradiol (E2) and testosterone (T) homeostasis could potentially affect fitness and may be used in determining the physiological health status of pollutant exposed individuals (Gaikwad, 2013).

Previous studies have reported that sex steroids are affected by PFAS (Olsen et al., 1998; Shi et al., 2007; Joensen et al., 2013; López-Doval et al., 2014; Zhao et al., 2014; Salgado et al., 2015; Kang et al., 2016). Despite varying results on effects, the majority of the studies showed that PFAS exposure leads to reduction in plasma or testis T concentrations (Shi et al., 2007; Joensen et al., 2013; López-Doval et al., 2014; Zhao et al., 2014; Kang et al., 2016). The effects of PFAS on E2 levels have shown greater divergence (Olsen et al., 1998; Salgado et al., 2015; Kang et al., 2016). Importantly, most *in vivo* studies have applied exposure scenarios with individual PFAS and at high concentrations that are neither environmentally nor physiologically relevant. Thus, more studies are needed to assess the endocrine disrupting potency of PFAS at concentrations that are relevant for the environment and organismal physiology.

Field studies can be challenging when assessing effects of contaminants, given that confounding factors, including other environmental stressors might affect the targeted endpoints. The results from our previous study on Bank voles from a skiing area were based on free living voles and natural exposure conditions (Grønnestad et al., 2020). Therefore, we have conducted a study to validate our observations in wild Bank voles under controlled laboratory conditions applying mice as a model species. Thus, the aim of the current study was to investigate response variables of the brain-dopaminergic and steroid hormone systems of A/J mice in relation to PFAS exposure in controlled laboratory conditions. The PFAS composition and concentrations in the feed used for dietary exposure were based on field results from our study at a Nordic skiing area (Grønnestad et al., 2019). Our hypothesis is that environmentally relevant PFAS mixture in the feed will disrupt brain dopaminergic and steroid hormone systems in A/J mice after dietary exposure, and that these disruptions may potentially lead to severe consequences for health, general physiology, cognitive behavior, reproduction and metabolism in exposed individuals.

2. Materials and methods

2.1. Ethical considerations

This study was conducted in accordance with local and national regulations on animal experimentation at the Section for Experimental Biomedicine, Norwegian University of Life Sciences (NMBU), in Oslo, Norway. The facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/). Approval was obtained by the Institutional Animal Care and Use Committee at NMBU and the Norwegian Food Safety Authority (application ID: FOTS 15446). The animals followed a health-monitoring program recommended by the Federation of European Laboratory Animal Science Association (FELASA, http://www.felasa.eu/) and were kept under strict specific pathogen free (SPF) conditions.

2.2. Feed design and chemicals

The design of the PFAS mixture in the experimental feed was based on results from our previous study, where PFAS was analyzed in different matrices in a skiing area in Trondheim, Norway (Grønnestad et al., 2019). The concentrations chosen were based on the highest concentration measured in earthworms for the most predominant PFAS deriving from ski wax (see Table 1) since earthworms are part of the Bank voles' diet. A previous exposure study (Berntsen et al., 2017), showed that about 66% of PFAS concentrations disappear during the production of the experimental feed. To adjust for this expected PFAS loss during the preparation of the diet, 3x the experimental end concentrations were added to the feed to obtain the desired daily exposure dose. The experimental feed was prepared at TestDiet (IPS products supplies, London, UK). PFAS were purchased from Chiron AS, Trondheim, Norway, and were dissolved in methanol. These were added to oil (Jasmin fully refined, Yonca GidaSan A.S., Manisa, Turkey) to the desired concentrations. The solvent (methanol) was evaporated under N_2-flow and oil containing the PFAS was incorporated into AIN-93G mouse pellet feed (see concentrations added in Table 1).

PFOS and PFOA is banned by the Stockholm Convention, and thus the feed production facility was not allowed to include these compounds

Table 1
PFAS concentrations added to the feed, desired experimental concentration (the concentrations detected in earthworms at a skiing area) and measured PFAS concentrations in the AIN-93G feed.

PFAS-mixture	Concentrations added to the feed	Experimental concentrations	Measured concentrations
PFNA	6	2.0	1.75
PFDA	9	3.0	2.96
PFUdA	9	3.0	2.98
PFDoDA	24	8.0	7.21
PFTrDA	48	16	11.4
PFTeDA	60	20	14.2
PFOA[a]	52	17.5	37.6
PFOS[a]	27	9.1	11.2

Values are given in ng/g feed. No PFAS were detected in control feed and, thus, it is not shown in the table.

[a] PFOS and PFOA were only added to the gel feed.

in the experimental feed. Thus, an additional experimental feed (gel feed powder provided by TestDiet) with PFOS and PFOA was prepared at the Department of Biology, NTNU, Norway. This was fed to the mice only once per week due to the short durability at room temperature, and limitation of resources. To obtain an approximate weekly exposure dose of PFOS and PFOA, the concentrations added to the feed were multiplied by 7, relative to the measured concentration in earthworms from a skiing area (Grønnestad et al., 2019). The gel feed (AIN-93G gel diet) was made according to the manufacturer's instructions. The gel powder has the same nutritional and ingredient list as the pellet feed. The powder was mixed with warm, near-boiling water until a homogenous mixture, then PFOS and PFOA was added while the mixture was still warm (70–90°C) to make sure the solvent would evaporate. This was mixed thoroughly, added to a plastic mold, and refrigerated to set. Once the gel was set, it was cut into pieces of 3 g/piece and stored at −20 °C. Desired experimental concentrations, concentrations added, and measured in AIN-93G pellet and gel feed are presented in Table 1.

