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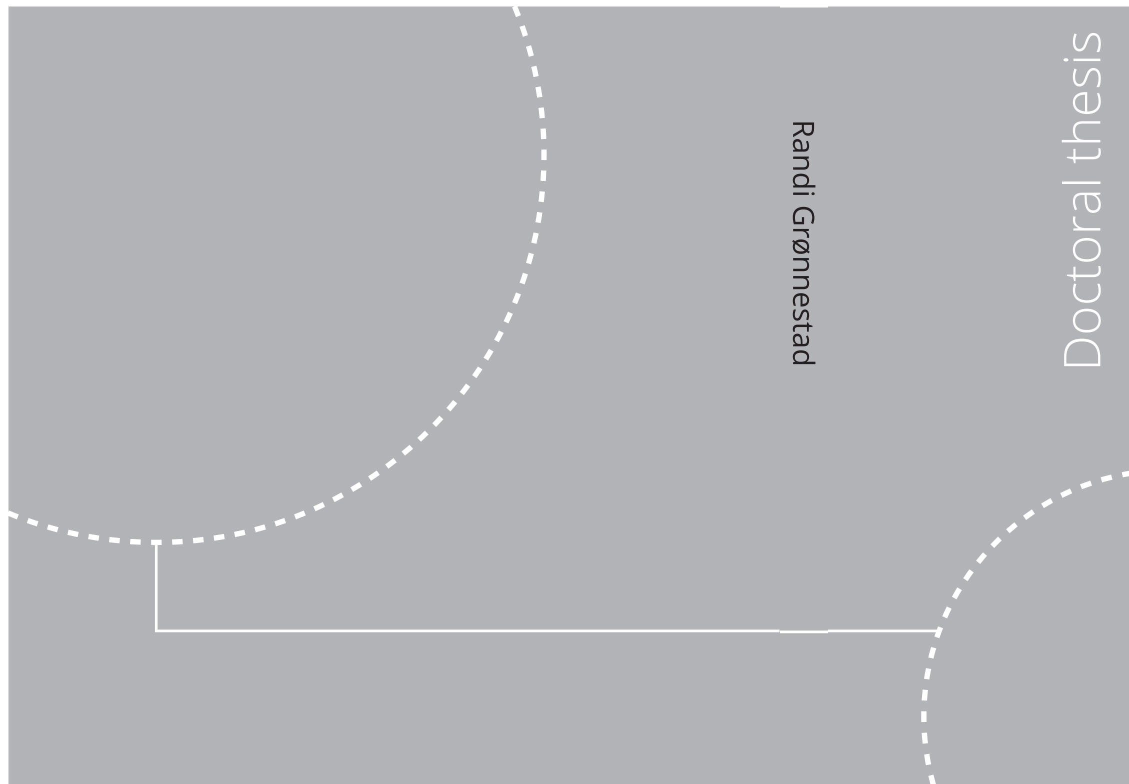

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Thesis for the degree of Philosophiae Doctor
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Randi Grønnestad

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Randi

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Summary

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic contaminants that are widespread in the environment. Due to the strong persistency and water and lipid repellency, PFAS have been used in a wide range of industrial processes and consumer products. The widespread use of PFAS leads to a continuous diffuse environmental and human exposure. PFAS have been used in fluorocarbon-based ski waxes since the 1980s and the chemical composition is continuously evolving. When the skis are being used, the wax will abrade from the ski sole, and be distributed in the environment. This can potentially lead to PFAS hot spots near ski resorts and ski tracks.

The aim of the current thesis was to investigate the environmental and biota occurrence, distribution, and toxicological effects of PFAS from ski products, studying a local environment and rodents near a ski arena. Soil, earthworm (*Eisenia fetida*) and bank vole (*Myodes glareolus*) samples were collected from a Norwegian skiing area (Granåsen, Trondheim, Norway) and from a reference area with no skiing activities (Jonsvatnet, Trondheim, Norway). PFAS concentrations and composition (referred to as PFAS profile) were analyzed and compared with previous studies on commercial fluorinated ski waxes. Possible PFAS-related effects on the dopaminergic and steroid hormone systems and relative liver weight (hepatosomatic index; HSI) in the wild bank voles were also investigated. These results were further reproduced in a controlled laboratory exposure study using A/J mice (*Mus musculus*).

The summarized PFAS concentrations (∑PFAS) were significantly higher in bank voles from the skiing area compared to the reference area, and 35% higher in earthworms from the skiing area, compared to the reference area. The perfluorocarboxylic acid (PFCA) profile in samples from the skiing area resembled that of the previously analyzed commercial ski waxes, dominated by long-chained PFCAs, while the samples from the reference area were dominated by short-chained PFCAs. This indicates that animals inhabiting skiing areas are exposed to higher PFAS concentrations than animals inhabiting areas with no skiing activities, and that these PFAS most likely are derived from fluorinated ski wax.

Significant effects on the dopaminergic system were detected in both the bank voles from the field and the A/J mice exposed in the laboratory. However, the observed effects on dopamine (DA) concentrations were contradictory in the two studies, suggesting that potentially different molecular mechanisms could be affected. The bank voles from the skiing area had significantly higher brain DA concentrations, compared to the reference area. There was a negative association between PFAS and transcription level of monoamine oxidase (*mao*), encoding the Mao enzyme which is important for DA-metabolism. Thus, PFAS may have reduced the levels of Mao enzymes, leading to lower DA metabolism and thus a build-up of DA in the brain. The PFAS exposed male A/J mice, however, had significantly lower DA concentrations in their brain, compared to control mice, with parallel lower transcription levels of tyrosine hydroxylase (*th*), which encodes an

enzyme that is important for DA synthesis. Thus, PFAS may have reduced the levels of Th enzymes, resulting in lower synthesis of DA and thus decreased DA concentrations in the brain.

Alterations of the dopaminergic system in small mammals can lead to cognitive disturbances and may affect the modulation of fear and anxiety, thermoregulation processes, defense responses and reproductive pathways. Excessive or deficient levels of DA have been hypothesized to contribute to a broad spectrum of mood, motor, and thought abnormalities. Thus, the possible PFAS-related alterations of the dopaminergic system and associated signaling pathways might potentially produce neurological disfunctions that may affect individual fitness of the exposed rodents.

Reduced testosterone (T) concentrations were detected in the muscle tissue of male bank voles from the skiing area, compared to the reference area. In the A/J mice, however, there were no effects of PFAS exposure on T, 17β-estradiol (E2) or 11-Ketostestosterone (11-KT) concentrations in either muscle- or plasma samples. The fact that there was no effect on the steroid-hormone system of A/J mice suggests that the observed associations between T and PFAS in the field study could potentially be due to other, non-measured variables in the nature, rather than PFAS alone. However, this discrepancy could also be caused by a range of other differences between the field and laboratory conditions, including different species, age or interactions with other pollutants and/or stressors.

In the A/J mice, the HSI was significantly higher in exposed males, compared to control males. In the bank voles, however, there was no difference in HSI between the skiing area and reference area. This indicates that the PFAS mixture that is based on the composition of fluorinated ski waxes could potentially produce toxicological and/or physiological responses in the liver, but it does not appear to increase the liver weight at the concentrations, or conditions that the wild bank voles were exposed to in the current study.

The liver PFAS concentrations reported in the bank voles from the skiing area and PFAS-exposed A/J mice, were within the range of concentrations reported in the plasma of professional waxing technicians. This indicates that the effects observed on the dopaminergic and endocrine systems in rodents could possibly be of concern for humans that are exposed to PFAS from ski wax products, although species-specific differences in toxicokinetics and toxicodynamics should be considered. The results presented in this thesis should be used to inspire future research on mixture effects of PFAS, preferably at environmentally- or human relevant concentrations, on the liver weight and the complex dopaminergic and steroid hormone systems.

Oppsummering (summary in Norwegian)

Per- og polyfluorerte alkylstoffer (PFAS) er menneskeskapte kjemikalier som finnes overalt i naturen. På grunn av de vann- og fettavvisende egenskapene, og siden de er så persistente, har de blitt brukt i en mengde industrielle prosesser og forbruksvarer. Den utbredte bruken av PFAS i diverse produkter fører til en kontinuerlig diffus eksponering for mennesker og miljøet. PFAS har blitt brukt i fluorkarbon-basert skivoks siden 1980-tallet, og den kjemiske sammensetningen i skivoksen utvikler seg stadig. Når skiene brukes, vil voksen etter hvert slites bort fra skisålen og bli liggende igjen i miljøet. Dette kan potensielt føre til områder med høy PFAS-forurensning i nærheten av alpinanlegg og skispor.

Målet med dette prosjektet var å undersøke nivåene, sammensetningen og mulige effekter av PFAS i miljøet og biota i et skiområde. Dette ble undersøkt ved å ta jord-, meitemark- og klatremusprøver fra et skiområde (Granåsen, Trondheim, Norge), og sammenligne med prøver fra et referanseområde uten skiaktivitet (Jonsvatnet, Trondheim, Norge). PFAS-konsentrasjoner og PFAS-profiler ble analysert og sammenlignet med tidligere studier på kommersiell fluorholdig skivoks. Forskjellige effektparametere relatert til det dopaminergiske systemet, steroidhormonsystemet og relativ levervekt (hepatosomatisk indeks; HSI) ble også analysert. De målte effektene fra det naturlige miljøet ble videre forsøkt reprodusert i et kontrollert laboratorieeksperiment ved bruk av A/J mus.

De summerte PFAS-konsentrasjonene (∑PFAS) var signifikant høyere i klatremus fra skiområdet sammenlignet med referanseområdet, og 35% høyere i meitemark fra skiområdet, sammenlignet med referanseområdet. Sammensetningen (profilen) av perfluorerte karboksylsyrer (PFCA) i prøvene fra skiområdet lignet på profilen til de tidligere analyserte kommersielle skivoksene, der alle var dominert av langkjedede PFCA. Prøvene fra referanseområdet var imidlertid dominert av kortkjedede PFCA. Disse resultatene indikerer at dyr som lever i skiområder er eksponert for høyere PFAS-konsentrasjoner enn dyr som lever i områder uten skiaktiviteter, og at disse PFAS-ene mest sannsynlig stammer fra fluorholdig skivoks-bruk.

Det ble detektert signifikante forskjeller i dopamin (DA)-nivåer mellom klatremusene fra Granåsen og klatremusene fra referanseområdet, og mellom A/J musene som var eksponert for PFAS, og kontrollmusene. De observerte effektene på DA-konsentrasjonene var imidlertid motsatt i de to artene, og forskjellige molekylære mekanismer ser ut til å være påvirket. I klatremusene var det høyere konsentrasjoner av DA i hjernen til mus fra skiområdet, sammenlignet med referanseområdet. I skiområdet var det en negativ sammenheng mellom PFAS-konsentrasjonene og transkripsjonen av monoaminoksidase (*mao*), som koder for et enzym som er viktig for DA-metabolismen. Dette kan potensielt ha ført til lavere DA-metabolisme og dermed en opphopning av DA i hjernen til klatremusene. Hos A/J musene, derimot, var det lavere DA-konsentrasjoner i mus som var eksponert for PFAS, sammenlignet med kontrollmusene. Det var også lavere gen-

transkripsjon av tyrosin hydroksylase (*th*), som koder for et enzym som er viktig for å syntetisere DA. PFAS-eksponeringen kan ha ført til lavere nivåer av enzymet Th, som resulterer i lavere syntese av DA fra tyrosin og dermed reduserte DA-konsentrasjoner i hjernen.

Effekter på det dopaminergiske systemet kan potensielt føre til forstyrrelser i reguleringen av frykt og angst, termoreguleringsprosesser, evnen til å forsvare seg, i tillegg til at det kan påvirke reproduksjonssystemet. Forhøyede, eller reduserte nivåer av DA har også blitt antatt å bidra til humørsvingninger og motoriske- og kognitive endringer. Dermed kan de foreslåtte PFAS-relaterte endringene i det dopaminergiske systemet og de tilhørende signalveiene potensielt føre til nevrologiske forstyrrelser som kan påvirke de eksponerte gnagernes «fitness».

Det var lavere konsentrasjoner av testosteron i muskelvev til hannmus fra skiområdet, sammenlignet med referanseområdet. I A/J-musene var det imidlertid ingen effekter av PFAS-eksponering på konsentrasjonene av testosteron, østrogen, eller 11-ketotestosteron i verken muskelvev eller plasma i noen av kjønnene. Det faktum at det ikke var observert noen effekter på steroidhormonsystemet i A/J-mus, indikerer at de observerte assosiasjonene mellom testosteron og PFAS i klatremusene fra feltstudien kan være forårsaket av andre, ikke-målte variabler i naturen, snarere enn PFAS. Dette avviket kan også være forårsaket av en rekke andre forskjeller mellom felt- og laboratoriestudiet, inkludert forskjeller mellom arter, ulik alder eller interaksjoner med andre forurensende stoffer og /eller stressfaktorer.

PFAS-konsentrasjonene som ble målt i klatremus fra skiområdet og de PFAS-eksponerte A/J musene, ligger innenfor spekteret av konsentrasjoner som er rapportert i plasmaprøver tatt fra profesjonelle skivoksteknikere. Dette indikerer at de målte effektene på det dopaminergiske systemet hos gnagere muligens også kan observeres hos mennesker som er utsatt for PFAS fra skivoksprodukter. Men, man må ta høyde for at det er artsforskjeller i toksikokinetikken og -dynamikken som bør vurderes når man ekstrapolerer fra gnagere til mennesker. Resultatene som presenteres i denne avhandlingen bør brukes til å inspirere fremtidig forskning på blandingseffekter av PFAS, helst ved miljørelevante konsentrasjoner, på levervekt og på de komplekse dopamin- og steroidhormonsystemene.

Abbreviations

ANOVA	Analysis of variance
C	Carbon
Comt	Catechol-O methyltransferase
Cyp19	Aromatase
DA	Dopamine
Dat	Dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
Dr1	Dopamine receptor 1
Dr2	Dopamine receptor 2
d.w.	Dry weight
E2	17β-estradiol
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
EOF	Extractable organic fluorine
F	Fluorine
GLM	General linear model
Gnrh	Gonadotropin releasing hormone
HSI	Hepatosomatic index
HPG	Hypothalamus-Pituitary-Gonadal
HVA	Homovanillic acid
HPLC MS-MS	High pressure liquid chromatography tandem mass spectrometry
HPT	Hypothalamus-pituitary-thyroid
LOD	Limit of detection
LOQ	Limit of quantification
Mao	Monoamine oxidase
NMBU	Norwegian University of Life Sciences
NTNU	Norwegian University of Science and Technology
OCPs	Organochlorine pesticides

PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PFAS	Perfluoroalkyl substances
PFCA	Perfluoroalkyl carboxylates
PFDA	Perfluorodecanoic acid
PFDoDA	Perfluorododecanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFSA	Perfluoroalkyl sulfonates
PFTrDA	Perfluorotridecanoic acid
PFUdA	Perfluoroundecanoic acid
POPs	Persistent organic pollutants
qPCR	Quantitative polymerase chain reaction
T	Testosterone
Th	Tyrosine hydroxylase
TOP	Total oxidizable precursor
TWI	Tolerable weekly intake
URC	University of California Riverside
Vmat	Vesicular monoamine transporter
w.w.	Wet weight
11-KT	11-Ketotestosterone

List of papers

I. Grønnestad, R., Vázquez, B. P., Arukwe, A., Jaspers, V. L. B., Jenssen, B. M., Karimi, M., Lyche, J. L., Krøkje, Å. (2019). "Levels, patterns, and biomagnification potential of perfluoroalkyl substances in a terrestrial food chain in a Nordic skiing area". *Environmental Science & Technology,* 53: 13390-13397

II. Grønnestad, R., Schlenk, D., Krøkje, Å., Jaspers, V. L. B., Jenssen, B. M., Coffin, S., Bertotto, L. B., Giroux, M., Lyche, J. L., Arukwe, A. (2020). "Alteration of neuro-dopamine and steroid hormone homeostasis in wild bank voles in relation to tissue concentrations of PFAS at a Nordic skiing area". *Science of the Total Environment,* 756: 143745

III. Grønnestad, R., Johanson, S. M., Müller, M. H. B., Schlenk, D., Tanabe, P., Krøkje, Å., Jaspers, V. L. B., Jenssen, B. M., Ræder, E. M., Lyche, J. L., Shi, Q., Arukwe, A. "Effects of an environmentally relevant PFAS mixture on dopamine and steroid hormone levels in exposed mice". *Submitted manuscript*

Declaration of contribution:

RG conducted the statistical analyses and was the main author for all papers, with contributions and comments from the co-authors.

Paper I was initiated and planned by RG, in collaboration with ÅK, AA, VLBJ and BMJ. The field work and sample collection were conducted by RG and BPV. The extraction of samples for the chemical analysis was conducted by RG, while the LC-MS/MS analysis was conducted by MK and JLL.

Paper II was initiated and planned by RG, in collaboration with AA, ÅK, VLBJ, BMJ and DS. The effect analyses were conducted by RG, with help from LBB, SC, MG and DS. The qPCR was conducted by RG.

Paper III: RG, SMJ and MHBM initiated and planned the study with contribution from AA, ÅK, VLBJ and BMJ. RG calculated and made the experimental feed. SMJ conducted the mouse breeding and exposure study. RG, SMJ and MHBM conducted the sampling. DS, PT and QS conducted the DA analysis, while RG conducted the steroid analysis and qPCR. JLL and EMR conducted the chemical analysis.

Papers published during the PhD-period, but not included in the thesis:

IV. Müller, M. H. B., Polder, A., Brynhildsrud, O. B., Grønnestad, R., Karimi, M., Lie, E.; Manyilizu, W. B., Mdegela, R. H., Mokiti, F., Murtada, M., Nonga, H. E., Skåre, J. U., Solhaug, A., Lyche, J. L. (2019). "Prenatal exposure to persistent organic pollutants in Northern Tanzania and their distribution between breast milk, maternal blood, placenta and cord blood". *Environmental Research*, 170: 433-442

V. Grønnestad, R., Villanger, G. D., Polder, A., Kovacs, K. M., Lydersen, C., Jenssen, B. M., Borgå, K. (2018). «Effects of a complex contaminant mixture on thyroid hormones in breeding hooded seal mothers and their pups". *Environmental Pollution,* 240: 10-16

VI. Jansen, A., Müller, M. H. B., Grønnestad, R., Klungsøyr, O., Polder, A., Skjerve, E., Aaseth, J., Lyche, J. L. (2018). "Decreased plasma levels of perfluoroalkylated substances one year after bariatric surgery". *Science of the Total Environment*, 657: 863-870

VII. Grønnestad, R., Villanger, G. D., Polder, A., Kovacs, M. K.; Lydersen, C., Jenssen, B. M., Borgå, K. (2016). "Maternal transfer of perfluoroalkyl substances in hooded seals". *Environmental Toxicology and Chemistry*, 36(3): 763-770

1. Introduction

There are currently over 350,000 chemicals registered for production and use in the world (Wang *et al.* 2020). Many of these chemicals are found in every-day products. During the last five decades, it has become evident that high concentrations of several persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) and organochlorinated pesticides (OCPs), produce harmful health related effects in humans and wildlife (Colborn *et al.* 1994, Qing Li *et al.* 2006, El-Shahawi *et al.* 2010). Many of these pollutants have either been regulated, phased out of production or banned in consumer products (www.pops.int). However, numerous current-use chemicals have also been shown to be harmful, and proper regulations are missing (Wang *et al.* 2017). These include, but are not limited to, per- and polyfluoroalkyl substances (PFAS) and their precursors. PFAS are a group of chemicals that consist of over 4700 different compounds (OECD/UNEP 2018). The predominant routes of PFAS exposure for most people and terrestrial wildlife are through food and drinking water. However, consumer products also represent a significant exposure source for humans and can contaminate the environment (Sunderland *et al.* 2019, De Silva *et al.* 2021). Still, there is a paucity of information regarding the environmental distribution and possible effects of PFAS deriving from consumer products on humans and wildlife.

1.1. PFAS

PFAS (Figure 1) are ubiquitous and persistent anthropogenic chemicals in the environment (Houde *et al.* 2006, Glüge *et al.* 2020). They are defined as aliphatic substances that contain at least one perfluoroalkyl moiety (i.e., C_nF_{2n+1}-, or -C_nF_{2n}-) (Buck *et al.* 2011, OECD/UNEP 2018). Due to the strong electronegativity and small atomic size of fluorine, the perfluoroalkyl moiety of PFAS enhances the molecular properties of the compound, such as higher surface activity, stability, and/or water- and oil-repellency (KEMI 2015). These properties make PFAS suitable for the production of a wide range of both industrial and consumer products, such as textiles, carpets, cosmetics, impregnating agents and in some types of skiing products, such as ski waxes, gliders and powders (Kotthoff *et al.* 2015).

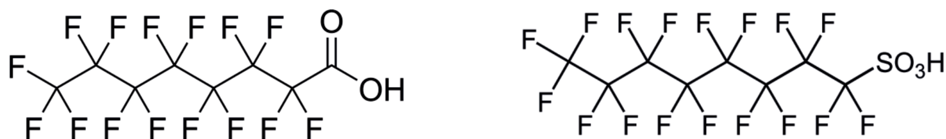

Figure 1. The chemical structure of the eight carbon perfuorooctanoic acid (PFOA, to the left) and perfluorooctane sulfonate (PFOS, to the reght).

The large scale production of PFAS started in the late 1940s (Prevedouros *et al.* 2005). However, little attention was devoted to these chemicals until the early 2000s, when studies showed that PFAS were ubiquitous in the environment (Kannan *et al.* 2001, Houde *et al.* 2006). After a production history of over half a century, perfluorooctane sulfonate (PFOS, Figure 1), perfluorooctanoic acid (PFOA, Figure 1) and their related precursors were in 2009 and 2019, respectively, listed under the Stockholm Convention on POPs, and perfluorohexane sulfonate (PFHxS) and its salts, is currently considered for listing (www.pops.int). In the developed countries, there have been several national/regional regulatory and voluntary initiatives established to mainly regulate PFOS and PFOA. However, there are still a wide range of PFAS in use and produced for the industrial and commercial market (OECD 2015). Currently, the most common industrial practice of phasing out one PFAS is to replace it with another (or several others) structurally similar PFAS (Wang *et al.* 2013). Such a strategy is easier and less costly than identifying a nonfluorinated substance to be used in the same or similar process (i.e., chemical replacement) or inventing a new process that does not require PFAS (i.e., functionality replacement) (Wang *et al.* 2015). However, this practice leads to the release of new, unregulated PFAS to the environment, rather than restricting the environmental contamination.

The carbon-fluorine bond (C-F-bond) is a strong, high energy bond that contributes to the stability of PFAS. PFAS are resistant against degradations by acids, bases, oxidants, reductants, photolytic processes, microbes, and metabolic processes (Prevedouros *et al.* 2005). The high persistence of PFAS leads to long-term exposure to these substances in the global environment (Cousins *et al.* 2016). Past and ongoing production and use of PFAS will lead to the accumulation and global distribution of persistent PFAS in the environment, with either slow mixing or sedimentation to the deep oceans, representing the main global environmental sinks (Prevedouros *et al.* 2005).

While legacy POPs accumulate in lipid rich tissues, PFAS are known to bind to proteins and accumulate mainly in blood, liver, kidneys and bile (Jones *et al.* 2003). The high solubility and protein-binding characteristics of PFAS challenge the conventional assessment of bioaccumulation potential that is either through bioconcentration factor (BCF) in aquatic species or models that are based on octanol–water partition coefficients (K_{ow}) (Vierke *et al.* 2012). Long-chain PFAS are defined as ≥8 carbon chain-length for perfluorocarboxylic acids (PFCAs), and ≥C6 carbon chain-length for perfluorosulfonic acids (PFSAs) (OECD 2021). These are generally considered bioaccumulative and can biomagnify in food webs (i.e., can be transferred up the food chain, where concentrations increase from one trophic level to the next via dietary accumulation) (Gobas and Morrison 2000). However, even when PFAS are not characterized as bioaccumulative (e.g. short-chain PFAS), the accumulation of these PFAS in the environment will lead to increasing exposure and uptake (Cousins *et al.* 2016).

1.2. PFAS in ski products

PFAS have been used in ski wax since the 1980s (Gotaas 2003). The global production of ski waxes is estimated to be 120 tons per year. Around 60% of the total market is produced in the European Economic Area (EEA), and the other main producers are USA, Japan and Russia (Heggelund 2021). In 2020, the global ski wax market was valued at 183.6 million USD and is estimated to grow at a compound annual growth rate (CAGR) at 2.6 during the next 5 years (MarketWatch 2021). Ski waxes can be divided into hydrocarbon-based and fluorocarbon-based (fluorinated) waxes (Breitschädel *et al.* 2014). While most recreational skiers use hydrocarbon-based glide waxes (approximately 70% of the total market) due to the high price of fluorinated waxes, the fluorinated waxes are favored by competitive skiers, or serious amateurs, because they have extremely hydrophobic properties (Heggelund 2021). Thus, fluorinated waxes function as water repellants from the bottom of the skis, allowing for increased glide over the snow, compared to hydrocarbon-based waxes (Breitschädel *et al.* 2014). However, application of these products (Figure 2) and abrasion of the waxes from the ski sole results in deposition of PFAS to the nearby environments (Plassmann and Berger 2013).

Figure 2. Professional ski waxing technicians applying ski wax for the biathlon World Cup 2020 in Oberhof, Germany. Photo: NordicFocus.

According to Kotthoff *et al.* (2015), ski wax has the highest concentrations of both PFCAs and PFSAs, compared to a wide range of other PFAS-containing consumer products. In November 2019, The International Ski Federation (FIS) announced that they would implement a ban of PFAS-containing ski wax products in all competitions from the 2020/21 season (FIS 2020). However, because of limitations of the testing devices that would be used during the competitions, the ban was postponed until 2021/22 (FIS 2020). The industry has for several years claimed that they have switched to formulations that contain chemicals based on shorter perfluoroalkyl chains. However, analytical results show that this is not the case (Fang *et al.* 2020). A recent study by Fang *et al.* (2020), analyzed eleven of the best-selling PFAS-containing ski wax products on the Norwegian market in 2019. They reported that PFOA levels in nine of the eleven ski waxing products analyzed were above the EU limit of 25 ng/g, which came into force on 4th July 2020 (EU Commissions delegated regulations No. 2020/784). The ski wax with the highest PFOA levels had a concentration that was 1215 times higher than the EU restrictions (Fang *et al.* 2020).

Based on concerns regarding the high persistence of PFAS and the lack of knowledge on chemical structures, properties, uses, and toxicological profiles of most PFAS currently in use, it has been argued by more than 200 scientists in "the Madrid Statement" that the production and use of PFAS should be limited (Blum *et al.* 2015). The Madrid Statement argues for stopping the use of PFAS where they are deemed not essential or when safer alternatives exist. Cousins *et al.* (2019) defines ski waxes as "non-essential" use of PFAS. Non-essential use is defined as "Uses that are not essential for health and safety, and the functioning of society. The use of substances is driven primarily by market opportunity". Recently, the Norwegian Environment Agency recommends avoiding the use of fluorinated ski waxes (Heggelund 2021). Furthermore, the Norwegian Ski Federation recommends the use of fluorine-free wax products at all cross-country ski races, and it is prohibited in the "under 16 years of age" classes (Skiforbundet 2017).

For most PFAS, there is either limited or non-existent understanding on the release, occurrence and accumulation patterns in the environment and biota over time (McGuire *et al.* 2014). Additionally, the concept of mixture toxicity is not adequately considered in the individual chemical-based paradigm often employed in various countries. Despite known structural similarities among many PFAS, there is nearly a complete lack of empirical knowledge on mixture toxicity for the ongoing simultaneous, chronic, low-level exposure to a large number of known and unknown PFAS (Wang *et al.* 2017).

1.3. Potential effects of PFAS exposure

Analysis of serum samples from professional ski waxing technicians have shown elevated concentrations of PFCAs, compared to the general population (Freberg *et al.* 2010, Nilsson *et al.* 2010). This is of great concern, since human studies have shown that PFAS can lead to several adverse health effects, such as increased cholesterol levels (Nelson *et al.* 2010), thyroid hormone

disruption (Thibodeaux *et al.* 2003), immunotoxicity (Yang *et al.* 2002, Keil *et al.* 2008, DeWitt *et al.* 2012), metabolic effects (Abbott *et al.* 2012, Jiang *et al.* 2015) and increased risk of cancer (Cohn *et al.* 2020). Increasing evidence suggests that exposure to low doses of either PFOA or PFOS at an early developmental stage produce effects that persist through ontogeny (DeWitt 2015). Several PFAS have been identified as endocrine-disrupting chemicals based on their ability to interfere with normal reproductive function and hormonal signaling (Jensen and Leffers 2008) and some PFAS are classified in the European Union (EU) as toxic for the reproduction and the liver for humans (Sunderland *et al.* 2019). Laboratory studies also suggest that PFAS can be neurotoxic and can lead to neurochemical and neurobehavioral alterations (Johansson *et al.* 2008, Sunderland *et al.* 2019). However, there is limited information regarding the effects of PFAS on wildlife species inhabiting areas where fluorinated products are being used and released into the environment.

1.3.1. Dopamine

Due to the blood-brain barrier, the brain is rarely considered a significant target for POPs (Staddon and Rubin 1996, Gebbink *et al.* 2008). However, PFAS have been found to accumulate and reach high concentrations in the brain of polar bears (*Ursus maritimus)* (Greaves *et al.* 2013, Pedersen *et al.* 2015), North Atlantic pilot whales (*Globicephala melas*) (Dassuncao *et al.* 2019), and has even been detected in the human brain (Maestri *et al.* 2006). Studies of large mammals suggest that PFAS can potentially be neurotoxic in exposed individuals (Dassuncao *et al.* 2019). In polar bears, brain PFAS levels were found to correlate with neurotransmitter alterations (Pedersen *et al.* 2015). Neurotoxicity studies in rodents showed that PFAS produced neurobehavioral alterations (Johansson *et al.* 2009, Lee and Viberg 2013) and developmental and motor deficits (Onishchenko *et al.* 2011). Some studies have suggested that the dopaminergic system is a potential target for environmental contaminants (Hallgren and Viberg 2016).

Dopamine (DA) is a hormone and neurotransmitter that plays several important roles in the brain and body. It is derived from the amino acid tyrosine, 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 catabolism occurs through metabolism of DA to the inactive 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (Mao), and then to homovanillic acid (HVA) by catechol-O methyltransferase (Comt) (Ashcroft 1969). A simplified illustration of the dopaminergic system is shown in Figure 3. DA controls many functions including cognition, mood, reward, fear, anxiety, vascular and reproductive functions (Nakajima *et al.* 2013, Goschke and Bolte 2014). DA is also involved in appetite, learning and certain aversive memory processes (Schultz 2013, Volman *et al.* 2013).

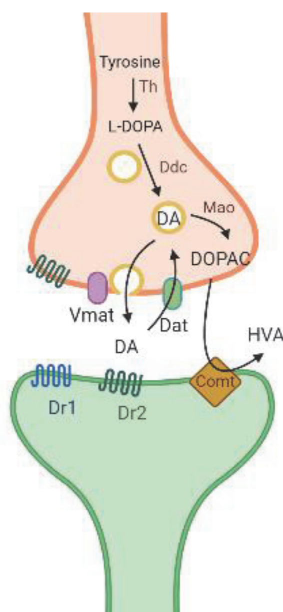

Figure 3. Illustration of the dopaminergic system showing the synthesis and catabolism pathways of DA, in addition to receptors and DA transporters. DA: dopamine, L-DOPA: L-3,4-dihydroxyphenylalanine, DOPAC: 3,4-dihydroxyphenylacetic acid, HVA: homovanillic acid, Mao: monoamine oxidase, Comt: catechol-O-methyltransferase, Ddc: DOPA decarboxylase, Th: tyrosine hydroxylase, Dat: dopamine active transporter, Vmat: vesicular monoamine transporter, L-DOPA: L-3,4-dihydroxyphenylalanine, Dr1: dopamine receptor 1, Dr2: dopamine receptor 2. Illustration created in biorender.com.

Laboratory studies on the effects of PFAS exposure on the dopaminergic system show diverging results. For example, northern leopard frogs *(Lithobates pipiens)* exposed to PFOS and PFOA (Foguth *et al.* 2019), and mice (*Mus musculus)* exposed to PFOS showed decreased DA levels (Long *et al.* 2013). On the other hand, 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. This raises the question of whether the exposure of rodents to PFAS in skiing areas could affect the dopaminergic system.

1.3.2. Steroids

Previous studies have reported that PFAS may affect sex steroid levels (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). Sex steroids (androgens, estrogens, and progestogens) are steroid hormones that interact with vertebrate steroid hormone receptors (Guerriero 2009). Sex steroids are produced by the gonads (ovaries or testes) (Brook 1999), by adrenal glands, or by conversion from other sex steroids in other tissues through enzymatic processes (Simpson and Davis 2001). Estrogens and

androgens are involved in growth and normal functioning of the reproductive organs, development of secondary sexual characteristics, and behavioral patterns in vertebrate species (Gaikwad 2013). Thus, the balance in various steroid metabolic pathways has been shown to be associated with reproductive health. Consequently, measurement of steroid hormones may assist in determining the physiological health status of organisms (Gaikwad 2013). However, most *in vivo* studies have used exposure scenarios with individual contaminants and at high concentrations that are neither environmentally nor physiologically relevant. Thus, more studies are needed to assess the endocrine disrupting potential of PFAS at environmental concentrations and in mixtures.

During reproductive development, estrogen (17β-estradiol: E2) and testosterone (T) biosynthesis is regulated through the hypothalamus–pituitary–gonadal (HPG) axis (Figure 4) (Zohar *et al.* 2010). The hypothalamus produces gonadotropin-releasing hormone (GnRH), and its release controls biosynthesis of the gonadotropins (GtHs): luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both LH and FSH regulate ovarian and testicular development, maturation, and release, and also control gonadal hormone synthesis, including E2 and T. T and E2 can also be modulated by the conversion of T to E2 by the aromatase enzyme (cyp19) in the brain, or other extragonadal sites, such as breast and adipose tissue (Simpson and Davis 2001).

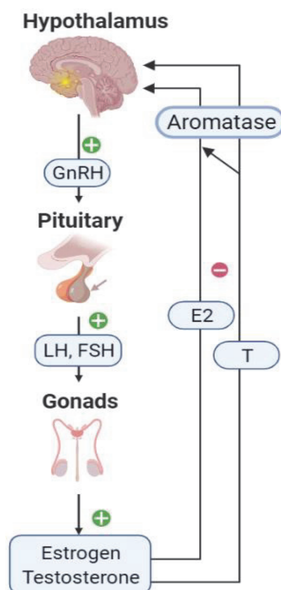

Figure 4. Illustration of the HPG-axis (hypothalamus-pituitary-gonadal-axis). GnRH: gonadotropin-releasing hormone, LH: luteinizing hormone, FSH: follicle-stimulating hormone, E2: estradiol, T: testosterone. Illustration created in biorender.com.

1.3.3. Hepatotoxicity

The liver is the primary organ for both endogenous and exogenous substance metabolism in mammals (Jiang *et al.* 2015). Since most PFAS are amphiphilic, they mainly partition to protein-rich tissues (Jones *et al.* 2003), and the liver has been identified as the primary organ for PFAS accumulation (Goecke-Flora and Reo 1996, Aas *et al.* 2014). It is therefore natural that the liver is a target for PFAS toxicity (EFSA 2018). PFAS are known to lead to increased relative liver weight or enlarged livers (hepatomegaly) and overexpression of fatty acid β-oxidation-related genes both *in vitro* and in experimental animal models. For example, increased relative liver weight has been observed following PFOA and PFOS exposure in rats (Kudo *et al.* 2006, Cui *et al.* 2008) and mice (Yu *et al.* 2016). PFOA has been reported to cause hepatocellular hypertrophy, hepatic triglyceride accumulation, increased peroxisomal β-oxidation and lipid droplets in hepatic nuclei in mice (Kudo and Kawashima 1997, Lau *et al.* 2007, Son *et al.* 2008, Wang *et al.* 2013). However, there is a paucity of studies on effects of PFAS on the liver weight and possible hepatotoxicity in wildlife and laboratory animals at environmentally relevant concentrations.

1.4. Rodents as model organisms

Rodents are excellent model organisms for toxicity studies (OECD 2008). When chemical exposures are linked to documented health effects in humans, the cause-and-effect relationship and clarification of the mechanism and mode of action is generally derived from experimental studies using mammal-based models (Bryda 2013). Furthermore, adverse health effects of pesticides, pharmaceuticals and industrial chemicals in humans are generally studied in surrogate animals, such as rodents. The use of murine models in human health research has several advantages, including a high similarity in genes, small body size, and short generation time, compared to larger mammals. Furthermore, there is a detailed understanding of the mouse biology and genetics acquired from their long-time use as research animals (Bryda 2013).