2.3. Animals and husbandry

A/J mice bred in-house were used in the present study. At 3 weeks of age, whole litters were randomly assigned to either control or exposed group, resulting in 20 (10/10 males/females) and 18 mice (8/10 males/females) within the two groups, respectively. AIN-93G control or exposed pellet diets were provided *ad libitum* six days per week. The control and exposed gel diets were given to the mice once per week (3 g/mouse) during the entire 10-week experimental period. Fig. 1 shows an illustration of the exposure regime.

All mice were housed in groups (2–6 mice per cage) in closed Type III individually ventilated cages (IVC) (Allentown Inc, USA), with standard aspen bedding, red polycarbonate houses and cellulose nesting material (Scanbur BK, Karlslunde, Denmark). Tap water was provided *ad libitum* to the experimental animals and changed twice per week, while water containers were changed weekly. Cages, bedding and nesting material were changed every fortnight. The animal room was provided with a 12:12 light:dark cycle at room temperature (20 ± 2 °C) and relative humidity (45 ± 5%). See more details in supplementary information (SI).

2.4. Sample collection

Mice were sacrificed at 13 weeks of age after being exposed to the PFAS or control diets for 10 weeks. Body weight was recorded prior to euthanasia by cardiac puncture and cervical dislocation under anesthesia (isoflurane gas obtained from Baxter, San Juan, Puerto Rico). Blood was collected from the heart using a sterile 1 mL syringe with a hypodermic needle (23G x 5/8") flushed with ethylene diamine tetraacetic acid (EDTA) disodium salt solution (Honeywell International Inc, Charlotte, USA), cooled down on ice and spun at 5000 rpm for 10 min (Hermle Z160M, Hermle Labortechnik, Wehingen, Germany). Plasma was collected and frozen on liquid nitrogen.

The liver was removed, and the weight was recorded. The entire calf muscles from both hind legs were removed, and the brain was separated into its two hemispheres. All samples were collected in 1.8 mL cryotubes, frozen on liquid nitrogen and stored at −80 °C until analysis.

2.5. Chemical analysis

PFAS concentrations in the liver of 12 mice (6 control and 6 PFAS exposed mice, n = 3 of each sex) were analyzed at the Laboratory of Environmental Toxicology at NMBU, Norway. In addition, samples of exposed and control pellet and gel diets were analyzed to verify PFAS exposure concentrations and possible background contamination. The analytical procedures are described in Grønnestad et al. (2016). The samples were analyzed for perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA) and perfluorooctane sulfonate (PFOS).

For the extraction, 1 g of feed (pellet and gel) and 0.4 g of liver was weighed for chemical analysis. A brief description of the method (Grønnestad et al., 2016) is as follows: internal standards (^{13}C-labeled equivalents, Wellington Laboratories, Table S1 in SI) were added prior to ultrasonic extraction with methanol. Clean-up was accomplished using active carbon (EnviCarb).

The final extracts were analyzed by high-performance liquid chromatography mass spectroscopy (HPLC-MS/MS) consisting of an Agilent 1200 HPLC and an Agilent 6460 triple quadrupole. Separation was performed on a Luna Omega C18 column (10 cm x 4,6 mm x, 3 μm) from Phenomenex. The injected volume was 5 μL.

The limits of detection (LODs) were calculated as 3*standard deviation (SD) of the procedural blanks and the limits of quantification (LOQs) were calculated as 10*LOD. Where no PFAS were detected in the blank samples, LOQs were determined as 10*signal-to-noise ratio (S/N) (LOD and LOQ are given in Table S2 in SI). The relative recoveries ranged from 80 to 93%. Samples were not corrected for recoveries.

2.6. Dopamine analysis

The DA analysis was conducted at the Department of Environmental Sciences, University of California, Riverside, USA. For the measurement of brain concentrations of DA and its metabolites (DOPAC and HVA),

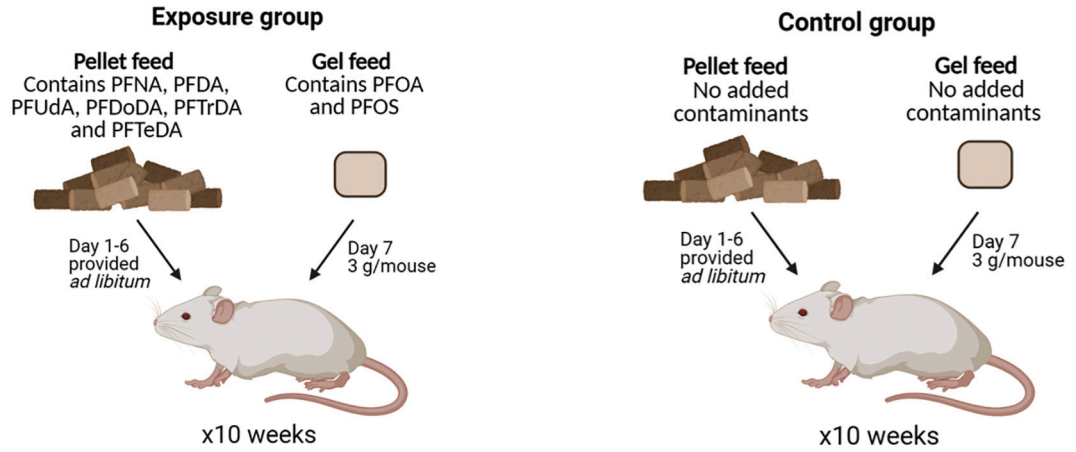

Fig. 1. Illustration of the exposure regime, showing the different types of feed (pellet and gel), when they were provided to the mice (from day 1 to 7 of each week), and the different types of PFAS they contain (only the exposure group). Illustration created in biorender.com.