Regarding environmental toxicology studies, rodents are good model study organisms in the field because they are relatively easy to catch and handle. In addition, they have a relatively small home range (Haupt *et al.* 2010), so it is easier to trace back the source and route of the potential contamination detected within the animals, compared to larger mammals. bank voles (*Myodes glareolus*), which are used in the current study, have been used in several monitoring studies on organic contaminants and metal pollution (Sawicka-Kapusta *et al.* 1990, Leffler and Nyholm 1996, Gdula-Argasińska *et al.* 2004, Martiniaková *et al.* 2010, Ecke *et al.* 2020).

2. Aims, objectives and hypotheses

The overall aim of this thesis was to characterize the environmental and biota occurrence, distribution, and toxicological effects of PFAS from skiing products, studying the environment and animals in a skiing area, and comparing with a non-skiing reference area. The effects identified in the natural environment were further reproduced in a controlled laboratory experiment using laboratory mice. The overall aim was divided into specific objectives with associated hypotheses:

Objective 1: Determine the concentrations of various PFAS in a skiing area, using soil, earthworm, and bank vole samples (**Paper I**).

> **H1:** There will be significantly higher PFAS concentrations in soil, earthworm and bank vole samples from the skiing area, compared to the reference area.

Objective 2: Investigate PFAS profiles in the different samples to determine the source of PFAS contamination in a skiing area (**Paper I**).

> **H2:** PFAS profiles in samples from the skiing area will be similar to the PFAS profiles in previously analyzed commercial ski waxes, and dissimilar to the PFAS profiles from the refence area.

Objective 3: Investigate the effects of PFAS on the dopaminergic and steroid systems of wild bank voles in a skiing area, relative to a reference area (**Paper II**).

> **H3:** Bank voles from the skiing area will show alterations of the dopaminergic and steroid hormone systems, compared to the reference area, and these effects will be associated with the measured PFAS concentrations, representing potential health consequences.

Objective 4: Investigate the effects of environmentally relevant concentrations of a PFAS mixture on the dopaminergic and steroid systems in A/J mice, under controlled laboratory conditions (**Paper III**).

> **H4:** Exposure to a PFAS mixture will alter the dopaminergic and steroid hormone systems of exposed A/J mice, compared to the control group, and these alterations will parallel observations from the field.

Objective 5: Investigate the possibility of extrapolating the results from the field and laboratory (i.e., the effect studies in **Paper II** and **III**) to possible effects on humans.

> **H5:** PFAS bioaccumulation and toxicological effects observed in the field will show similar responses in the laboratory, forming a significant basis for extrapolation to humans.

3. Methods

3.1. Study design

The current project was divided into two work packages: 1) the field study and 2) the laboratory exposure study. The field study was performed to measure the concentrations of PFAS in a skiing area and a reference area, and to investigate possible toxicological effects on the bank voles inhabiting these areas. The laboratory study was performed in order to assess the reproducibility of the observations from the field, under controlled laboratory conditions, and removing potential confounding factors in the environment.

3.2. Field study

3.2.1. Study area

The study area was “Granåsen skisenter” (Figure 5, 63º 22’N, 10º18’E), which is located approximately 10 km southwest from the city center of Trondheim (Norway). Granåsen is the main arena for winter sports in Trondheim and surrounding cities. The arena hosts a range of regional, national and international competitions in cross-country skiing. Thus, Granåsen includes several cross-country ski tracks that are used for training and competitions by professionals, amateurs, and recreational skiers.

As a reference site, a natural forest area not used for ski-sports, was chosen in the vicinity of an organic farm next to Lake Jonsvatnet (Figure 5, 63º20’N, 10º33’E). This site is approximately 15 km southeast from Trondheim city center. The two study areas have quite similar vegetation, consisting of mainly mosses and different species of *Ericaceae,* commonly known as the heath family.

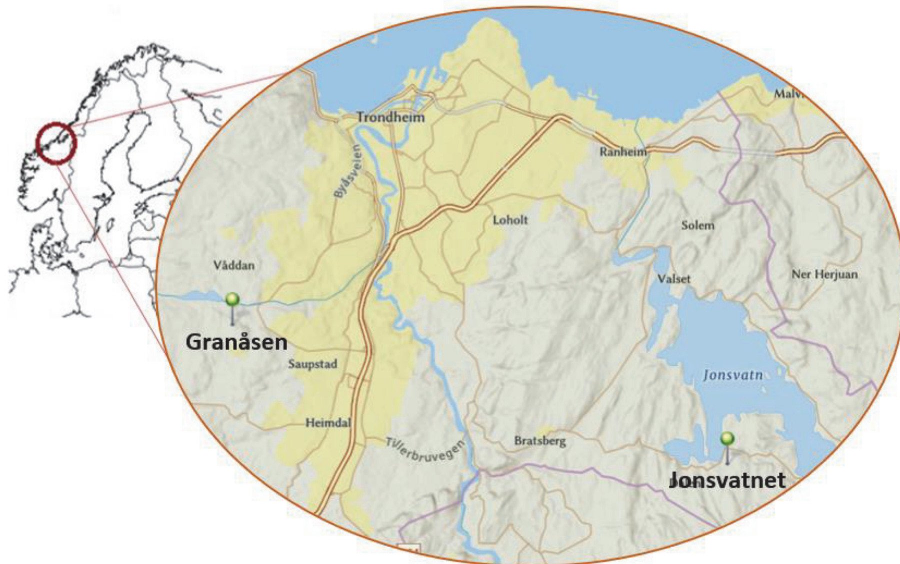

Figure 5. Map of the study areas. Granåsen is the skiing area and Jonsvatnet is the reference area (marked with green pins). Modified figure retrieved 03.10.2019, from ArcGis.

3.2.2. Earthworms (Eisenia fetida)

Earthworms are considered one of the most suitable model organisms for monitoring and assessing soil pollution, as they are integral soil macro-invertebrates (Cortet *et al.* 1999, Gao and Luo 2005). Earthworms constitute an important part of the diet of local rodent and bird species and serve as the gateway for chemical movement from the contaminated soils and ground water into the terrestrial food chain (Cortet *et al.* 1999). Thus, earthworm was chosen as a test organism due to its critical role at the base of the investigated terrestrial food chain and its constant contact and ingestion of soil. Earthworms have a life expectancy of 1-5 years (Venter and Reinecke 1988). They are susceptible to chemicals, and provide information on the bioavailability of soil contaminants (Gao and Luo 2005).

3.2.3. Bank voles (Myodes glareolus)

The bank vole (Figure 6) is a rodent that lives in woodland areas and is around 10 centimeters in length and weighs between 15.4 and 36.0 grams. It is found in much of Europe and in northwestern Asia. It can live for eighteen months to two years in the wild and is mostly herbivorous, feeding on buds, bark, seeds, nuts, leaves and fruits and occasionally insects and other small invertebrates, such as earthworms, and may take eggs from ground-nesting birds. The bank vole is found in forests, especially in deciduous and mixed woodland with scrub, low

plants and leaf litter (Konig 1973). They are active both by day and night and do not hibernate in the winter. They are therefore more susceptible to be exposed to PFAS deriving from ski wax during the whole year, compared to other rodents that go into winter hibernation. Females maintain territories which may overlap somewhat, and males occupy larger territories covering those of several females. The home range of females is usually between 500 and 2,000 m^2 (Haupt *et al.* 2010), while the home range of adult males can reach up to 4,000m^2 (Korn 1986). The breeding season lasts from late April to September and the gestation period averages 21 days. The pups are weaned at 20 to 25 days and the females become sexually mature by six weeks with the males reaching maturity by eight weeks. There may be up to four litters per year (Konig 1973). bank voles play an important part in the diet of various predators including the red fox (*Vulpes vulpes*), the least weasel (*Mustela nivalis*), the common kestrel (*Falco tinnunculus*), the rough-legged buzzard (*Buteo lagopus*) and the tawny owl (*Strix aluco*) (Lundrigan 2003).

The bank vole was chosen as a model species because it is an important intermediate species in the terrestrial food chain (Koivula *et al.* 1999). In addition the species has a relatively small home range, so it could be expected that their contaminant levels are representative of the area where they were caught.

Figure 6. Pictures from the field work. Left: shows how the cage traps were set up in the field. Right: shows a cage trap with a bank vole. Photos: Randi Grønnestad.

3.2.4. Sampling

The collection of bank voles was performed in May/June 2017. The catching, handling, anesthesia, sampling and euthanizing of the bank voles were approved by the Norwegian Food Safety Authority (Mattilsynet; references no. 2017/76552) and by the Norwegian Environmental Agency (Miljødirektoratet; reference no. 2017/4061). Permissions for the collection of bank voles were also given by the land-owners. All traps were live traps of type "Ugglan" baited with rye bread dipped in sunflower oil and peanut butter (Figure 6). Once captured, the animals were brought back to the animal facilities at NTNU for sampling. The bank voles were sacrificed by cervical dislocation. The animals were weighed and visually sexed. The livers and brains were dissected, weighed and snap-frozen in liquid nitrogen and stored at -80°C. The eyes were dissected for later age determination (see **Paper I** for method) and stored in 10% formalin. In total, 21 and 31 individuals were caught at the Granåsen and Jonsvatnet areas, respectively, during a period of 5 weeks. For more details on sampling and handling, see **Paper I**.

The sampling of earthworms was performed in June of 2018 by digging 5-10 cm into the soil, using a metal spade, and collecting the animals in sealed plastic bags. The earthworms were collected in the same areas as the bank voles were captured. They were frozen at -80 °C until analysis. The short time between collection and freezing did not allow them to empty their guts, as this would be more representative of how they serve as bank voles' prey.

The soil samples were collected in May/June 2017 and June 2018 from the Granåsen and Jonsvatnet areas. The upper layer (constituting from 3-10 cm depth and an area of approximately $1m^2$) of soil was collected and dried. Five samples per year (2017 and 2018) in Granåsen and Jonsvatnet were chosen for chemical analysis. Only soil from locations where both earthworms and bank voles had been sampled were selected for analysis.

3.3. Laboratory exposure study

In the *in vivo* laboratory exposure study, A/J mice were used as a model species (Figure 7). This is a common mouse strain in toxicity studies because they are inbred, meaning that there is little genetic difference, and thus less variation between individuals compared to outbred strains. In addition, they are relatively calm and easy to breed. They have a strong tendency to develop tumors when presented with common carcinogens and are therefore often used in cancer research (Falconer and Bloom 1962). The life-span of A/J mice in specific pathogen free (SPF) fostered conditions is around 512 days in males and 558 days in females (Festing and Blackmore 1971).

The exposure study was conducted 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 SPF conditions (Figure 7).

Figure 7. Left: The animal facilities at NMBU and Silje Modahl Johansson, who was coordinating the breeding and husbandry of the exposure. Photo: Randi Grønnestad. Right: A/J mouse. Photo: Andrea Johanna Eickstedt

3.3.1. Feed design and chemicals

The composition and concentrations of PFAS in the feed were chosen based on the results from **Paper I**, to reflect the highest concentration found in earthworms from the skiing area, since earthworms are part of the diet of bank voles. The most predominant detected PFAS that were also detected in ski wax (mainly long-chained PFAS) were used in the PFAS mixture. PFOS and PFOA could not be added to the regular pellet feed by the manufacturer because of their regulatory status and were therefore prepared separately in gel feed. See **Paper III** for more details on the design of the feed composition. The desired experimental concentrations and the measured PFAS concentrations are presented in Table 1. See Figure 8 for an overview of the feed and exposure regime.

Table 1. Desired experimental concentration (the concentrations detected in earthworms at a skiing area) and measured PFAS concentrations in the feed. Values are given in ng/g feed. No PFAS were detected in control feed, hence not shown in the table. This table is obtained from **Paper III** and modified.

PFAS-mixture	Desired experimental concentrations	Measured concentrations
PFNA	2.0	1.75
PFDA	3.0	2.96
PFUdA	3.0	2.98
PFDoDA	8.0	7.21
PFTrDA	16	11.4
PFTeDA	20	14.2
PFOA*	17.5	37.6
PFOS*	9.1	11.2

*only added to the gel feed

3.3.2. Husbandry and sample collection

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 males/10 females) and 18 mice (8 males /10 females) within the two groups, respectively. Control or exposed pellet feed was 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. All mice were housed in groups (2-5 mice per cage) in closed Type III individually ventilated cages (IVC). Figure 8 shows an illustration of the exposure regime.

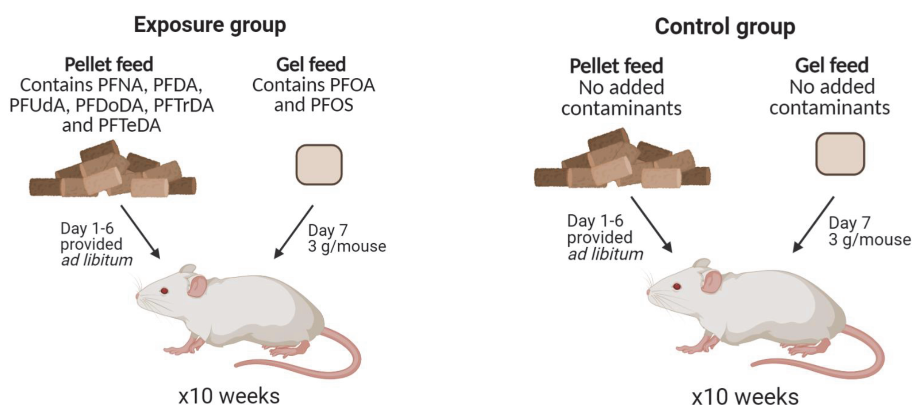

Figure 8. Illustration of the exposure regime, showing the different types of feed (pellet and gel), when they were provided to the mice (from day 1-7 of each week), and the different types of PFAS they contain. Figure from **Paper III.**

The mice were sacrificed at 13 weeks of age after being exposed to the PFAS or control feed for 10 weeks. Body weight was recorded prior to euthanasia by cardiac puncture and cervical dislocation under anesthesia (isoflurane gas). Blood was collected from the heart, cooled down on ice and spun at 6000 rpm for 10 minutes. Serum was extracted and frozen in liquid nitrogen. The liver, brain and calf muscles were removed, weighed and frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

3.4. Analytical procedures

3.4.1. Contaminant analyses

The PFAS concentrations were analyzed using liquid chromatography - tandem mass spectrometry (LC-MS/MS) at the Environmental Toxicology Laboratory, NMBU, Oslo, Norway. See Table 2 for an overview of the different PFAS analyzed for in the studies. Extraction and analysis of PFAS in soil, earthworms and bank vole liver is described in detail in **Paper I**. The analysis of PFAS in A/J mice liver and feed is described in **Paper III**.

Table 2. Classification, names, acronyms and chemical structures of the different PFAS analyzed for in the current project. Chemical structures are retrieved from Nakayama *et al.* (2019)

PFAS group	Compound name	Acronym	Structure
Perflurorocarboxylic acids (PFCAs)	Perfluorobutanoic acid	PFBA	
	Perfluorohexanoic acid	PFHxA	
	Perfluoroheptanoic acid	PFHpA	
	Perfluorooctanoic acid	PFOA	
	Perfluorononanoic acid	PFNA	
	Perfluorodecanoic acid	PFDA	
	Perfluoroundecanoic acid	PFUdA	
	Perfluorododecanoic acid	PFDoDA	
	Perfluorotridecanoic acid	PFTrDA	
	Perfluorotetradecanoic acid	PFTeDA	
Perfluorosulfonic acids (PFSAs)	Perfluorobutane sulfonate	PFBS	
	Perfluorohexane sulfonate	PFHxS	
	Perfluorooctane sulfonate	PFOS	
Perfluoroalkane sulfonamides (FASAs)	Perfluorooctane sulfonamide	PFOSA	
	N-methyl perfluorooctane sulfonamide	MeFOSA	
	N-ethyl perfluorooctane sulfonamide	EtFOSA	
N-alkyl perfluoroalkane sulphonamido ethanols (FASEs)	2-(N-methyl perfluorooctane sulfonamido)-ethanol	MeFOSE	
	2-(N-ethyl perfluorooctane sulfonamido)-ethanol	EtFOSE	

3.4.2. Dopamine (DA) analyses

Brain DA analyses of both bank voles and A/J mice were conducted at the Department of Environmental Sciences, University of California, Riverside (UCR), USA, using ultra performance liquid chromatography – tandem mass spectrometry (UPLC MS/MS). The method is described in detail in **Paper II** and **III**.

3.4.3. Steroid analyses

Steroid (T and E2) analysis in bank vole muscle tissue was conducted at the Department of Environmental Sciences, UCR, USA (method description in **Paper II**). Steroid analysis (T, E2 and 11-KT) in plasma and muscle tissue of A/J mice was conducted at the Department of Biology, NTNU, Norway (method description in **Paper III**). Steroid analyses were conducted using enzyme immunoassay (EIA) kits.

3.4.4. Gene expression (qPCR)

Gene expression was analyzed at the Department of Biology, NTNU, Norway. RNA was isolated from brain and liver tissue using Direct-zol^{TM} RNA extraction kit. The transcript expression analysis related to the genes of interest was performed using quantitative (real-time) polymerase chain reaction (qPCR). Genes analyzed were dopamine receptor 1 and 2 (*dr1* and *dr2*), monoamine oxidase (*mao*), vesicular monoamine transporter (*vmat*), dopamine active transporter (*dat*), catechol-*O*-methyltransferase (*comt*), tyrosine hydroxylase (*th*), estrogen receptor α and β (*esr1* and *esr2*), aromatase (*cyp19*) and gonadotropin releasing hormone (*gnrh)*. Detailed descriptions of the methods are presented in **Paper II** and **III**.

3.4.5. Hepatosomatic index (HSI)

The liver and body weight of both bank voles and A/J mice were recorded, and the hepatosomatic index (HSI) was calculated as liver weight/body mass*100.

3.5. Data handling and statistical analysis

All statistical analyses were conducted in R (the R project for statistical computing, version 3.5.3 for **Paper I** and version 3.6.3 for **Paper II** and **III**). An α-value ≤0.05 was considered statistically significant for all tests. The plots for **Paper I** were created in Excel (Microsoft 365), while the plots for **Paper II** and **III** were created in R. All data were tested for normality with Shapiro Wilk's test, and homogeneity of variance with Levene's test.

In **Paper I**, two sample Student's t-tests were used to test for significant differences between the skiing and reference areas. There was no significant difference in PFAS concentrations between years for the soil samples (t-test, $p > 0.05$), so the 2017 and 2018 samples were combined for statistical analysis. There was no effect of sex (t-test, $p > 0.05$) or age (t-test, $p > 0.05$) on bank vole liver PFAS levels; therefore, the contaminant data were not separated into subgroups for statistical analysis.

In **Paper II**, two sample Student's t-tests were used to test for significant differences between the measured endpoints in the skiing and reference areas. Multivariate analyses (principal component analyses; PCA) were carried out to investigate for possible relationships between the response variables (DA-related variables or steroid-related variables) and the explanatory variables (individual PFAS) in the skiing area (Granåsen). Based on the visualization of possible relationships from the PCAs, general linear models (GLM) were used to quantify the amount of variance explained (R^2) by the respective single explanatory variables.

In **Paper III**, mixed effect ANOVA models were run (with the packages "nlme" and "multcomp" in R) to test for significant differences between control and exposure groups for the measured endpoints. The ID of the mothers was used 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 potential litter effects. The residuals of the models were visually inspected to test for normality.

4. Results and discussion

In this thesis, PFAS concentrations and the PFAS profiles in a skiing area were analyzed and compared to a non-skiing reference area. The analyses included soil, earthworm and bank vole samples. Possible PFAS-related effects in the wild bank voles were also investigated, and further attempted to be reproduced in a laboratory exposure study using laboratory A/J mice.

This section will present the main results of this thesis and discuss the relevance of the obtained results in a broader context.

4.1. PFAS contamination in a skiing area

Paper I investigated the concentrations and profiles of PFAS in a Nordic skiing area in different environmental matrices, including soil, earthworms and bank voles, relative to their levels and profiles at a reference area with no skiing activities.

There was no significant difference in the mean summarized PFAS concentrations (∑PFAS) in the soil samples from the Granåsen skiing area and Jonsvatnet reference area (Figure 9). Given that the observed PFAS occurrence and concentration in the soil did not support the proposed hypothesis (**HI**), it was speculated that this discrepancy could be attributed to the fact that the soil samples were collected from the forest areas near the ski tracks, and not directly in the ski tracks. Differences in soil types and soil microbial communities between the two areas could also have implications on the detected concentrations (Wang *et al.* 2009). It is also possible that this result could be due to PFAS being washed out with rainwater, as these soil samples were collected in June, several months after the snow had melted. Thus, the PFAS could be diluted in the area. The fate of PFAS in melting snow and how they impact underlying soil has not been precisely predicted. However, a laboratory simulation by Plassmann *et al.* (2011) found that short-chained PFCAs partition in early meltwater fractions, while long-chained PFCAs partition in late meltwater and particle fractions that may be more likely to deposit in underlying soil. Therefore, sampling ground water or near-by water reservoirs and deeper soil may have given more information regarding the degree of PFAS contamination in the local environment near ski tracks.

In the earthworms, the mean ∑PFAS concentrations were 35% higher in Granåsen than in Jonsvatnet (Figure 9). However, this difference was not significant due to the large individual variation. Still, the concentrations of several individual PFAS were significantly higher in Granåsen compared to Jonsvatnet (See Figure 1b in **Paper I**). For the bank voles, significantly higher ∑PFAS concentrations were detected in Granåsen, compared to Jonsvatnet (Figure 9). These findings are in accordance with the proposed hypothesis (**H1**). These results indicate that animals inhabiting areas near ski tracks, ski slopes, etc., are exposed to higher concentrations of PFAS, than animals inhabiting areas with no skiing activities, and that these PFAS may biomagnify further up in the food chain.

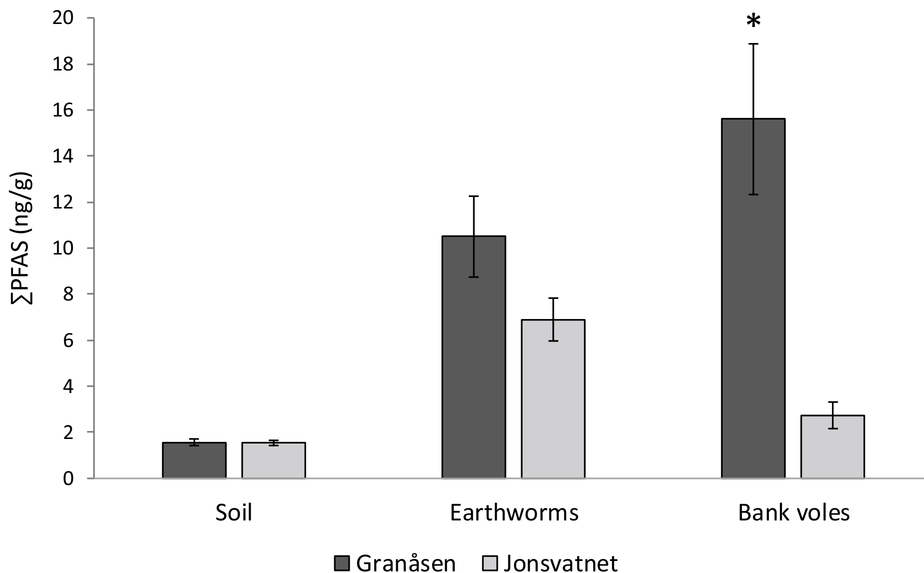

Figure 9. ∑PFAS concentrations in soil (ng/g d.w.) during 2017 and 2018 at Granåsen (n = 10) and Jonsvatnet (n = 10), earthworms (ng/g w.w.) during 2018 at Granåsen (n = 13) and Jonsvatnet (n = 13), and bank voles during 2017 at Granåsen (n = 21) and Jonsvatnet (n = 31). Error bars indicate standard deviation (SD). Asterisk (*) indicate significant site differences (t-test); * = $p < 0.05$,

Although the global production of fluorinated ski wax is low (approximately 36 tons/annum), relative to the overall production of PFAS containing products (Paul *et al.* 2009, Abbott *et al.* 2012), and although fluorinated ski wax does not contribute greatly to global PFAS emissions, it could have a great impact on the PFAS pollution in the local environments near ski tracks. In addition to the use of fluorinated ski waxes under the ski sole, PFAS is also released in the environment during the application process. Approximately 80% of the ski wax applied is lost during the application, where approximately half of this is estimated to be recaptured (e.g., via vacuuming) and disposed to waste. The other half is lost to the environment (Heggelund 2021). In addition, it is important to take into account the PFAS that are released into the environment during the production and disposal of fluorinated waxes.

The composition of PFAS (PFAS profiles) are often used to determine the sources of PFAS contamination (Hu *et al.* 2018). Paper I compared the PFAS profiles in the samples from the skiing area to the reference area, and with previously analyzed ski waxes (Freberg *et al.* 2010, Kotthoff

et al. 2015). Since the previously analyzed ski waxes reported mainly PFCA concentrations, only the PFCAs were used for the comparisons. The PFCA profiles were relatively similar in commercial fluorinated ski wax samples (Freberg *et al.* 2010, Kotthoff *et al.* 2015) and samples from Granåsen (soil, earthworms and bank voles), which were all dominated by the long-chained PFCAs (C8-C14, Figure 10). The long-chained PFCAs made up 70-100% of the total PFCA burden in all these samples, while in the samples from Jonsvatnet reference area, they made up only 25-40% (Figure 10). It was evident that the PFCA profiles measured at Granåsen were more similar to the ski wax profiles than the PFCA profiles measured at Jonsvatnet, which is in accordance with the proposed hypothesis (**H2**). These results indicate that there are different sources of contamination to the two areas, and that fluorinated ski wax, most likely, is a significant source of PFAS contamination in the skiing area.

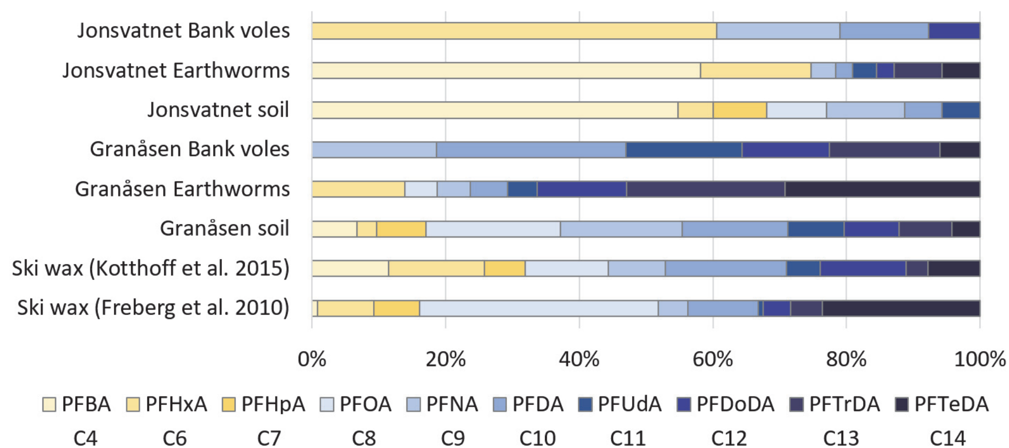

Figure 10. PFCA profiles showing the contribution (%) of each PFCA to the total median PFCA concentration in soil, earthworm, and bank vole samples from Granåsen and Jonsvatnet and in ski wax samples from two different studies (Kotthoff *et al.* 2015) and (Freberg *et al*. 2010). Yellow color indicates short-chained PFCA, blue color indicates long-chained PFCA. C4 to C14 indicates carbon chain-length. Figure modified from **Paper I.**

In a recent study from a skiing resort in Maine, USA (Carlson and Tupper 2020), high PFAS concentrations were reported near the skiing area, and these levels were related to fluorinated ski waxes. This emphasizes that environmental contamination of PFAS from ski waxes represent a problem at all places where there is a high frequency of skiers using fluorinated waxes. The concentrations reported in soil samples from the ski resort in Maine were much higher than the concentrations reported in the Granåsen ski area in Norway (**Paper I**). This indicates that, even though Granåsen is a popular skiing arena in Norway, it is small compared to many ski resorts in more populated countries. Consequently, many ski resorts or ski areas in the world are potentially much more contaminated by PFAS from skiing products than the current study ski area.

The fact that the samples from the ski area in 2017 and 2018 (**Paper I**) showed a very similar PFCA profile to the studies published on ski wax samples from 2009 (Freberg *et al.* 2010), indicates that the PFAS formulations of the waxes have not changed considerably. This was supported by the findings of Fang *et al.* (2020) and Carlson and Tupper (2020). For example, Carlson and Tupper (2020) reported that ski waxes currently in use contain hazardous PFAS, such as PFOA and long-chained PFCAs, despite regulatory actions. A study by Fang *et al.* (2020), which analyzed eleven of the best-selling PFAS-containing ski wax products on the market in Norway (2019), showed that the samples were still dominated by long-chain PFAS, indicating that the PFAS levels detected in the Granåsen ski area does not only represent previous use, but most likely also current-use ski waxes. Importantly, as shown in **Paper I**, and the studies mentioned above, ski products contain a wide array of PFAS (mainly long-chained), not only PFOA, PFOS and their precursors, which are the regulated ones. Until recently, one of the management practices of PFAS were to change the composition from long-chain PFAS to shorter chain PFAS technology. Although short chained PFAS are less bioaccumulative and have less potential to biomagnify in the food chain (Conder *et al.* 2008), short chain PFAS partition more easily to water phases and can even accumulate in plants (Ghisi *et al.* 2019). They can therefore end up in ground water, drinking water and food sources, and might be even harder to eliminate, once released into the environment, compared to longer chain PFAS. Consequently, the transfer to more C6 technology in ski wax may not necessarily be an improved strategy. This emphasizes that PFAS should, as proposed by Wang *et al.* (2017) and Kwiatkowski *et al.* (2020), be regulated as a group rather than managing each PFAS individually.

In the current thesis, targeted analyses were used to investigate concentrations of individual PFAS and to explore the PFAS profile in the different matrices and different areas. The PFAS that were analyzed were chosen based on previous studies on ski wax (Freberg *et al.* 2010, Kotthoff *et al.* 2015) and on established methods for extracting and determining PFAS concentrations. However, the great number of PFAS, (more than 4700 are distributed on the global market (OECD/UNEP 2018)) including known and unknown PFAS used directly in industrial processes and consumer products, replacement compounds, impurities, and degradation products, makes it practically

impossible to target each individual substance in the environment (Wang *et al.* 2017). Non-target screening using high resolution mass spectrometry (HRMS) can be used to identify new compounds, but this method is time consuming and therefore expensive on a routine basis (Benotti *et al.* 2020). Thus, non-specific inclusive approaches have been developed to estimate the total mass of PFAS in samples. Two approaches that have been increasingly used the recent years are the total oxidizable precursor (TOP) assay, and the measurement of extractable organic fluorine (EOF) (McDonough *et al.* 2019, Nakayama *et al.* 2019). Some of these methods should be included in future studies, in addition to the targeted analysis, to be able to create a more accurate picture of the degree of PFAS contamination in the skiing area, or any other area contaminated by PFAS.

4.2. Effects of PFAS on the dopaminergic system

The aim of **Paper II** was to investigate the possible effects of PFAS exposure on the dopaminergic system of wild bank voles at Granåsen skiing area, compared to Jonsvatnet reference area. In summary, it was detected that bank voles living at a Nordic skiing area contaminated by PFAS from ski products had higher total brain DA concentrations compared to the reference area (Figure 11), with accompanying positive associations between brain DA concentrations and the concentration of several PFAS (Figure 12). The multivariate analysis showed a negative relationship between most PFAS and the gene transcription of *dr1,* encoding DA receptor 1, in bank voles from the skiing area (Figure 12). As for the DA metabolism, the ratio between DA and its metabolites (DOPAC and HVA) is often used as a measure of DA turnover. The DOPAC/DA ratio was lower in bank voles from Granåsen, compared to Jonsvatnet (Figure 11), and showed a negative association with several PFAS (Figure 12). There was no difference in HVA/DOPAC between the two areas. This suggests that PFAS altered the intra-neuronal metabolism of DA via the Mao enzyme, which metabolizes DA to DOPAC. This assumption was supported by an observed negative association between *mao* transcription level and PFAS (Figure 12). These results indicate that PFAS alter the dopaminergic system of voles inhabiting areas near skiing tracks and is in accordance with the proposed hypothesis (**H3**).

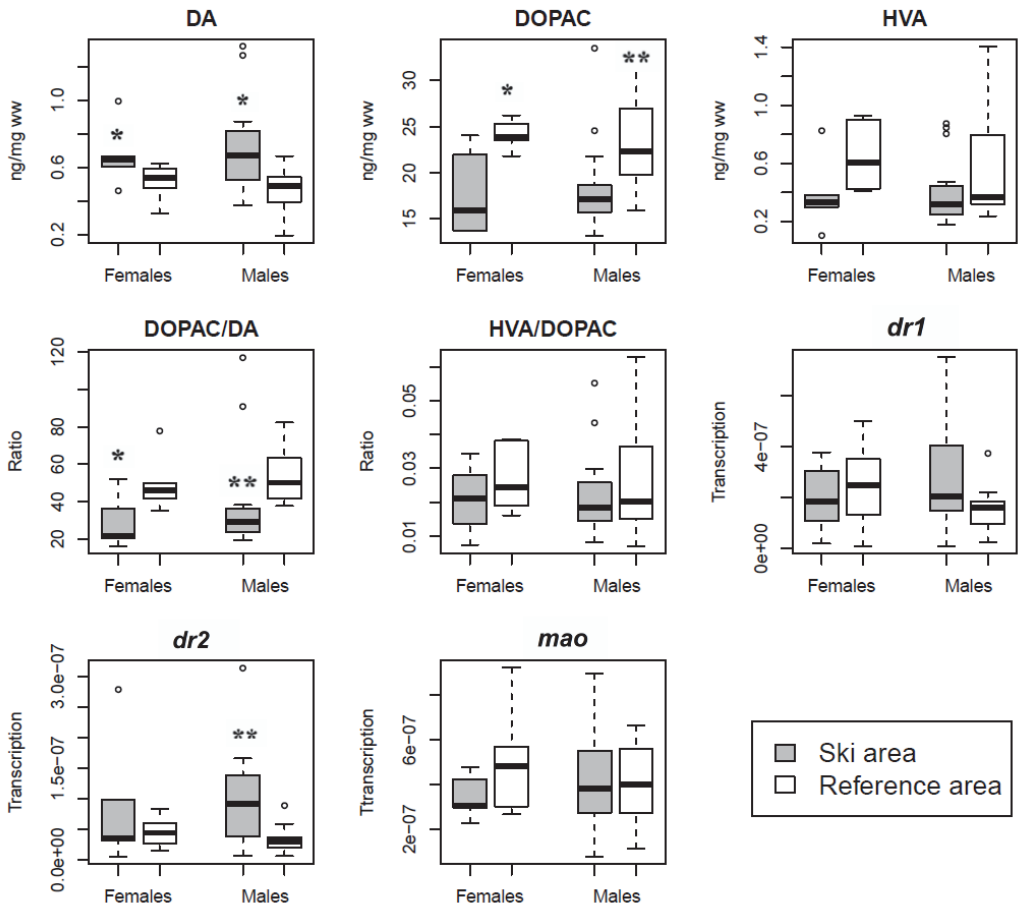

Figure 11. Boxplots of variables related to the dopaminergic system in male and female bank voles from Granåsen skiing area (n = 5 females, 16 males) and Jonsvatnet reference area (n = 6 females, 16 males). Variables are dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), DOPAC/DA ratio, HVA/DOPAC ratio, dopamine receptor 1 (d*r1)*, dopamine receptor 2 (*dr2)* and monoamine oxidase (*mao)*. Asterisks indicate the significance level: *p<0.05, **p<0.01. Figure from **Paper II**.

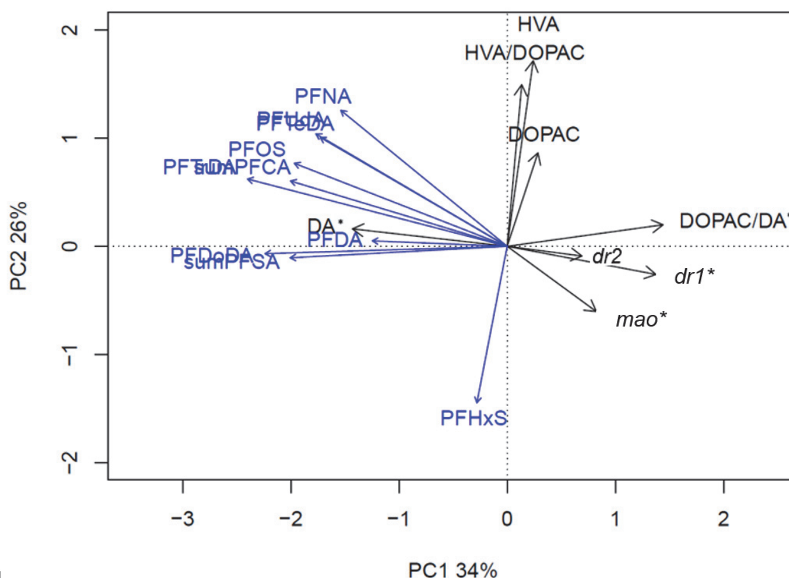

Figure 12. Biplot of PFAS concentrations and dopamine variables (DA, DOPAC, HVA, DOPAC/DA, HVA/DOPAC, *dr1, dr2, mao*) in bank voles from Granåsen skiing area (n=21). Explanatory variables are projected as passive arrows (blue). The % of the total variance explained by each principal component (PCs) is given on each axis. Concentrations of all variables are log transformed and standardized to unit variance. Direction and length of arrows indicate respective strength and increasing variance of loading. Asterisks indicate response variables with significant associations to one or more PFAS. Figure from **Paper II**.