samples were prepared using methods based on Tareke et al. (2007), Bertotto et al. (2018), and Najmanová et al. (2011) with slight modifications. Samples were kept on ice during handling and extraction. The samples were homogenized prior to extraction. Approximately 200 mg brain tissue (right brain-half) was placed into a 2 mL centrifuge tube, and internal standards (deuterated dopamine: DA-d4 and deuterated HVA: HVA-d5) were added to yield 1 ng DA-d4 and 2 ng of HVA-d5 per 1 mg of tissue. A mixture of solvents composed of ice cold 0.1 M HCl (0.4/100 mg brain tissue), acetonitrile (0.5 mL/100 mg brain tissue), and 27 mM EDTA in H_2O (0.1 mL/100 mg brain tissue) were then added to the centrifuge tube. The brain tissue was then homogenized with a pestle tissue homogenizer on ice for 1 min. The sample was centrifuged at 6500 ×*g* for 20 min at 4°C. The supernatant was then filtered through a 0.22 μm polypropylene syringe filter and transferred to autosampler vial inserts for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. See SI for more details.

Deuterated dopamine (DA-d4) was used as an internal standard for DA. Relative recoveries ranged from 84% to 106% for Da-d4. The LOD was calculated from 3* S/N and was set as 0.005 ng/mg.

2.7. Steroid analysis

Both plasma and muscle tissue were used for steroid analysis. In our previous study (Grønnestad et al., 2020), we used muscle tissue as a proxy for the free fraction of steroids in blood, since we did not have sufficient blood sample for this purpose. Hence in the current study, we evaluated whether changes in steroid concentrations are similar in muscle tissue and plasma, to validate muscle tissue for measurement of steroid levels in rodents, when plasma samples are not available.

Approximately 250 mg muscle tissue was added to 4x volume of homogenizing buffer (0.1 M Na-phosphate, 0.15 M KCl, 1 mM EDTA, 1mM Dithiothreitol (DTT) and 10% glycerol, pH 7.4). This was incubated on ice for 30 min followed by homogenization with a pistil homogenizer and centrifugation at 15,000 ×*g* for 20 min at 4°C. The supernatant was used for steroid extraction.

Steroid hormones were extracted using di-ethyl ether which was added to a volume of 4x sample volume (400 μL plasma sample or 500 μL muscle supernatant was used). The organic phase was separated using liquid nitrogen, evaporated overnight, and reconstituted with the initial sample volume with enzyme immunoassay (EIA) buffer provided in the EIA kit (Cayman Chemicals).

The extracts of muscle tissue and plasma were used for the measurement of E2, T and 11-ketotestosterone (11-KT) using EIA kit from Cayman Chemicals (Ann Arbor, MI, USA). All assay solutions were prepared according to kit instructions with ultrapure water. Absorbance readings were performed on a spectrophotometer (Cytation 5 Imaging Reader, Biotek) at 412 nm. Steroid hormone concentrations were calculated by extrapolating sample absorbance on a logarithmic standard curve using the analysis tool provided by the kit's manufacturer.

2.8. RNA extraction and quantitative (real-time) polymerase chain reaction (qPCR)

Total brain RNA was isolated from frozen tissues using Direct-zol™ RNA extraction kit. Quality of RNA was confirmed by NanoDrop (see SI) and formaldehyde agarose gel electrophoresis and spectrophotometric analysis (see Fig. S1 in SI).

Transcript expression analysis related to the dopaminergic and steroid pathways were performed using quantitative polymerase chain reaction (qPCR). Briefly, cDNA was synthesized from 1 μg total RNA according to instructions provided with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). PCR reaction mix (20 μL) containing 5 μL of 1:6 diluted cDNA, 0.5 μM each of the forward and reverse primer pair sequences (Table S3 in SI) were amplified using Mx3000P real-time PCR machine (Stratagene, La Jolla, CA). See Khan et al. (2019), for the detailed protocol for qPCR analysis of gene expression patterns. Transcripts tested include - dopamine receptor 1 and 2 (*dr1* and *dr2*), monoamine oxidase a and b (*maoa* and *maob*), vesicular monoamine transporter (*vmat*), dopamine active transporter (*dat*), catechol-*O*-methyltransferase (*comt*), tyrosine hydroxylase (*th*), estrogen receptor alfa and beta (*esr1* and *esr2*), aromatase (*cyp19*) and gonadotropin releasing hormone (*gnrh)*.

2.9. Statistical analysis

The program R (version 3.6.3, the R project for statistical computing) was used for the statistical analysis and to make boxplots. Mixed effect ANOVA models were run (with the packages "nlme" and "multcomp") to test for significant differences between control and exposure groups for the measured endpoints. We used "mother id" as a random effect to account for the fact that several of the replicates in the present study cannot be considered completely independent, due to litter effects. The residuals of the models were visually inspected to test for normality. Where the residuals were not normally distributed, the data were log transformed, to yield a better fit. Full factorial two-way ANOVA with "gender" and "exposure groups" as factors, and "mother id" as random factor were performed to test for interactions between gender and exposure group. If there was no interaction, the model was run without an interaction term. If there was an interaction, the data were split by gender, and the difference between means in exposure groups were analyzed using a mixed effect one-way ANOVA. The significance level was set at 0.05.

Prior to correlation tests, normal distribution was tested with Shapiro Wilk's test. Where the assumptions of normality were not met, the data were log-transformed prior to analyses. Log transformation of the data yielded a normal distribution. Pearson correlations were performed to test for correlations between steroid levels in plasma and muscle tissue, and to test for correlations between the gene expression of *cyp19* and *esr1* and *esr2* mRNA. This was performed on males and females separately.

3. Results and discussion

3.1. Biometric measurements

The average body mass at the termination of the experiment was 22.3 (± 2.1) g and 26.0 (± 2.2) g for PFAS-exposed females (n = 10) and males (n = 8), respectively, and 21.5 (± 1.8) g and 27.8 (± 4.2) g for control females (n = 10) and males (n = 10), respectively. The body mass was significantly higher in males than in females ($p < 0.001$, $F = 31.1$). Dietary exposure to PFAS did not affect the body mass of A/J mice, showing no significant difference between the control and exposed group ($p = 0.61$, $F = 0.26$).