The aim of **Paper III** was to reproduce the results from the field study (**Paper II**) under controlled laboratory conditions. The effects of PFAS exposure on the dopaminergic system in A/J mice was investigated. The PFAS composition and concentrations were based on the PFAS detected in earthworms from Granåsen (**Paper II**). Dietary exposure to an environmentally relevant PFAS mixture led to lower brain DA concentrations in male mice, compared to the control group (Figure 13). This is in accordance with the proposed hypothesis (**H4**). Lower transcript levels of *th* mRNA were detected, while there was no effect on the transcript levels of *mao* or *comt*, which are important for DA catabolism. The results from the current study indicate that the concentrations and composition of PFAS that are observed in earthworms at a Norwegian skiing area alter the neuroendocrine system of male A/J mice.

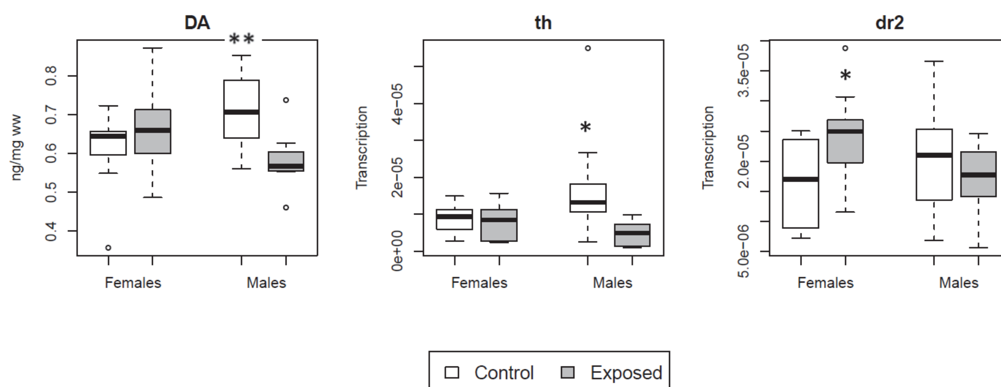

Figure 13. Boxplot of variables related to the dopaminergic system in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice. Asterisks indicate significant difference between exposure groups (*p<0.05, **p<0.01).

Overall, significant effects of PFAS exposure on the dopaminergic system were detected in both the bank voles and A/J mice. However, the measured effects on DA concentrations were opposite in the two studies, indicating that different mechanisms or pathways could be affected. **Paper II** proposed that PFAS exposure reduced the levels of the Mao enzymes, which in turn led to lower DA metabolism, and thus a build-up of DA in the brain. **Paper III**, however, proposed that PFAS exposure reduced the levels of Th enzymes, which in turn led to recused synthesis of DA from tyrosine, eventually leading to decreased DA concentrations in the brain. The differences in response pattern between the two studies could be caused by differences in PFAS concentrations that the animals were exposed to. This was also suggested in **Paper II**, where opposite effects of PFAS exposure on the *mao* mRNA were detected in bank voles from the current study, compared to previous findings in polar bears (Pedersen *et al.* 2015) which had much higher PFAS concentrations. Although the concentrations used for the feed in the laboratory exposure study were based on the concentrations reported in the earthworms at the skiing area, ∑PFAS concentrations measured in the exposed A/J mice were over 200-fold higher than the ∑PFAS concentrations measured in the free-living bank voles from the skiing area. Bank voles are mainly herbivores, and earthworms are only a small part of their diet. Therefore, the bank voles may probably be exposed to lower PFAS doses than the A/J mice, that were exposed to a dose equal to a diet that consisted of earthworms only. In addition, differences in the amount of food ingested may also account for the dose that the animals are exposed to. The bank voles in nature are also exposed to other stressors that could limit their food intake, while the A/J mice had an unlimited access to food. Further, voles and A/J mice could have significant differences in toxicokinetic variables. There are large interspecies differences in toxicokinetics of PFAS, which may result in

substantial uncertainty in the dosimetry extrapolation from one species to another (Chou and Lin 2019). Thus, it is possible that species differences exist between A/J mice and bank voles that could ultimately affect the measured liver concentrations. In addition, factors such as mixture toxicity could affect the observed outcomes in the field study, as there could be interactions with other, non-measured PFAS or other pollutants. Chemical interactions, such as synergism or potentiation may occur, where the observed effects are larger than their expected, additive effects. Oppositely, inhibition or antagonism may occur, which reduces the combined toxicity (IGHRC 2009). Different contaminants may also act by different mechanisms at different sites (i.e., independent action) (Reffstrup *et al.* 2010).

Another explanation for the different responses in the DA system for the two studies could be related to age of exposure. As discussed in **Paper III**, a previous study (Hallgren and Viberg 2016) suggested that there could be differences in response to PFAS exposure within the same individuals at different ages. Hallgren and Viberg (2016) found that *th* transcription increased in neonates and decreased in adult male mice after 24h exposure to PFOS. They suggested that the hippocampal release of DA may be augmented by PFOS exposure during development. The opposing results on *th* transcription in adults, compared to in the neonates may reflect a compensatory effect of an overexpression of the gene during development, which results in the depression of transcription of *th* later in life (Hallgren and Viberg 2016). This could also possibly explain the opposing results in the A/J mice and bank voles. Although the average age in the bank voles was approximately the same as the A/J mice, there was a larger 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. The fact that the wild bank voles were, most likely, also exposed in utero, while the A/J mice were only exposed from 3-13 weeks of age could also account for some of the differences. Since PFAS are known to be transferred from mothers to offspring (Hinderliter *et al.* 2005, Grønnestad *et al.* 2016), enabling the possibility of a combined *in utero* and lactational exposure scenario, in addition to exposure via diet, would resemble a more natural exposure scenario.

It should be mentioned that the current study analyzed the DA concentrations of an entire brain hemisphere, and not in specific brain regions. DA is synthesized and released in several different regions of the brain (Björklund and Dunnett 2007), and these regions 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 brain specific regions.

The increased or reduced concentrations of DA reported in PFAS exposed bank voles (**Paper II**) and A/J mice (**Paper III**), could lead to alterations in modulation of fear and anxiety (de la Mora *et al.* 2010), thermoregulation processes (Hasegawa *et al.* 2000), aversive events (Sweidan *et al.* 1991), as well as in reproductive pathways (Henderson *et al.* 2008). Excessive or deficient levels of DA

have also been hypothesized to contribute to a broad spectrum of mood, motor, and thought abnormalities. Genes related to the dopaminergic system, such as *dat* and *dr2*, have been implicated in aggressive behavior from animal and human studies (Zai *et al.* 2012). Consequently, PFAS exposure could alter neurological functions related to these emotional states. In addition, DA is involved in cognitive functions, attention and flexibility responses to stimuli (Seamans and Robbins 2010). Thus, the possible PFAS-related changes on the dopaminergic system and associated signaling pathways might potentially produce neurological disfunctions that may affect individual fitness of the exposed rodents.

4.3. Effects of PFAS on steroid hormones

In Paper II, the steroid hormone concentrations of wild bank voles were evaluated, in relation to tissue concentrations of various PFAS at Granåsen skiing area. Previous studies have shown that DA homeostasis can indirectly regulate cellular E2 and T biosynthesis by modulating the synthesis and release of Gnrh, or by affecting the aromatase activity (Chang *et al.* 1990, Yu *et al.* 1991, Xing *et al.* 2016). There were no associations between DA and T or E2 in the bank voles. However, there was a trend towards lower T concentrations in muscle tissue from male bank voles in the skiing area, compared to the reference area, while there were no differences in females. Specific PFAS associations were observed with a negative relationship between T concentrations in muscle tissue and several PFCAs (Figure 14). This was in accordance with the proposed hypothesis (H3).

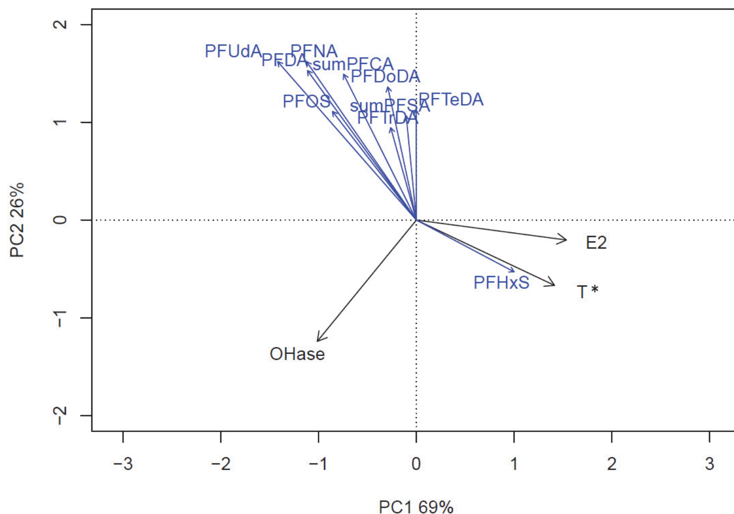

Figure 14. Biplot of PFAS concentrations and steroid hormone variables (estrogen: E2, testosterone: T and liver steroid hydroxylase activity: OHase) in bank voles from Granåsen skiing area (n=21). Explanatory variables are projected as passive arrows (blue). The % of the total variance explained by each principal component (PCs) is given on each axis. Concentrations of all variables are log transformed and standardized to unit variance. Direction and length of arrows indicate respective strength and increasing variance of loading. Asterisk indicates response variables with significant associations to one or more PFAS.

In **Paper III**, no significant differences in the T, E2 or 11-KT concentrations between exposed and control mice were detected, in either plasma or muscle samples (Figure 15). Transcript patterns of genes related to both the steroid and dopaminergic system were analyzed, such as estrogen receptor 1 and 2 (*esr1* and *esr2*) in the brain, gonadotropin releasing hormone (*gnrh*) and aromatase (*cyp19*). No differences in transcripts between the two exposure groups were detected, indicating that the changes in DA concentrations in the male mice did not translate to downstream effects on the related steroidogenic pathways. This is not in accordance with the proposed hypothesis (**H4**). It should be mentioned that the A/J mouse strain has been reported to have high plasma T levels (Hampl *et al.* 1971), hence, they may be more resilient to external factors affecting the plasma T concentrations, compared to other mouse strains.

The fact that similar effects on the steroid-hormone system in bank voles and A/J mice were not observed, indicates that the observed effects in the field study could be caused by other, non-measured variables in the nature or interactions with other pollutants not included in the exposure study. There could also be an effect of concentration differences and differences in age and species, as discussed for DA above, or interactions with other stressors or contaminants in the environment.

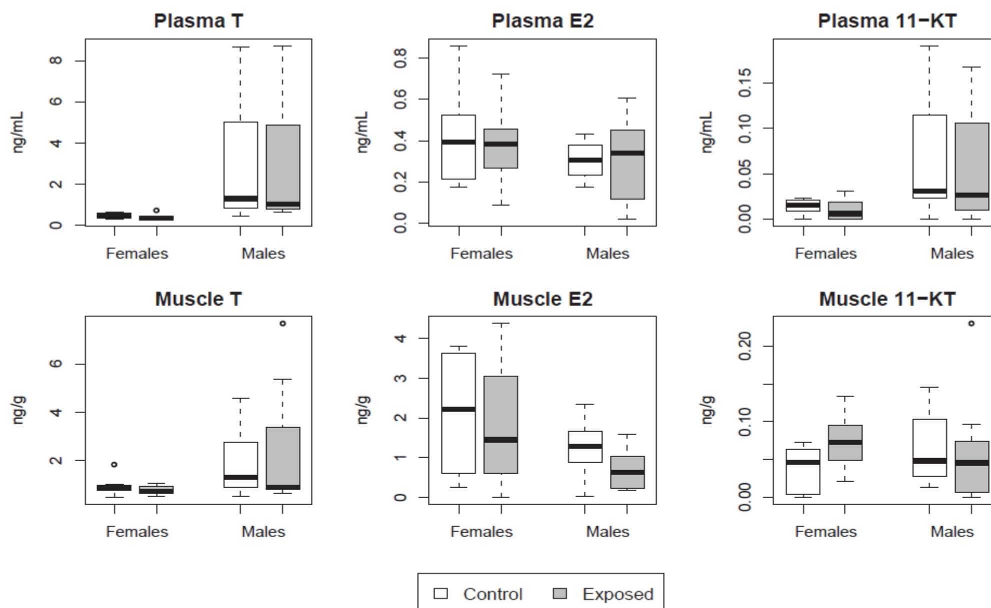

Figure 15. Boxplot of sex steroid concentrations measured in plasma (ng/mL) and muscle tissue (ng/g) in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice.

4.4. Effects of PFAS on the relative liver weight (HSI)

In the laboratory exposed A/J mice (**Paper III**), dietary exposure to PFAS mixture altered the HSI, which was significantly higher in exposed males, compared to the control males (Figure 16a). In the bank voles from the field study, however, there were no significant differences in the HSI between the skiing and reference areas (Figure 16b, two-way ANOVA, p=0.4, F=0.8), and no apparent associations between the PFAS concentrations in the skiing area, and HSI (see PCA Figure A1 in appendix). Several rodent studies 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, EFSA 2018, Frawley *et al.* 2018). A report by the European Food Safety Authority (EFSA 2018) reported that for exposed rodents, increases in relative liver weight were observed at doses above 0.15 mg PFOS/kg/day and 0.64 mg PFOA/kg/day (EFSA 2018). These doses are far higher than the PFAS and PFOA doses used in the current exposure study with A/J mice, suggesting that the PFAS mixture used in **Paper** III, which consist of 8 PFAS, could potentially be more toxic than individual PFAS (PFOS or PFOA) at higher doses.

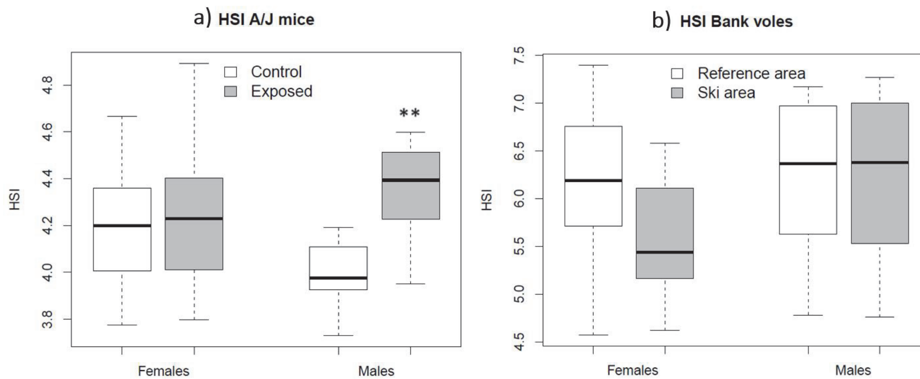

Figure 16. Boxplot of hepatosomatic index (HSI) in **a)** control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice, and **b)** bank voles from the ski area (n = 5 females, 16 males) and reference area (n= 6 females, 16 males). Asterisks indicate significant difference between exposure groups (*p<0.05, **p<0.01).

Reasons for the discrepancy in HSI results observed in the field and laboratory studies could be related to different PFAS concentrations, or as mentioned in the previous chapters; related to species, age, other biotic or abiotic factors, other pollutants/stressors, or the interactions of these factors. In addition, the A/J mice, on average, have a smaller relative liver weight, compared to the bank voles (Figure 16), which potentially could affect their sensitivity to PFAS exposure.

Previous studies have shown that an increase in liver mass is partly attributed to proliferation of peroxisome, smooth endoplasmic reticulum, and mitochondria (Pastoor *et al.* 1987, Berthiaume and Wallace 2002, Cui *et al.* 2008). Fatty acid metabolism is the major metabolic pathway being affected by PFAS exposure, raising the concern of fat deposition in the liver (fatty liver), as observed in mice in Kudo and Kawashima (1997) and Tan *et al.* (2013). Some of these cellular processes might have played significant roles in the observed changes in relative liver weight in the A/J mice from **Paper III**. The results indicate that an environmentally relevant PFAS mixture produced effects on liver physiology of A/J mice with potential effects on general health and fitness of the mice. PFAS does, however, not seem to affect the liver weight of wild bank voles at the concentrations or conditions that the wild voles were exposed to in the skiing area.

4.5. Sex-specific sensitivity to PFAS exposure

The findings from the current project indicate that male rodents are somewhat more sensitive to PFAS exposure than females. **Paper II** reported an association between PFAS and muscle T concentrations in male bank voles from the skiing area, but no effects on the estrogen concentrations in females. **Paper III** reported effects of PFAS exposure on the brain DA concentrations, transcription of *th* and liver weight (HSI) in male mice. None of these effects were observed in females. The only effect that was observed in females, that was not observed in males, was increased *dr2* transcription in PFAS exposed A/J female mice. It is therefore proposed that male rodents may be more sensitive to specific effects from PFAS exposure, compared to females.

A few other studies have suggested that male mice might be more sensitive to PFAS exposure than females. For example, a study on the immunotoxicological effects of developmental exposure to PFOS demonstrated that male mice pups (B6C3F1 strain) appeared to be more susceptible to effects on the immunological tests, compared to females (Keil *et al.* 2008). Another study on the effects of PFOS on motor behavior in mice (C57BL/6/Bkl strain) reported a decrease in exploratory activity, increased number of resting periods, and decreased muscle strength. These effects were more pronounced in males, while in females, they were either attenuated or absent (Onishchenko *et al.* 2011). A report by EFSA showed that male rodent offspring were more sensitive than females, when analyzing the expression of genes relevant for signal transduction in the brain after PFOS and PFOA exposure (EFSA 2018).

In bank voles, there was no significant difference in PFAS concentrations between males and females (**Paper I**). Thus, the difference in effects of PFAS exposure on the steroids between the sexes cannot be attributed to differences in PFAS concentrations. In the A/J mice (**Paper III**), however, there were higher ∑PFAS concentrations in the liver of exposed males than females. However, when normalizing the concentrations to body weight, there was no pronounced difference in ∑PFAS concentrations between the sexes. A study on polar bears suggest that there are no differences in brain PFAS concentrations between sexes (Greaves *et al.* 2013), while a study using mice found that liver and brain PFAS concentrations correlate (Liu *et al.* 2009), which would favor higher PFAS concentrations in the brain of male mice, as observed in the liver. It can therefore not be ruled out that the different responses to PFAS exposure between males and females are due to males accumulating higher PFAS concentrating in their brain.

The differences in effects of PFAS exposure between the two rodent species, and between sexes within the same species, indicates that several of the molecular mechanisms related to PFAS exposure are sex-, species- and/or dose specific, in addition to the differences in external conditions between field and controlled laboratory studies.

4.6. Relevance to humans

The different experiments in this thesis were not designed to address or evaluate the effects of PFAS from ski wax in humans. However, the mouse model that was used in **Paper III** is often used in studies related to effects on humans, and since humans are also exposed to similar PFAS mixtures as in the current studies when applying ski waxing products and spending time in skiing areas, it was inevitable to attempt to extrapolate the results from rodents to humans.

The use of mice in human health research has several advantages including high gene similarities and detailed understanding of the mouse biology and genetics acquired from their long-time use as research animals (Beck *et al.* 2000). However, a challenge in the risk assessment of PFAS is the large interspecies differences in toxicokinetics that results in substantial uncertainty in dosimetry and toxicity extrapolation from laboratory animals to humans (Chou and Lin 2019). Similar external PFOS dosages in animals may result in substantially different internal dosimetry in humans. In addition, there are physiological and biochemical differences among mice, voles and humans that must be accounted for when conducting interspecies extrapolation. For example, PFOS elimination is highly variable between species. The half-lives of PFOS range from days in rats (7–82 days) (Chang *et al.* 2012, Kim *et al.* 2016) and mice (30–42 days) (Chang *et al.* 2012), to weeks in monkeys (15–24 weeks) (Seacat *et al.* 2003, Chang *et al.* 2012), and years in humans (3.3–6.9 years) (Olsen *et al.* 2007, Worley *et al.* 2017). With this in mind, it is still interesting to attempt to extrapolate from rodents to humans.

The risk of human exposure to PFAS from ski wax is greatest when glide waxes containing PFAS are heated up, scraped and brushed off (Heggelund 2021). The exposure route in this case is mainly through the airways. Norwegian and Swedish studies have shown that concentrations of PFOA and PFNA were 25-100 times higher in professional ski wax technicians than in the general population (Daae 2009, Nilsson *et al.* 2010). The PFAS concentrations reported in plasma of professional waxing technicians (Freberg *et al.* 2010, Nilsson *et al.* 2010) were within the range of concentrations reported in the liver of bank voles from the Granåsen ski area (**Paper I**). For some of the technicians who had worked in the field for >10 years, some of the PFAS were within the range of concentrations reported in the liver of PFAS exposed A/J mice (**Paper III**). Both studies reported significant effects on the dopaminergic system of the rodents. Considering that the PFAS concentrations were measured in the plasma in humans, and that the liver normally is the main tissue for PFAS accumulation in mammals (Aas *et al.* 2014), it is possible to expect higher PFAS concentrations in their liver. However, it should be mentioned that the mice and voles used in the current study have been exposed to PFAS from a young age, while the ski technicians were exposed as adults. This could affect the susceptibility to the effects of PFAS exposure, as exposure during early life stages has been shown to produce more adverse effects (DeWitt 2015).

In recent years, professional ski wax technicians have become much better at using personal protective equipment, but much of the ski preparation process is carried out in poorly ventilated

spaces, especially in the non-professional part of the sport. The Norwegian Institute of Public Health (FHI) published a study showing that the concentration of dust and PFAS in conjunction with the application of ski waxes by amateur skiers is comparable with the concentration which has been shown to present health problems for professional ski wax technicians (Hetland 2017). The fact that the half-life of PFAS is so long in humans (up to 7 years)(Olsen *et al.* 2007, Worley *et al.* 2017), indicates that the internal exposure, and thus possible effects of PFAS from ski wax in humans, could be relatively long-term. This is concerning and is a good incentive for further regulations on the use of fluorinated ski waxes.

In 2020, EFSA set a safety threshold for tolerable weekly intake (TWI) for the sum of PFOA, PFOS, PFNA, and PFHxS at 4.4 ng/kg body weight per week ($ng/kg_{bw}/week$) for humans (EFSA 2020). In the laboratory exposure study (**Paper III**), the A/J mice were exposed to an average dose of approximately 0.0016 $ng/kg_{bw}/week$ of PFNA, 0.0005 $ng/kg_{bw}/week$ of PFOS and 0.005 $ng/kg_{bw}/week$ of PFOA (but no PFHxS). This was calculated based on a body weight of 23g for the mice. The sum of these 3 PFAS is thus 0.0071 $ng/kg_{bw}/week$, while the sum of all PFAS that the mice were exposed to was 0.043 $ng/kg_{bw}/week$. This dose is far below the toxicity threshold set by EFSA. Yet, significant effects of PFAS exposure on the dopaminergic system and relative liver weight of male mice were detected in A/J mice. The fact that the TWI of PFOS has been adjusted from 150 $ng/kg_{bw}/week$ in 2013, to 13 $ng/kg_{bw}/week$ in 2018 (EFSA 2018), and again to 1.1 $ng/kg_{bw}/week$ in 2020 (EFSA 2020), emphasizes that PFOS (and other PFAS), may be more toxic to human health than previously anticipated.

The observed effects from the laboratory exposure study (**Paper III**) did not parallel the results from the field study (**Paper II**) and thus contradicts the proposed hypothesis (**H5**). This adds more uncertainty when extrapolating the results from rodents to humans. When human causality is only based on non-human data, an unknown degree of uncertainty is introduced into the hazard and risk estimations. Thus, animal data should be judged cautiously and not as the tipping point when reaching a conclusion on human causation (James *et al.* 2015). However, as analysis of DA concentrations in relation to pollution in human brain is not easily-, or commonly conducted, and cannot be conducted as a controlled exposure study, risk assessment on effects of PFAS on the DA system needs, to some extent, to be based on mouse models. As put forth in the evidence-based toxicology framework ("the Hill criteria of plausibility"), animal data should be used as supporting information when identifying the mechanisms linking cause and effect (Hill 1965) and to facilitate future epidemiological and mechanistic studies (James *et al.* 2015). Thus, the results presented herein should be used to inspire future research on mixture effects of PFAS, preferably at low and environmentally- or human relevant concentrations, and to facilitate mechanistic investigations into the complex dopaminergic and steroid hormone systems.

5. Concluding remarks

Based on the objectives of the present thesis, the following conclusions can be drawn:

1. PFAS contamination in a skiing area (objectives 1 and 2):

∑PFAS concentrations were significantly higher in bank voles from the skiing area, compared to the reference area, and 35% higher in earthworms from the skiing area, compared to the reference area. The PFCA profiles in the samples from the skiing area resembled that of the previously analyzed ski waxes, dominated by long-chained PFCAs, while the samples from the reference area were dominated by short-chained PFCAs. This indicates that animals inhabiting skiing areas are exposed to higher ∑PFAS concentrations than animals inhabiting areas with no skiing activities, and that these PFAS most likely are derived from ski wax.

2. Effects of PFAS on the dopaminergic and steroid hormone systems in rodents (objectives 3 and 4):

Significant effects on the dopaminergic system were detected in both the bank voles and A/J mice. However, the observed effects on DA concentrations were opposite in the two species, and different molecular mechanisms appear to be affected. In bank voles, PFAS appeared to reduce the transcript level of *mao*, and thus possibly Mao enzyme levels. This could in turn lead to lower DA metabolism, resulting in a build-up of DA in the brain. In A/J mice, however, it seems like PFAS exposure reduced the transcript level of *th*, encoding the Th enzyme, which in turn leads to lower synthesis of DA from tyrosine and thus decreased DA levels in the brain.

There was a trend towards lower T concentrations in male bank voles from the skiing area, compared to the reference area. In the A/J mice, however, there were no effects of PFAS exposure on the T, E2 or 11-KT concentrations in either muscle- or plasma samples in either sex. The fact that there was no effect on the steroid-hormone system in A/J mice indicates that the observed associations between T and PFAS in the field study could be caused by other, non-measured variables in the nature, by species differences or interactions with other pollutants, rather than PFAS alone.

3. Relevance to humans (objective 5):

The effects of PFAS exposure on A/J mice did not parallel the results in bank voles, which adds more uncertainty when extrapolating the results from rodents to humans. However, the PFAS concentrations reported in the bank voles from the skiing area and PFAS-exposed A/J mice, were within the range of concentrations reported in plasma of professional waxing technicians. This indicates that the effects observed on the dopaminergic and endocrine systems in rodents could possibly be of concern to humans that are exposed to PFAS from ski wax products, although species differences in toxicokinetics and toxicodynamics must always be considered.

6. Considerations and future perspectives

It should be mentioned that, even though statistically significant differences were detected between variables in the PFAS exposure group and control group, and between the skiing area and the reference area, it does not necessarily translate to biological effects in the exposed individuals. The fact that the increased and decreased DA concentrations in **Paper II** and **Paper III**, respectively, did not translate to effects on the steroid system, indicates that changes in DA concentrations were either not great enough to affect the homeostasis of this system or involve other physiological pathways not investigated in these studies. However, since the dopaminergic system is involved in numerous physiological functions, both in the brain and the body, it is challenging to detect all the possible downstream effects that can directly or indirectly be attributed to the changes in DA concentrations. In addition, several physiological processes under the control of the dopaminergic system, are transiently coordinated. Therefore, it is possible that the current study's exposure and sample regime might have missed these transient responses.

Since there are few peer reviewed publications on PFAS contamination near ski tracks, there is little basis for comparison. Therefore, more studies are needed to evaluate end explore just how contaminated these areas are, and whether there is a need for remediation action in some of these areas. Sampling of ground water, or near-by lakes, ponds, etc. would give a better indication of the fate of PFAS in skiing areas.

There is a need for more studies on effects of PFAS at environmentally relevant concentrations on the dopaminergic and endocrine systems. To elucidate the molecular mechanisms behind the effects, it is also necessary to perform more *in vivo* and *in vitro* studies on PFAS exposure, both individual PFAS and in combination. Further focus should be given to understanding PFAS as a group, or as several subgroups, including identifying the drivers of mixture toxicity. This knowledge could then be used in the development of effect-oriented chemical and biological analysis and predictive models to evaluate the total burden of simultaneous exposure to multiple PFAS, as well as the justification of best grouping methodologies for PFAS (e.g. based on mode-of-action, as suggested in the WHO/IPCS framework) (Meek *et al.* 2011).

To protect the environment and human health from PFAS contamination, it is important to continue developing safer practices and policies for ski wax use. Future studies should also include non-target screening for PFAS, TOP or EOF assay, to detect new PFAS and be able to better estimate the total PFAS contamination to an area.

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Appendix

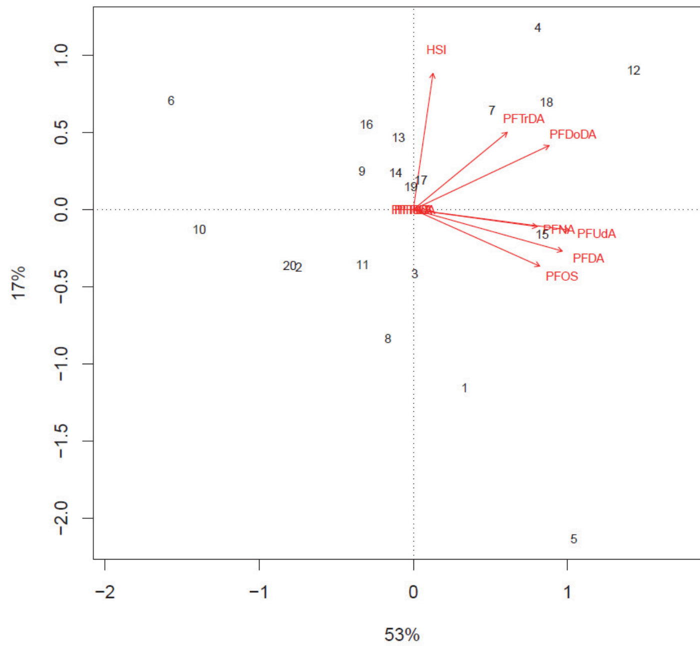

Figure A1. Biplot of PFAS concentrations and hepatosomatic index (HSI) in bank voles from Granåsen skiing area (n=21). The % of the total variance explained by each principal component (PCs) is given on each axis. Concentrations of all variables are log transformed and standardized to unit variance. Direction and length of arrows indicate respective strength and increasing variance of loading.

Paper I

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Levels, Patterns, and Biomagnification Potential of Perfluoroalkyl Substances in a Terrestrial Food Chain in a Nordic Skiing Area

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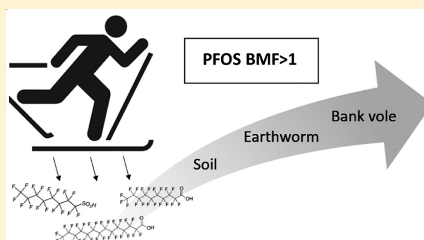
Supporting Information

ABSTRACT: Perfluoroalkyl substances (PFASs) are used in a wide range of consumer products, including ski products, such as ski waxes. However, there is limited knowledge on the release of PFASs from such products into the environment and the resultant uptake in biota and transport in food webs. We investigated levels, patterns, and biomagnification of PFASs in soil, earthworms (*Eisenia fetida*), and Bank voles (*Myodes glareolus*) from a skiing area in Trondheim, Norway. In general, there was higher PFAS levels in the skiing area compared to the reference area with no skiing activities. The skiing area was dominated by long-chained perfluorocarboxylic acids (PFCAs, ≥70%), while the reference area was dominated by short-chained PFCAs (>60%). The soil PFAS pattern in the skiing area was comparable to analyzed ski waxes, indicating that ski products are important sources of PFASs in the skiing area. A biomagnification factor (BMF) > 1 was detected for Bank vole$_{whole}$/earthworm$_{whole}$ for perfluorooctansulfonate in the skiing area. All other PFASs showed a BMF < 1. However, it should be noted that these organisms represent the base of the terrestrial food web, and PFASs originating from ski wax may result to higher exposure in organisms at the top of the food chain.



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INTRODUCTION

Perfluoroalkyl substances (PFASs) are ubiquitous and persistent anthropogenic chemicals in the environment.[1] They are a group of surface-active compounds that are applied in a wide range of consumer products, such as textiles, carpets, impregnating agents and in some types of ski products, such as ski waxes, gliders, and powders.[2] The global production of ski waxes is estimated to be several tons per year.[3] During the last decade, the production and use of ski waxing products have increased considerably, and the chemical composition of these products is continuously evolving.[4] In cross-country and downhill skiing, these products are applied to increase performance, as the fluorinated molecules enhance the glide on the water film between the ski and snow surface.[5] However, abrasion of these products from the ski sole results in deposition of the PFASs to the nearby environments.[6] Because PFASs are very persistent, they can remain in the environment for decades, creating PFAS-hotspots in the skiing areas.[7] However, little is known about the environmental levels of PFASs in these areas and their uptake in biota and transport in food webs.

In recent times, there has been an increasing focus on PFASs in consumer products, their toxicity, persistence in nature, and potential spread to the environment.[2,8,9] Particularly, the two most toxic congeners, namely, perfluorooctanoic acid (PFOA) and perfluorooctansulfonate (PFOS), have received much attention. In the year 2000, the US Environmental Protection Agency (USEPA) banned PFOS, and in May 2009, it was added to Annex B of the Stockholm Convention on persistent organic pollutants (POPs: www.pops.int). In 2010, the maximum content of PFOS allowed in products was reduced to equal or below 10 mg/kg in the Commission Regulation (EU) No. 757/2010.[10] In Norway, the use of PFOS was banned in firefighting foams, textiles, and impregnation agents (max. content 0.005%) in 2004 (FOR-2004-06-01-922, 2004). In addition, a maximum content of 0.1 mg/kg PFOS is allowed in other types of products (FOR-2009-06-22-827, 2009). A similar restriction for PFOA is under development. However, several PFASs continue to be manufactured as the industry has not yet found suitable replacements for these compounds.

According to Kotthoff et al.,[2] ski waxes had the highest concentrations of both perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs), compared to a wide range of other consumer products. Despite the legislative focus, PFOA and PFOS were the main contributors to total PFAS levels in most of the consumer products.[2] Studies on blood serum from professional ski waxing technicians have shown elevated concentrations of PFCAs, compared to the general population.[5,11] This is of great concern, since studies have shown that

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PFASs can, among others, lead to several adverse health effects, such as hormone imbalance,[12,13] immune suppression,[14,15] and alterations of lipid homeostasis.[16,17] This has resulted in an increased focus on the levels and possible effects of PFASs on human health.[11,18,19] However, there is limited or no data regarding the effects of PFASs on wildlife species inhabiting areas where these products are being used and released into the environment.

Herzke et al.[20] reported high PFAS levels in earthworms in skiing areas in Oslo, Norway, compared to a reference site. Although only five samples were used for this study, these results gave reason for concern and follow-up studies were recommended. Furthermore, snow chamber studies revealed that PFCAs elute in concentrated peaks from the melting snow,[21] potentially affecting biota during their most vulnerable stage of development in the spring.

Therefore, the aims of the current study were (1) to investigate the levels and patterns of PFASs in a Nordic skiing area in different environmental matrices, including soil, earthworms (*Eisenia fetida*), and Bank voles (*Myodes glareolus*), and to compare these levels and patterns to a reference area with no skiing activities and (2) to investigate to which extent these contaminants biomagnify in the food chain. These data will be useful in regulatory aspects of PFASs in ski wax, providing better insights into the sources and exposure routes in the environment.

■ MATERIALS AND METHODS

Study Area. The study area was "Granåsen skisenter" (63°22′N, 10°18′E), located approximately 10 km from the Trondheim city center (Norway, Figure S1 in the Supporting Information). Granåsen is the main arena for winter sports in Trondheim and hosts an annual ski jumping World Cup event in addition to a range of other regional, national, and international competitions in cross-country skiing. Thus, Granåsen offers several cross-country ski tracks that are used for training and competitions by amateurs and hobby skiers.

As a reference site, a natural forest area not used for ski-sports was chosen in the vicinity of an ecological farm next to Lake Jonsvatnet (63°20′N, 10°33′E). This site is approximately 15 km away from Trondheim city center (Figure S1 in the Supporting Information). The lake supplies drinking water to the Trondheim and surrounding communities. The two study areas have quite similar vegetation, consisting of mainly mosses and different species of *Ericaceae*.