The hepatosomatic index (HSI: liver weight/body mass*100) was significantly higher in males exposed to the PFAS-mixture, compared to control males (Fig. 2, two-way ANOVA, $p = 0.01$, $F = 6.35$). This is in accordance with several rodent studies that have identified the liver as the primary target organ for both acute and chronic exposure to PFAS (Cui et al., 2008; Dong et al., 2012; Yu et al., 2016). A report by The European Food Safety Authority (EFSA, 2018) showed that for PFOS, increases in relative liver weight were observed in rodents from 0.15 mg/kg_{bw}/day and for PFOA, increased absolute and relative liver weight and hepatic peroxisomal β-oxidation were observed at 0.64 mg/kg_{bw}/day (EFSA, 2018). These doses are far higher than the PFAS and PFOA doses used in the current study, suggesting that the PFAS mixture used in the current study could potentially be more hepatotoxic, than individual PFAS (PFOS or PFOA) at higher doses. This should be further investigated.

3.2. PFAS concentrations

The PFAS concentrations measured in the exposure feed are shown in

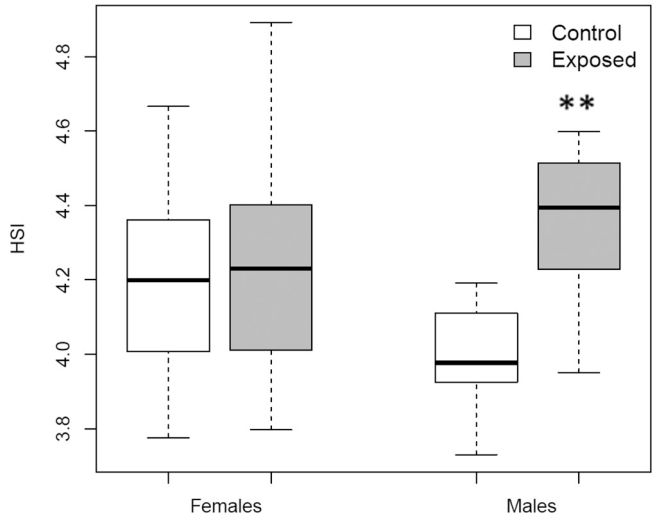

Fig. 2. Boxplot of hepatosomatic index (HSI) in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice.

Table 1. PFAS were not detected in the control feed. The concentrations measured in the PFAS feed were relatively similar to the desired experimental concentrations (Table 1). The only exception was PFOA, where the measured concentration was 115% higher than the experimental concentration. This is most likely due to human error during the preparation of the feed, since PFOA was not detected in the control feed.

The concentrations of PFAS in the liver of exposed and control mice are shown in Table 2. All PFAS included in the exposure mixture were higher in the exposure group, compared to control. The ∑PFAS concentrations were higher in the liver (ng/g liver) of exposed males, compared to exposed females (only 3 samples per gender, so it is not possible to run statistics). However, when converting the concentrations to body weight (ng/g bw), there were no pronounced sex differences. This indicates that the sex differences in PFAS concentrations is most likely due to the males consuming more of the PFAS-containing feed than the females. However, previous studies have shown that female rats have higher excretion rate of PFOS and PFOA, compared to males (Hanhijärvi et al., 1982; Heuvel et al., 1991). Given that excretion rate was not evaluated in the current study, we cannot rule out possible sex-related differences in the metabolism and excretion of different PFAS in A/J mice.

Table 2
Mean PFAS concentrations ± SD in liver of exposed and control A/J mice.

	LOQ	PFAS exposed		Control	
		Males (n = 3)	Females (n = 3)	Males (n = 3)	Females (n = 3)
PFOA	1.073	416.4 ± 111	181.8 ± 146.3	nd	nd
PFNA	0.045	286.3 ± 29.2	192.7 ± 28.7	1.40 ± 0.16	0.942 ± 0.17
PFDA	0.258	465.1 ± 43.6	344.7 ± 48.3	0.739 ± 0.06	0.554 ± 0.11
PFUdA	1.225	448.6 ± 42.7	353.2 ± 53.3	nd	nd
PFDoDA	0.046	937.3 ± 95.9	744.3 ± 121.4	0.165 ± 0.03	0.109 ± 0.03
PFTrDA	0.070	1047.4 ± 67.2	933.5 ± 148.2	0.164 ± 0.04	0.087 ± 0.08
PFTeDA	0.033	518.2 ± 72	573.8 ± 54.6	0.053 ± 0.02	0.035 ± 0
PFOS	0.173	186.0 ± 66.4	137.0 ± 109.5	1.76 ± 0.26	2.19 ± 0.39
∑PFAS		4605 ± 86.9	3461 ± 567.2	4.27 ± 0.47	4.6 ± 1.08

Values are given in ng/g ww. LOQ: limit of quantification. nd: not detected.