Study Matrices. Chemical analyses of soil are useful for detecting the concentration of contaminants in the environment,[22] and earthworms (*E. fetida*) are considered one of the most suitable model organisms for monitoring and assessing soil pollution as they are integral soil macroinvertebrates.[23,24] Earthworms constitute an important part of the diet of local rodent species and serve as the gateway for chemical movement from the contaminated soils into the terrestrial food web. Thus, earthworm was chosen as a test organism because of its critical role at the base of the investigated terrestrial food web and its constant contact and ingestion of soil. Earthworms are susceptible to chemicals, providing information on the bioavailability of soil contaminants.[24] We chose Bank voles as a model organism because it is an important intermediate species in the terrestrial food chain, being preyed upon by raptors and carnivorous mammals,[25] and feeds on roots, seeds, buds, and berries, in addition to earthworms and other invertebrates. In addition, they have a relatively small home range, so we could expect that their contaminant levels are representative of the area where they were caught.

Sampling. The soil samples were collected in June 2017 and 2018 from the Granåsen and Jonsvatnet areas. The upper layer (constituting 3−10 cm depth and an area of approximately 1 m^2) of soil was collected and dried (40 °C for 48 h). Five samples per year in Granåsen and Jonsvatnet were chosen for chemical analysis. Only soil from locations where both earthworms and Bank voles had been sampled were selected for analysis.

The sampling of earthworms was performed in June of 2018 by digging 5−10 cm into the soil, using a metal spade and collecting the animals in sealed plastic bags. They were immediately frozen at −80 °C until analysis. The short time between collection and freezing did not allow them to empty their guts, as this would be more representative of how they serve as Bank voles' prey.

The catching, handling, anesthesia, sampling, and euthanizing of the Bank voles were approved by the Norwegian Food Safety Authority (Mattilsynet; references no. 2017/76552) and by the Norwegian Environmental Agency (Miljødirektoratet; reference no. 2017/4061). Permissions for the collection of Bank voles were also given by the land owners. The sampling and handling were performed in accordance with the regulations of the Norwegian Animal Welfare Act and EU legislation (3R). The collection of Bank voles was performed in June 2017. All traps were live traps of type "Ugglan" baited with rye bread dipped in sunflower oil and peanut butter (all food products were sold as "ecological food material"). The Bank voles were sacrificed by cervical dislocation. The animals were weighed, measured, and sexed. The livers were dissected and snap-frozen in liquid nitrogen and stored at −80 °C. The eyes were dissected for later age determination and stored in 10% formalin. In total, 21 and 31 individuals were caught at the Granåsen and Jonsvatnet areas, respectively. For more details on sampling and handling, see the Supporting Information.

Age Determination. The age of the Bank voles was determined using the weight of the dried eye lenses.[26] The lenses were dried at 80 °C for 24 h, and each lens was weighed to the nearest 0.1 mg. The mean weight of the two lenses was used to calculate approximate age. The calculations of age were done according to Kozakiewicz,[26] using the following formula: $Y = 0.013x + 4.610$, where y = lens weight and x = age (days).

Because the growth rates of the eye lenses are larger during the first three months of their life,[26] the Bank voles which were estimated to be less than 3 months old were recalculated using the following formula: $Y = 0.063x + 1.050$.

Chemical Analysis. The PFAS concentrations were analyzed at the Environmental Toxicology Laboratory, Norwegian University of Life Sciences (NMBU), Oslo, Norway. The analytical procedure of PFASs is described by Grønnestad et al.[27] The samples were analyzed for the following PFASs: ten PFCAs: perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), and perfluorotetradecanoic acid (PFTeDA), three PFSAs: perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), and PFOS, and five perfluoroalkane sulfonamide derivatives (FASAs): perfluoro-1-octane sulfona-

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mide (FOSA), *N*-methyl perfluoro-1-octane sulfonamide (*N*-MeFOSA), *N*-ethyl perfluoro-1-octane sulfonamide (*N*-EtFOSA), 2-(*N*-methyl perfluoro-1-octane sulfonamido) ethanol (*N*-MeFOSE), and 2-(*N*-ethyl perfluoro-1-octane sulfonamido) ethanol (*N*-EtFOSE).

Extraction of Biota. 0.5 g of Bank vole liver or whole earthworm was weighed for chemical analysis. Brief description of the method is as follows: internal standards (^{13}C-labeled equivalents, 20 ng/mL; Wellington Laboratories, Table S1 in Supporting Information) were added prior to double extraction with methanol. Cleanup was accomplished using active carbon (EnviCarb). See more detailed description in Supporting Information.

Extraction of Soil Samples. The dried soil sample (5 g) was weighed for the chemical analysis. The method for soil extraction was similar to that of biota; however, an additional step with addition of 2 mL of 200 mM sodium hydroxide (NaOH) prior to the extraction and 200 μL of 2 M hydrochloric acid (HCl) after extraction was included in the procedure.

Analysis. The final extracts were analyzed by separation on high-performance liquid chromatography with a Discovery C18 column (15 cm × 2.1 mm × 5 μm, Supelco, Sigma-Aldrich, Oslo, Norway), connected to a precolumn; Supelguard Discovery C18 column (2 cm × 2.1 mm × 5 μm, Supelco, Sigma-Aldrich, Oslo, Norway). Detection and quantification were accomplished with a tandem mass spectrometry (MS−MS) system (API 3000, LC/MS/MS System). The injected volume was 5 μL.

External standards were used to produce a standard curve from which the PFAS levels were calculated, using the instrument control and data processing program Mass Hunter Quantitative analysis Version B.05.02 (Agilent Technologies). The limits of detection (LODs) were calculated as 3 × SD of the procedural blanks (see blank values Table S5 in the Supporting Information), and the limits of quantification (LOQs) were calculated as 10 × LOD. Where no blanks were detected, LOQs were determined as 10 × signal-to-noise ratio (S/N). For the soil and earthworm samples, individual LOQs were determined for each sample because of matrix effects. Further information on the chemical analyses and LOQs can be found in the Supporting Information (Tables S1−S4).

Quality Assurance. The Environmental Toxicology Laboratory is accredited by the Norwegian Accreditation as a testing laboratory according to the requirements of NS-EN ISO/IEC 17025 (TEST 137).

For each series of maximum 30 samples, three blank samples, one blind, and four recovery samples were run. Mean of procedural blanks, consisting of internal standards and solvents, was subtracted from each series separately because of variation between series. The relative recovery rate in Bank voles ranged from 84 to 128% for the PFCAs, 78−129% for the PFSAs, and 86−115% for the FASAs. For the earthworm samples, recoveries ranged from 110 to 140% for the PFCAs, 99−115% for the PFSAs, and 106−141% for the FASAs. For the soil sample, recoveries ranged from 91 to 140% for the PFCAs and 97−124% for the PFSAs. It was not possible to analyze the FASAs in the soil samples because of poor response of the internal standards.

Contaminants with concentrations above LOQ in more than 50% of samples were included in the statistical analyses, and missing values (i.e., <LOQ) were assigned a random value between the LOQ and zero.

Calculations of the Biomagnification Factor. Because bioaccumulation of PFASs are highly tissue and substance specific,[28] the most appropriate approach for calculating biomagnification factors (BMFs) is to use whole-organism concentrations for both predator and prey.[29] In the earthworms, whole-body concentrations were analyzed. However, in the Bank voles, only liver concentrations were analyzed, and the liver mass accounted for 5−7% of the total body mass. Thus, to provide indications on the potential of PFASs to biomagnify at the base of a terrestrial food chain, individual whole-body concentrations were calculated for the Bank voles and used for estimation of BMFs. Because the PFAS concentrations generally are higher in liver tissue than in other tissues,[30] we assumed that the PFAS concentrations in the rest of the tissues on average were 10% of that in the liver. This was based on calculations of whole-body concentrations of PFASs in mice.[31] Whole-body concentrations were thus estimated as C_{whole} = (liver fraction × C_{liver}) + (fraction of other tissues × C_{liver} × 0.1).

The BMF was calculated as the ratio between Bank $vole_{whole}/earthworm_{whole}$ for individual PFASs at the Granåsen and Jonsvatnet sites of values above LOQ.

Statistical Analysis. The program R (version 3.5.3, the R project for statistical computing) was used for the statistical analysis. Normal distribution was tested with Shapiro Wilk's test, and homogeneity of variance was tested with Levene's test. Data were log-transformed prior to data analyses to reduce deviation from normality and homogeneity of variance. Two sample Student's *t*-tests were used to test for significant differences between the skiing and reference areas. The significance level was set at 0.05, and all tests were two-tailed.

There was no significant difference in PFAS concentrations between years for soil samples (*t*-test, p = 0.1 for Granåsen and p = 0.09 for Jonsvatnet), so the 2017 and 2018 samples were pooled for statistical analysis. There was no effect of gender (*t*-test, p = 0.7 for Granåsen and 0.6 for Jonsvatnet) or age (*t*-test, p = 0.8 for Granåsen and p = 0.3 for Jonsvatnet) on Bank vole liver PFAS levels; therefore, the contaminant data were not separated into subgroups for statistical analysis.

RESULTS AND DISCUSSION

PFASs in Soil and Earthworms. There was no significant difference (*t*-test, p = 0.8) in the mean-summarized PFAS concentrations (ΣPFAS) in the soil samples from the Granåsen skiing area and the Jonsvatnet reference area (Figure 1a), showing concentrations of 1.57 and 1.54 ng/g d.w. (dry weight), respectively. In the earthworms (Figure 1b), the mean ΣPFAS levels were 35% higher at Granåsen than Jonsvatnet (10.5 and 6.92 ng/g w.w. (wet weight), respectively). However, this difference was not significant (*t*-test, p = 0.08) due to large individual variation (see Table S3 in the Supporting Information).

For the PFCAs, PFDA was the most predominant compound in the soil samples from Granåsen, while the long-chained PFTeDA was the most predominant compound in the earthworms from Granåsen. At Jonsvatnet, PFBA was the dominating compound in both soil and earthworm samples. A study from the Antarctic Peninsula found that PFBA was found in 80% of lichen samples,[32] indicating that PFBAs are present in quite pristine areas. For the PFSAs in both soil and earthworms, PFOS was the dominating compound, representing a significant portion of the PFSA group. The FASA derivatives were FASA derivatives were

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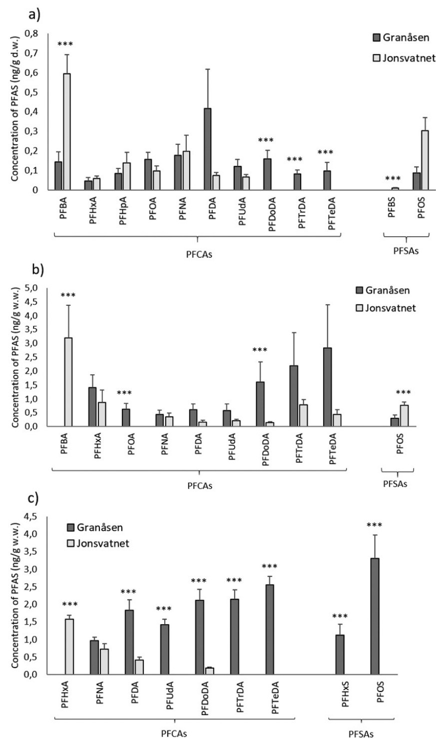

Figure 1. PFAS concentrations in (a) soil (ng/g d.w.) during 2017 and 2018 at Granåsen (n = 10) and Jonsvatnet (n = 10), (b) earthworms (ng/g w.w.) during 2018 at Granåsen (n = 13) and Jonsvatnet (n = 13), and (c) Bank voles (*M. glareolus*) during 2017 at Granåsen (n = 21) and Jonsvatnet (n = 31). Error bars indicate standard error of the mean (SE). Asterisks (*) indicate significant site differences (t-test); * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. PFASs with missing bars have levels <LOQ.

below LOQ in earthworms in both study areas (see Table S3 in the Supporting Information) and could not be analyzed in soil samples.

Concentrations measured in soil were below 1 ng/g d.w. for all individual PFASs at Granåsen (Figure 1a). This concentration is low, compared to similar soil studies in other areas near skiing tracks.[20,33,34] The ΣPFAS levels in soil from a skiing area in Oslo, Norway, was 10.3 ng/g d.w. in 2016[33] and 7.1 ng/g d.w. in 2017,[34] compared to 1.57 ng/g d.w. at Granåsen. According to the Norwegian guidelines on classification of environmental quality of soil, concentrations of 100 ng/g d.w. of PFOS represent the threshold for clean soil (FOR-2004-06-01-931, § 2, attachment 1). This indicates that in both the skiing area in Trondheim and Oslo, the levels are several orders of magnitude below the threshold for contaminated soil.

For the earthworms (Figure 1b), the PFAS concentrations at Granåsen were below the concentrations reported in the Oslo skiing area.[20] The ΣPFAS concentrations in earthworms from Granåsen, 10.5 ng/g w.w., were lower than the concentrations reported in Oslo, where concentrations ranged from 34.8 in 2015 to 70 ng/g w.w. in 2017.[20,33,34] Recently, an LC_{50} (lethal concentration at which 50% of the population is killed) of approximately 478 mg/kg was reported for PFOS in earthworms,[35] and this LC_{50} value is several orders of magnitude above the levels measured in the present study (<0.011 mg/kg). Nevertheless, there are potential and other severe effects, besides mortality, that can be observed at lower PFOS concentrations. For example, Zheng et al.[35] reported DNA damage in earthworm coelomocytes at their lowest test concentration of 50 mg PFOS/kg. Elsewhere, Xu et al.[36] observed that exposure to soil PFOS concentration of 10 mg/kg (their lowest test concentration) produced DNA damage and oxidative stress in earthworms. Therefore, although the individual PFOS concentrations reported in earthworms from skiing areas in Norway are below concentrations that produce acute toxicity (i.e., mortality), it is not possible to conclude on other long-term chronic effects. In addition, we must consider mixture toxicity scenarios, which might lower the toxicity thresholds.

PFASs in Bank Voles. To our knowledge, there are no previous studies of PFAS levels in Bank voles at skiing areas.

The mean ΣPFAS concentration was 5.7 times higher in Bank voles from Granåsen, compared to Jonsvatnet (15.6 ng/g w.w. and 2.74 ng/g w.w. at Granåsen and Jonsvatnet, respectively, Figure 1c). This difference was statistically significant (t-test, p = 0.02). There was no difference in the sex ratio (F/M = 33/67 at Granåsen and 34/66 at Jonsvatnet) or age distribution (t-test, p = 0.2) between the two areas. Thus, the differences in PFAS concentrations between the two areas are not caused by differences in these biological factors. The FASA derivatives were below LOQ in both areas (see Table S4 in the Supporting Information).

PFAS levels in Bank voles from forest and subalpine biotopes in Sweden have previously been reported.[37] However, that particular study was not linked to skiing areas, and mean concentrations of PFAS in the biotopes varied from 5.8 ng/g w.w. to 18.7 ng/g w.w., with the highest concentrations in Våladalen.[32] It should be noted that there are skiing areas in Våladalen (https://www.valadalen.se/en/cross-country-skiing), but no information is provided on the exact sampling locations of the voles in relation to these skiing areas.[32] Several studies have reported PFAS levels in terrestrial animals, however, these are mainly from areas near factories, where PFASs are produced or used.[38,39]

The concentrations of the long-chained PFCAs (C10−C14) were significantly higher in Bank voles from Granåsen compared to Jonsvatnet (t-test, PFDA: $p < 0.001$, PFUdA: $p < 0.001$, PFDoDA: $p < 0.001$, PFTrDA: $p < 0.001$, PFTeDA: $p < 0.001$, Figure 1c), while no difference between the two areas was observed for PFNA (C9, t-test, p = 0.25). For the short-chained PFHxA (C6), the levels were significantly higher at Jonsvatnet than Granåsen (t-test, $p < 0.001$). The higher levels of PFHxA in Jonsvatnet than Granåsen could potentially reflect a local source for short chained PFCAs near Jonsvatnet. In the soil and earthworm samples, there was no significant differences in PFHxA concentrations between the two study areas, while there were significantly higher concentrations of the short-chained PFBA at Jonsvatnet, compared to Granåsen. This suggests that there is not a local release of specific short-chained PFCAs to the environment near Jonsvatnet but rather a probable source of PFCA precursors, such as fluorotelomer alcohols (FTOH). Biotransformation of FTOH could explain the higher PFHxA levels in Bank voles from Jonsvatnet because this is one of the major metabolites of FTOH metabolism in

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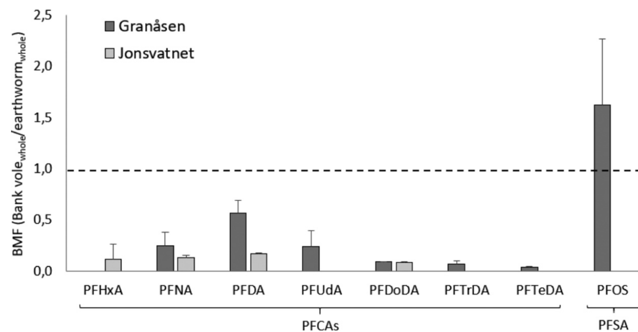

Figure 2. BMF for Bank $vole_{whole}$/$earthworm_{whole}$ (whole body concentration, w.w./w.w.) for individual PFASs in Granåsen and Jonsvatnet. Ratios are calculated from estimated (Bank vole) and measured (earthworm) average PFAS concentrations. Concentrations of PFUdA, PFTrDA, PFTeDA, and PFOS were below LOQ in the Bank voles at Jonsvatnet, and PFHxA was below LOQ at Granåsen and could not be calculated. The horizontal dashed line indicates the BMF threshold. Error bars indicate SD of the ratio (Bank vole/earthworm).

rats and other small rodents.[40] A similar observation was reported from the Antarctic Peninsula, where several PFCA compounds (e.g., PFBA, PFHxA, and PFHpA) were reported.[41] These findings suggest that the PFCAs most likely originated from FTOHs[41] because increasing trends of PFCA precursors (i.e., FTOHs) were previously observed in the Arctic with doubling times of 2.3−3.3 years between 2006 and 2012.[42]

The observed differences in PFHxA could potentially also be due to differences between locations in soil microbial communities, affecting degradation of PFCA precursors.[43−45] Furthermore, there could be differences in rate of removal between the two environments. Short-chained PFCAs readily leach from soil, and the occurrence may vary rapidly between sites, depending on the soil type. The differences in bioavailability to earthworms at the two sites or differences in the bioaccumulation pattern between earthworm and Bank vole at the two sites can probably explain these variabilities.