PFAS concentrations were not measured in the brain due to limited sample size. However, a study on PFOS exposed KM mice showed that the ratio between liver and brain concentrations varied with duration after birth (Liu et al., 2009). While brain PFOS concentrations decreased with increasing time, the liver concentrations increased with increased duration since birth, even though the mice were exposed to PFOS once a week. The PFOS concentrations in the mouse brain were around 25% of the liver concentrations at 7 days after birth, while it was only 5% of the liver concentrations at 35 days after birth (Liu et al., 2009). In a different study using 8 weeks old rats, brain PFOS concentration was around 5–10% of the liver concentration (Sato et al., 2009). Elsewhere, several other studies have shown that the abundance of PFAS in the brain increases with carbon chain length (Greaves et al., 2012; Dassuncao et al., 2019). For example, a study in North Atlantic Pilot whales (*Globicephala melas)* showed that brain PFOS concentration was around 11% of the liver concentration, while the brain concentration of PFTrDA (C13) was 85% of the liver concentrations, and PFTeDA (C14) concentration was higher (133%) in the brain than the liver (Dassuncao et al., 2019). This shows that PFAS accumulation in brain could be highly compound-, age- and species specific. All individual compounds in the mixture used in the current study are long-chained PFAS with carbon chain length of up to C14, which could favor a larger proportion of PFAS accumulation in the brain. In another study using mice, a correlation between liver and brain PFOS levels was observed, while no such relationship was observed between brain and plasma concentrations (Liu et al., 2009). This accumulation scenario indicates that the organ-specific differences in PFAS accumulation may parallel sex-specific differences in concentration. Still, it should be mentioned that a study on ∑PFAS concentrations in different brain regions of East Greenland Polar bears (*Ursus maritimus)* (Greaves et al., 2013) detected sex differences in PFAS concentrations only in the cerebellum and the authors did not consider sex a confounding factor for PFAS accumulation in the brain.

3.3. Dopamine

Dietary exposure to an environmentally relevant PFAS mixture led to lower total brain DA concentrations in male mice, as compared to control mice (Fig. 3, $p = 0.008$, $F = 10.3$), whereas in the females, no significant difference was observed between control and PFAS exposed groups (Fig. 3, $p = 0.3$, $F = 1.04$). These results suggest that the brain dopaminergic system could be more sensitive to PFAS exposure in male rodents compared to females. However, this discrepancy between sexes could also be related to possible higher PFAS concentrations in the male brain, compared to female brain, as discussed above. The observed effects in the current study were in contrast to the effects reported in the free-living Bank voles from our previous study (Grønnestad et al., 2020), where we reported elevated DA concentrations with increasing tissue PFAS burden. These contradicting results may indicate potential species- or dose-specific differences in DA-related effects after PFAS exposure. However, there could also be other factors affecting the results, such as interaction with other contaminants or stressors in the field.

Diverging results in DA responses after PFAS exposure at different concentrations in different species have also been reported in previous studies (Long et al., 2013; Pedersen et al., 2015; Salgado et al., 2016; Yu et al., 2016; Foguth et al., 2019). For example, adult male rats exposed to PFOS showed increased DA concentrations in the hippocampus and prefrontal cortex (Salgado et al., 2015; Salgado et al., 2016) and male Balb/c mice exposed to PFOA showed increased brain DA concentrations, compared to control (Yu et al., 2016). However, Northern leopard frogs (both male and female) exposed to PFOS and PFOA showed decreased brain DA concentrations (Foguth et al., 2019) and C57BL6 mice (both male and female) exposed to PFOS showed decreased DA concentrations in the hippocampus (Long et al., 2013). In all of the above-mentioned rodent studies, animals were exposed to only one PFAS compound (PFOS or PFOA) and the doses were in the mg/kg/day

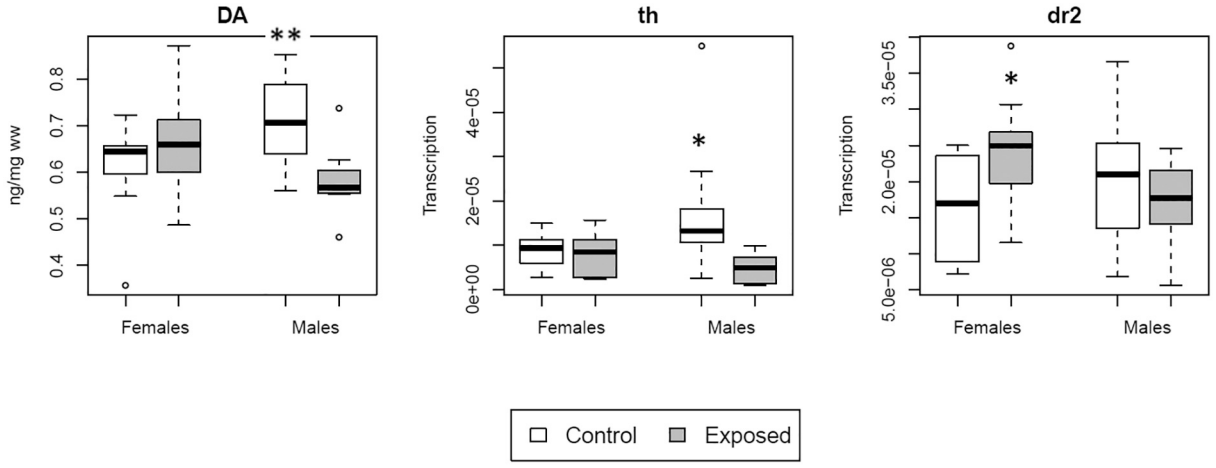

Fig. 3. Boxplot of variables related to the dopaminergic system in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice. Asterisks indicate significant difference between exposure groups (*p < 0.05, **p < 0.01).

range. The A/J mice used in our study were exposed to a mixture consisting of eight PFAS at the estimated dose of ng/kg/day range, which is much lower than the rodents in the above-mentioned studies.

It should be noted that the current study analyzed DA concentrations of the entire brain hemisphere, and not in specific regions. The prefrontal cortex, hippocampus, substantia nigra pars compacta, ventral tegmental area and nucleus accumbens are some of the primary sites of DA production in the brain (Björklund and Dunnett, 2007). These areas and systems may be differentially affected by PFAS exposure or may show different responses in different species or at different concentrations. Future studies should therefore investigate effects of PFAS concentrations in specific brain regions.

DA is derived from the amino acid, tyrosine, which is converted to L-DOPA by the enzyme Th and further metabolized to DA. Th is considered the rate-limiting step of DA synthesis (Daubner et al., 2011). In the current study, *th* transcript was significantly lower in PFAS exposed males, compared to control males (Fig. 3, p = 0.05, F = 4.79). There were no significant differences in *th* transcript expression between female control and exposure groups (Fig. 2, p = 0.6, F = 0.23). The reduced DA levels observed in PFAS-exposed males may be attributed to reduced Th enzyme levels, which thus, limits the synthesis of DA. A previous study on PFOS treated male mice found that, adult mice had decreased hippocampal *th* mRNA after 24h exposure (Hallgren and Viberg, 2016). However, the authors also reported increased *th* expression in PFOS treated neonates, suggesting that the hippocampal release of DA may be augmented by PFOS exposure during ontogeny. The opposing results on *th* expression in adults, compared to the neonates may reflect a compensatory effect of an overexpression of the gene during ontogeny, potentially producing the dysregulation of *th* transcription later in life (Hallgren and Viberg, 2016). In addition, this could partly explain the discrepancy between the current findings in the controlled laboratory study and the data from Grønnestad et al. (2020), which is a field study. Even though the average age in the Bank voles from Grønnestad et al. (2020) was approximately the same as the A/J mice at the time of euthanasia, there was more variation between individuals in the field study, as some of the Bank voles were born during the sampling season, while some were estimated to be born the previous season. Furthermore, the mice from the current study were exposed 3–13 weeks after birth, while the wild Bank voles, most likely, also experienced *in utero* exposure.

Once released in the synaptic cleft, DA can bind to one of its two receptor families: dopamine receptor 1-like (Dr1) or dopamine receptor 2-like (Dr2) (Beaulieu and Gainetdinov, 2011). Dr1 activates cyclic AMP-dependent protein kinase, stimulating the DA neuron, while Dr2 plays an important role in regulating DA neuronal activity through synthesis, release, and uptake. In addition, activation of Dr2 decreases the excitability of DA neurons and release of DA (Jaber et al., 1996). Herein, *dr2* expression was significantly higher in PFAS-exposed females, compared to control females (Fig. 3, p = 0.04, F = 5.3), whereas there was no effect of PFAS exposure on *dr2* transcript in males (Fig. 3, p = 0.4, F = 0.7) or on *dr1* transcript in either sex (Fig. S2 in SI). Since the DA concentrations were altered in PFAS exposed males, but not in the females, we would expect to detect effects on the DA receptor transcript expression levels in males, not in the females. Nevertheless, it should be noted that, based on our knowledge, the current study is the first rodent exposure experiment using an environmentally relevant PFAS mixture. Thus, given that transcript expression profiles are transient molecular events, our data only provides a preliminary overview of the expression pattern at a given exposure time and calls for extended studies accounting for concentration and exposure time differences with both individual PFAS compounds, as well as mixture scenarios.

The vesicular monoamine transporter (Vmat) facilitates the transport of DA into synaptic vesicles, which releases the neurotransmitter into the synapse, representing an essential regulator of monoaminergic neuronal function (Fleckenstein and Hanson, 2003). The plasma membrane DA transporter (Dat), on the other hand, terminates the actions of DA by rapidly removing DA from the synapse (Shimada et al., 1991). Inhibition of DA reuptake via Dat increases the extracellular and synaptic concentrations and DA lifespan, leading to prolonged stimulation of DA receptors. *In vitro* studies have shown that PFOS can inhibit Vmat and Dat in dopaminergic cells (Patel et al., 2016). However, we did not observe any effects of the PFAS exposure on either *vmat* or *dat* expression in the current study (see statistics in Table S4 and Fig. S2 in SI).

The ratios between DA and its metabolites are generally used as a measure of DA turnover (Salgado et al., 2015). In our previous study on Bank voles (Grønnestad et al., 2020), we measured lower DOPAC/DA ratio in voles from the skiing area, compared to the reference area, with corresponding negative correlations between *mao* transcript and PFAS concentrations in the liver. We suggested that PFAS might lead to lower levels of Mao enzyme, which is responsible for the metabolism of DA to DOPAC, and thus lead to reduced DA turnover and a build-up of DA in the brain (Grønnestad et al., 2020). In the current study, we were not able to effectively detect DOPAC and HVA. We therefore do not have any measurement of metabolic levels and DA turnover. However, we did measure transcript levels of *mao* isoforms (a and b) and *comt*, showing no PFAS exposure related effects on these transcripts in the A/J mice brain (see statistics in Table S4 in SI). This indicates that the reduced DA concentrations observed in PFAS exposed males are, most likely, not due to increased metabolism of DA, but rather decreased DA synthesis, which is consistent with the elevated *th* expression. Furthermore, it

should also be noted that, it is common that transcript levels of some metabolic enzymes may increase or decrease after exposure to contaminants, without parallel increase or decrease of functional products (enzymes and proteins), requiring the need of caution when interpreting our results in term of functional effects (Regoli et al., 2005). Given that *mao* and *comt* mRNA, proteins and enzymatic activities could be target of transcriptional, post-transcriptional and/or post-translational PFAS effects, including, but not limited to reduced mRNA stability, reduced protein synthesis, folding alterations, and cofactor depletion, it is possible that we have partially evaluated potential PFAS effects on the DA metabolic pathway. Thus, future studies should also include analysis of functional enzymes and DA metabolites for better understanding of contaminant-induced alterations of the dopaminergic system. In addition to DA and DA metabolites, the dopaminergic brain regions ventral tegmental area and substantia nigra pars compacta, receive inputs from other neurotransmitters systems, including glutaminergic, γ-aminobutyric acid-ergic (GABAergic), cholinergic, and inputs from other monoaminergic nuclei (Yager et al., 2015). These systems were not analyzed in the present study, but could potentially be affected by PFAS exposure, and possibly alter overt dopaminergic system.

The apparent reduced synthesis of DA in PFAS exposed male mice in the current study could lead to alterations in fear and anxiety responses (de la Mora et al., 2010), thermoregulation processes (Hasegawa et al., 2000), cognitive functions and attention (Seamans and Robbins, 2010), as well as modulation of the reproductive pathways (Henderson et al., 2008). Excessive or deficient levels of DA have been shown to contribute to mood and motor abnormalities, and is often coupled to aggressive behavior in animal and human studies (Zai et al., 2012). The current study did not conduct any behavioral analysis of the exposed mice; however, behavioral analyses could possibly link the neurochemical changes in the mice to functional changes. This should be included in future studies investigating the effects of PFAS on the dopaminergic system. In addition to behavioral analysis, future studies should include histological analysis of the brain to determine whether transcript changes are directly responsible for changes in DA concentrations, or whether toxicity to DA neurons results in phenotypic alterations. For example, decreased Th and DA concentrations and increased post-synaptic DA receptors has been observed following loss of DA neurons (such as in patients with Parkinson's disease) (Lotharius and Brundin, 2002).

3.4. Steroid hormones

In the current study, we measured the E2, T and 11-KT concentrations in both plasma and muscle tissue of A/J mice. Plasma T and 11-KT concentrations were significantly higher in males than females (Fig. 4), while there was no difference in E2 concentrations between the sexes (see statistics in Table S4 in SI). T is the most important androgen that is produced from cholesterol in the gonads (Miller and Auchus, 2011). 11-KT belongs to a class of active androgens that can be converted from T and other precursors (Yazawa et al., 2008; Swart et al., 2013; Imamichi et al., 2016). Thus, we expected to detect higher concentrations of these androgens in males, compared to females. 11-KT was originally characterized as a teleost-specific hormone that is important for the male sexual phenotype and spermatogenesis in many teleost species (Miura et al., 1991; Kobayashi and Nakanishi, 1999; Nagahama, 2002). However, recent studies have noted that it is also present in mammals of both sexes (Imamichi et al., 2016), and that 11-KT is produced in human gonads and is one of the major androgens in mammals (Yazawa et al., 2008, Swart et al., 2013, Imamichi et al., 2016). The synthesis and functions of 11-KT in mammals are still not well understood. Since our previous study on Bank voles from a skiing area showed a negative trend between PFAS and T levels (Grønnestad et al., 2020), it was interesting to investigate whether 11-KT, an androgen that is synthesised from T, could be affected after PFAS exposure.

There were no effects of dietary PFAS exposure on the plasma E2, T

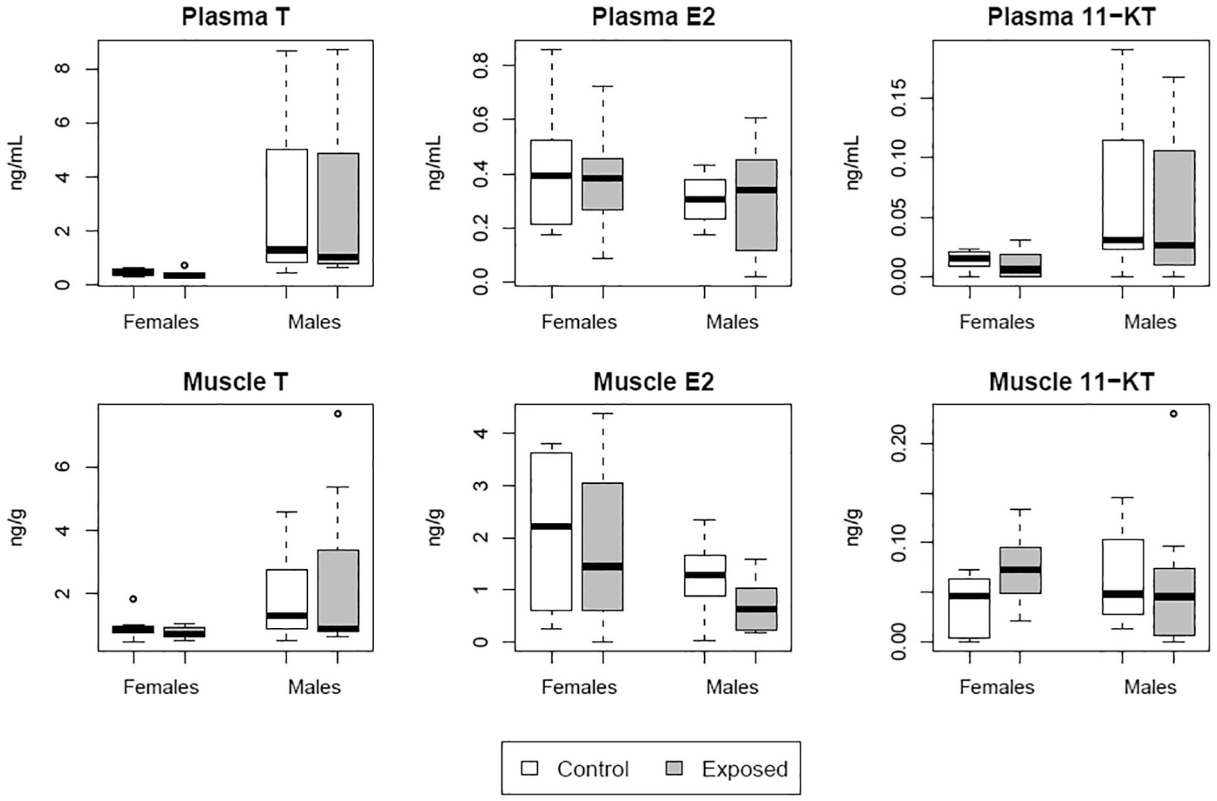

Fig. 4. Boxplot of sex steroid concentrations measured in plasma (ng/mL) and muscle tissue (ng/g) in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice.

or 11-KT concentrations in A/J mice (Fig. 4, see statistics in Table S4 in SI). However, there was a weak, borderline significant effect of PFAS exposure on E2 concentrations in the muscle tissue of male mice (Fig. 4, $p = 0.06$. $F = 3.7$), showing lower E2 concentrations in the muscle of PFAS exposed males, compared to control. Further, there was no significant difference in brain *gnrh* and *cyp19* (aromatase enzyme involved in the conversion from T to E2) expression of PFAS exposed mice, compared to control mice (Fig. S3 in SI). Lastly, no significant differences were observed in the expression of brain estrogen receptors (*esr1* and *esr2*), between PFAS exposed and control mice (see Fig. S3 and statistics Table S4 in SI), indicating that PFAS exposure may not directly affect transcription of brain ERs at the doses used in the current study. It is possible that our sampling regime might have missed transcriptional alterations after PFAS exposure, which are generally recognized to be transient molecular events (Swift and Coruzzi, 2017). There was, however, a significant positive correlation between *cyp19* and *esr2* gene transcription in the brain of both male and female mice (Males: $r = 0.56$, $p = 0.02$; Females: $r = 0.47$, $p = 0.03$). This could indicate that E2 synthesized from androgen in the brain activates *esr2* (ERβ) to a larger extent, than *esr1* (ERα).

In our previous study on Bank voles from a skiing area (Grønnestad et al., 2020), we reported a weak negative association between PFAS and muscle T levels in males. Since the T levels were not affected in the current study, it could indicate that the weak association we reported in the Bank voles was not due to the PFAS, but rather other unknown contaminants/stressors in the field, or the interactions with these. Other explanations for the different results between these studies could be, as mentioned above, related to differences in PFAS doses, life-stages and/or species differences in exposure regime. It should also be mentioned that, in the current laboratory experiment, male and female animals were kept separately, while in the wild, the Bank vole were interacting freely between genders, which could possibly affect the sensitive feedback mechanisms of the HPG-axis. Other studies on PFAS exposure have reported a negative association between PFAS exposure and T levels (Shi et al., 2007; Joensen et al., 2013; López-Doval et al., 2014; Zhao et al., 2014; Kang et al., 2016).

3.5. Correlations between steroid hormones in muscle tissue and plasma

In our previous study (Grønnestad et al., 2020), we used muscle tissue as a proxy for the free fraction of steroids in blood, since we did not have sufficient blood for this purpose. Previous studies have shown that whole-body homogenate or muscle are suitable tissues for measuring the cellular and circulatory levels of steroid hormones in fish (Arukwe et al., 2008; Preus-Olsen et al., 2014). There was a significant correlation between T concentration in plasma samples and muscle tissue ($r = 0.9$, $p < 0.001$, Fig. S4 in SI) and between E2 concentration in plasma samples and muscle tissue in males ($r = 0.54$, $p = 0.02$, Fig. S5 in SI), but not in females. There were no significant correlations for 11-KT levels in plasma and muscle tissue in either sex (Table S5 in SI).

Steroids are hydrophobic molecules and consequently do not readily dissolve in the blood; instead, almost all steroids in the circulation are bound to binding proteins (Schwarz and Pohl, 1992). The main binding proteins for steroids are sex hormone-binding globulin (SHBG) and albumin (Dunn et al., 1981; Kraemer et al., 1998). The physiological effects of the binding proteins vary. SHBG reduces the movement of steroids from the blood into other bio-compartments, and thus prevents the biological actions of steroids. On the other hand, albumin appears to allow for greater bioavailability of steroids (Pardridge and Mietus, 1979; Hobbs et al., 1992). There could be differences in the composition and concentrations of binding proteins in the plasma of males and females, which could affect how steroids are transported into other bio-compartments, such as the muscle tissue. Studies on humans have shown that serum albumin levels are lower in females than males between the ages of 20 and 60 years old (Weaving et al., 2016), while the serum level of SHBG is lower in males than females (Elmlinger et al., 2005). As SHBG reduces the movement of steroids from the blood into muscle, and SHBG levels in general, are higher in females than males, this could explain the differences in correlation between plasma and muscle tissue between sexes in the current study.

The positive correlations for T and E2 between plasma and muscle of male mice, indicate that muscle tissue can be suitable for analyzing sex steroid profiles in rodents and other species, particularly when working with small mammals with insufficient amount of blood in order to minimize the number of animals euthanized.

4. Conclusions

The current study shows that dietary exposure to an environmentally relevant PFAS mixture led to increased liver weight (HSI) in male mice, compared to control males. Brain DA concentrations were lower in male mice, compared to the control group. We detected lower transcript levels of *th* mRNA, while there was no significant effect on the expression of *maoa*, *maob* or *comt*, which are important for DA catabolism. These results indicate that the observed decrease in DA concentrations in males is most probably caused by reduced synthesis of DA due to effects of PFAS exposure on Th synthesis. We did not detect significant effects of PFAS exposure on T, E2 and 11-KT concentrations in either plasma or muscle tissue of the mice. There were positive correlations for T and E2 between plasma and muscle concentrations in male mice, indicating that muscle tissue can be suitable for analyzing sex steroid levels in rodents, although contaminant-related changes may be gender dependent. The results from the current study indicate that exposure to environmentally relevant concentrations and composition of PFAS (as measured in earthworms at a Norwegian skiing area) were able to affect the brain dopaminergic system of male mice with potential health consequences.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2021.115670.

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