PFOS was the most predominant compound in Bank voles at Granåsen (Figure 1c), and the levels were higher at Granåsen, compared to Jonsvatnet, where 72% of the samples had levels below the LOQ. This is in contrast to what was measured in the soil and earthworm samples (Figure 1a,b), where PFOS concentrations were higher at Jonsvatnet, compared to Granåsen, and where PFOS was not the predominant PFAS. In wildlife studies, PFOS is usually the congener found at the highest concentrations.[46−48] However, previous studies on ski products have reported that PFCAs are the major PFASs measured in these products, while PFOS is the only PFSA detected, although at lower concentrations than the PFCAs.[5] Nevertheless, PFOS was used in skiing products in Norway until phased out in 2004. Because PFOS is very persistent, the PFOS levels measured in Bank voles in the skiing area could reflect previous use. In addition, some precursors, such as perfluorooctanesulfonyl fluoride (POSF)-based compounds[49] and perfluoroalkane sulfonamido alcohols and acrylates, degrade to PFOS.[50] However, because the same pattern was not observed in soil and earthworms, other factors such as leaching from soil, differences in the biotransformation rate, or the bioconcentration rate might be playing significant roles.

The Canadian Environmental Protection Act[51] set the liver PFOS critical toxicity value at 14.4 μg/g, based on laboratory studies in rats. Hoff et al.[38] extrapolated the environmental toxicity value for mammals to 0.144 μg/g. In the present study, the concentrations of PFOS in wild Bank voles did not exceed this value in individual animals (the maximum measured PFOS concentration was 0.016 μg/g). Accordingly, the liver concentration of PFOS detected in the Bank vole population at Granåsen may not pose a toxicological risk to these small rodents. However, considering that Bank voles are subjected to a complex mixture of PFASs, where PFOS only represents about 21%, there is still reason for concern on the physiology, endocrine, reproductive, and general health of this species and other biota at skiing areas.

While most of the research on PFASs has focused on the effects of single compounds, especially PFOS and PFOA, several hundreds of other per- and polyfluorinated compounds are currently in use[52,53] and the knowledge about the potential toxicological effects of PFAS mixtures are limited or almost nonexistent.[54] This indicates that, although their concentrations in the environment and biota are not high, they could still pose significant risks to exposed individuals under complex mixture exposure scenarios. In addition, it should be noted that the measured concentrations reported herein were detected in young individuals collected in the early summer, just after the Bank voles have started their annual reproduction cycle. The reproductive period is an exceptionally vulnerable period for these rodents, and most of the studied individuals were less than two months old, indicating that they have been exposed in utero[55] and/or from an early life stage and throughout their ontogenetic developmental period. Thus, toxicity thresholds are probably lower, compared to observations in adult rodents because young developing animals are considered more susceptible to toxic effects, compared to adults.[56]

Biomagnification of PFASs. Through the process of biomagnification, PFASs can be transferred up the food chain, where concentrations increase from one trophic level to the next via dietary accumulation.[57] The PFAS concentrations were higher in earthworms (on a w.w. basis) compared to soil (on d.w. basis) in both study locations. When considering that water in the soil will dilute the PFAS soil concentrations, the present study shows a clear bioaccumulation of PFASs from soil to earthworms. Higher concentrations in earthworms than soil were also reported in the skiing area in Oslo.[20]

The results showed that based on estimated whole-body concentrations of PFASs in the Bank voles, the BMF of PFOS at Granåsen was 1.6 (Figure 2), while for all other PFASs, the

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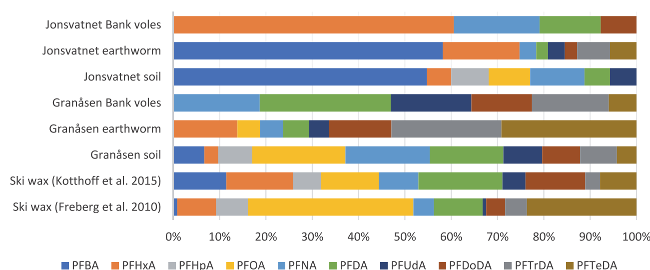

Figure 3. Overview of the contribution (%) of each congener to the total median PFCA concentration in soil, earthworm, and Bank vole samples from Gransåsen and Jonsvatnet and in ski wax samples from two different studies (Kotthoff et al. 2015[2]) and (Freberg et al. 2010[5]).

calculated BMFs were <1 in both study areas. This indicates that PFOS seems to biomagnify from earthworm to Bank vole in the skiing area, while none of the other PFASs biomagnify from earthworms to Bank vole. This is in contrast to the results from a study on a terrestrial food web (lichen-caribou-wolf),[28] which reported that several PFASs biomagnified. In that particular study, the trophic magnification factor (TMF) was the highest for PFOS and PFCAs with nine to eleven carbons. Although the BMFs for most substances except for PFOS in the present study where <1, the pattern (i.e., relative BMF) at Granåsen is comparable to the pattern found in the study on the lichen-caribou-wolf study.[28]

There may be several causes for the apparent lack of BMF of the PFASs, other than PFOS, in the present study. The Bank vole is mainly a herbivore,[58] with a diet consisting of roots, seeds, buds, and berries, in addition to earthworms and other invertebrates. On the other hand, the earthworms consume soil microorganisms, organic matter, dead leaves, and grass, and thus, the trophic levels of our study species may not be significantly different. It is therefore necessary that future studies should include organisms at a higher trophic level of the food web (e.g., carnivorous mammals or birds of prey), to properly answer whether these PFASs biomagnify in the terrestrial food chain. A recent study in the same area (county of Trøndelag) has found higher concentrations of PFASs in terrestrial birds of prey,[59] indicating that these PFASs are transported and biomagnified in terrestrial food chains.

Comparison between the PFAS Pattern. The PFCA pattern (Figure 3) is quite similar in ski wax and soil samples from Granåsen, especially for the ski wax analyzed in 2015.[2] The earthworm and Bank vole samples from Granåsen also have similar pattern to ski wax and soil samples, dominated by the longer-chained PFCAs. The long-chained PFCAs (C8–C14) make up 70–100% of the total PFCA burden in all these samples, while in the samples from Jonsvatnet, they make up only 25–40%. It is clear that the pattern measured at Granåsen is more similar to the ski wax profile than the pattern measured at Jonsvatnet (Figure 3). This strengthens the concern that ski products are a significant source of long-chained PFCAs at the local environments around skiing areas.

Studies from skiing areas found that the major PFAS congeners measured were C10–C14 PFCAs.[6] This is consistent with the findings from the present study, showing that the C10–C14 PFCAs were significantly higher at Granåsen than at Jonsvatnet. Although studies on ski products reported that PFOA is one of the main PFASs,[2,5] and it was present in the soil and earthworms at Granåsen; PFOA was not detected in the Bank voles at Granåsen. A possible reason for the low-detection frequency of PFOA in Bank voles could be the reduction of PFOA use in consumer products in Norway during the last decade, as PFOA is on Norway's priority list of chemicals, with an aim of stopping the release completely by 2020.[60] Although PFOA concentrations in soil and earthworm samples at Granåsen were below 1 ng/g, bioaccumulation of PFOA should be expected in the voles.[61] Thus, it is surprising that PFOA was not found in the Bank vole samples.

In summary, the different PFAS pattern in the two study areas clearly shows that there are different sources of PFASs to these two environments. However, the detected concentrations are far below toxicity threshold levels set in laboratory studies, indicating that individual PFASs in ski products may not pose a significant risk to the environment. Still, it should be taken into consideration that the reported concentrations were measured in organisms from the base of the food web, and because PFASs are persistent, and several of the PFASs biomagnify in food webs,[28] the levels could be much higher at a higher trophic level, such as top predators. In addition, they are exposed to a mixture of PFASs, rather than single contaminants, so the issue of mixture toxicity should also be considered and addressed in any risk environmental assessment program of contaminants from skiing areas.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02533.

Map of the study areas, internal standards used for quantification of PFASs, PFASs in soil samples from Jonsvatnet and Granåsen, PFASs in earthworm samples from Jonsvatnet and Granåsen, PFASs in Bank vole samples from Jonsvatnet and Granåsen, PFASs in blank samples, additional analytical details of sampling, chemical analyses, and data treatment (PDF)

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Author Contributions

The manuscript was written through contributions of all the authors. All the authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

LOD	limit of detection
LOQ	limit of quantification
BMF	biomagnification factor
TMF	trophic magnification factor
PFAS	perfluoroalkyl substances
PFCA	perfluorocarboxylic acid
PFSA	perfluorosulfonic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctansulfonate
POSF	perfluorooctanesulfonyl fluoride
PFBA	perfluorobutanoic acid
PFHxA	perfluorohexanoic acid
PFHpA	perfluoroheptanoic acid
PFNA	perfluorononanoic acid
PFDA	perfluorodecanoic acid
PFUdA	perfluoroundecanoic acid
PFDoDA	perfluorododecanoic acid
PFTrDA	perfluorotridecanoic acid
PFTeDA	perfluorotetradecanoic
PFBS	perfluorobutane sulfonate
PFHxS	perfluorohexane sulfonate
FASAs	perfluoroalkane sulfonamide derivatives
FOSA	perfluoro-1-octane sulphonamide
N-MeFOSA	*N*-methyl perfluoro-1-octane sulphonamide
N-EtFOSA	*N*-ethyl perfluoro-1-octane sulfonamide
N-MeFOSE	2-(*N*-methyl perfluoro-1-octane sulfonamido) ethanol
N-EtFOSE	2-(*N*-ethyl perfluoro-1-octane sulfonamido) ethanol
w.w.	wet weight
d.w.	dry weight

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SUPPLEMENTARY INFORMATION

Levels, Patterns and Biomagnification Potential of Perfluoroalkyl Substances (PFASs) in a Terrestrial Food Chain in a Nordic Skiing Area

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Additional analytical details of sampling, chemical analyses and data treatment.

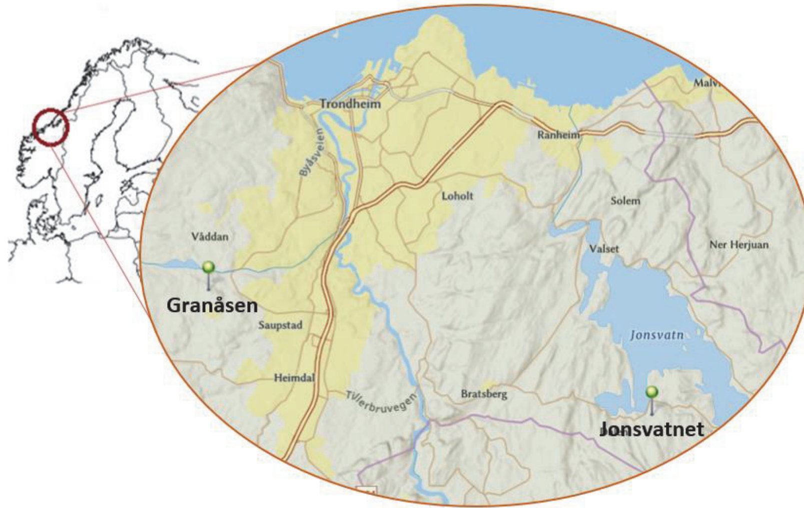

Figure S1. Map of the study areas. Granåsen is the skiing area and Jonsvatnet is the reference area. Modified figure retrieved from ArcGis. Esri. "National Geographic" [basemap]. Scale Not Given. "World Topographic Map". October 3, 2019. https://ntnut.maps.arcgis.com/home/webmap/viewer.html?webmap=50bac58b640448298cd6534a8f5b844e (October 3, 2019).

Table S1. Internal standards of PFASs used for quantification. All standards were added to the concentration of 20 ng/mL, Wellington Laboratories, USA.

Internal standard	**Compounds**
Perfluoro-n-(1,2-$^{13}C_2$) undecanoic acid	PFUdA
Perfluoro-n-(1,2-$^{13}C_2$) dodecanoic acid	PFDoDA, PFTrDA and PFTeDA
Perfluoro-n-(1,2-$^{13}C_2$) decanoic acid	PFDA
Perfluoro-n-(1,2,3,4,5-$^{13}C_5$) nonanoic acid	PFNA
Perfluoro-n-(1,2,3,4-$^{13}C_4$)octanoic acid	PFOA
Perfluoro-n-(1,2,3,4-$^{13}C_4$) heptanoic acid	PFHpA
Perfluoro-n-(1,2,3,4,6-$^{13}C_5$) hexanoic acid	PFBA
Perfluoro-n-(2,3,4-$^{13}C_3$) butanoic acid	PFHxA
Sodium perfluoro-1-hexane($^{18}O_2$) sulfonate	L-PFHxS and L-PFBS
Sodium perfluoro-1-(1,2,3,4,-$^{13}C_4$) octanesulfonate	L-PFOS
N-methyl-d3-perfluoro-1-octanesulfonamide	N-MeFOSA-M
N-ethyl-d3-perfluoro-1-octanesulfonamide	N-EtFOSA-M
Perfluoro-1-($^{13}C_8$) octanesulfonamide	FOSA-1
2-(N-methyl-d3-perfluoro-1-octanesulfonamido) etnane-d4-ol	N-MeFOSE-M
2-(N-ethyl-d5-perfluoro-1-octanesulfonamido) ethane-d4-ol	N-EtFOSE-M

Table S2. PFASs in soil samples from Jonsvatnet and Granåsen. Values are given in ng/g d.w.

	Jonsvatnet (n = 10)					Granåsen (n = 10)				
	Mean LOQ	**Df (%)**	**Range**	**Mean**	**SD**	**Mean LOQ**	**Df (%)**	**Range**	**Mean**	**SD**
PFBA	0.125	100	<LOQ-1.06	0.593	0.315	0.125	60	<LOQ-0.563	0.145	0.164
PFHxA	0.038	60	<LOQ-0.178	0.0586	0.0470	0.038	60	<LOQ-0.184	0.048	0.050
PFHpA	0.079	50	<LOQ-0.621	0.139	0.174	0.079	70	<LOQ-0.288	0.085	0.085
PFOA	0.047	60	<LOQ-0.216	0.0974	0.0793	0.047	90	<LOQ-0.403	0.156	0.120
PFNA	0.056	70	<LOQ-0.928	0.198	0.265	0.056	90	<LOQ-0.602	0.179	0.177
PFDA	0.053	80	<LOQ-0.154	0.0753	0.0485	0.053	80	<LOQ-1.96	0.417	0.632
PFUdA	0.044	70	<LOQ-0.122	0.0658	0.0443	0.044	60	<LOQ-0.294	0.134	0.112
PFDoDA	0.085	20	<LOQ	<LOQ	<LOQ	0.085	50	<LOQ-0.401	0.159	0.139
PFTrDA	0.083	20	<LOQ	<LOQ	<LOQ	0.083	50	<LOQ-0.203	0.090	0.067
PFTeDA	0.011	0	<LOQ	<LOQ	<LOQ	0.011	50	<LOQ-0.138	0.122	0.140
PFBS	0.005	70	<LOQ-0.0385	0.0093	0.0110	0.005	10	<LOQ	<LOQ	<LOQ
PFHxS	0.032	10	<LOQ	<LOQ	<LOQ	0.032	0	<LOQ	<LOQ	<LOQ
PFOS	0.042	100	0.0813-0.637	0.304	0.212	0.042	90	<LOQ-0.341	0.105	0.108
FOSA	4.1	NA	NA	NA	NA	4.1	NA	NA	NA	NA
N-MeFOSA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
N-EtFOSA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
N-MeFOSE	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
N-EtFOSE	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

**LOQ = limit of quantification, Df = detection frequency (%) above LOQ. Range = minimum concentration detected above LOQ to maximum concentration. SD = Standard deviation, NA = not analyzed*

Table S3. PFASs in earthworms from Jonsvatnet and Granåsen. Values are given in ng/g w.w.

**LOQ = limit of quantification, Df = detection frequency (%) above LOQ. Range = minimum concentration detected above LOQ to maximum concentration. SD = Standard deviation.*

	Jonsvatnet (n = 13)					Granåsen (n = 13)				
	Mean LOQ	**Df (%)**	**Range**	**Mean**	**SD**	**Mean LOQ**	**Df (%)**	**Range**	**Mean**	**SD**
PFBA	0.20	83	<LOQ-15.6	3.200	4.20	0.20	31	<LOQ	<LOQ	<LOQ
PFHxA	0.20	54	<LOQ-5.55	0.866	1.63	0.20	69	<LOQ-5.56	1.405	1.63
PFHpA	0.23	23	<LOQ	<LOQ	<LOQ	0.23	23	<LOQ	<LOQ	<LOQ
PFOA	0.28	15	<LOQ	<LOQ	<LOQ	0.28	54	<LOQ-2.47	0.625	0.758
PFNA	0.066	62	<LOQ-1.72	0.354	0.502	0.066	85	<LOQ-1.94	0.445	0.547
PFDA	0.056	54	<LOQ-0.642	0.164	0.211	0.056	85	<LOQ-2.74	0.613	0.775
PFUdA	0.076	62	<LOQ-0.708	0.211	0.223	0.076	100	0.060-2.46	0.628	0.866
PFDoDA	0.086	54	<LOQ-0.320	0.148	0.121	0.086	92	<LOQ-8.35	1.728	2.62
PFTrDA	0.076	92	<LOQ-2.35	0.783	0.703	0.076	100	0.291-15.7	2.356	4.41
PFTeDA	0.091	77	<LOQ-2.41	0.432	0.622	0.091	92	<LOQ-24.2	3.06	5.63
PFBS	0.33	23	<LOQ	<LOQ	<LOQ	0.33	8	<LOQ	<LOQ	<LOQ
PFHxS	0.35	8	<LOQ	<LOQ	<LOQ	0.35	0	<LOQ	<LOQ	<LOQ
PFOS	0.63	54	<LOQ-1.78	0.764	0.460	0.63	54	<LOQ-1.28	0.371	0.422
FOSA	0.24	0	<LOQ	<LOQ	<LOQ	0.24	0	<LOQ	<LOQ	<LOQ
N-MeFOSA	0.83	0	<LOQ	<LOQ	<LOQ	0.83	0	<LOQ	<LOQ	<LOQ
N-EtFOSA	0.28	0	<LOQ	<LOQ	<LOQ	0.28	0	<LOQ	<LOQ	<LOQ
N-MeFOSE	0.31	0	<LOQ	<LOQ	<LOQ	0.31	0	<LOQ	<LOQ	<LOQ
N-EtFOSE	0.40	0	<LOQ	<LOQ	<LOQ	0.40	0	<LOQ	<LOQ	<LOQ

Table S4. PFASs in Bank voles from Jonsvatnet and Granåsen. Values are given in ng/g w.w.

	Jonsvatnet (n = 31)					Granåsen (n = 21)				
	LOQ	**Df (%)**	**Range**	**Mean**	**SD**	**LOQ**	**Df (%)**	**Range**	**Mean**	**SD**
PFBA	1.9	0	<LOQ	<LOQ	<LOQ	0.44	0	< LOQ	< LOQ	< LOQ
PFHxA	1.2	72	<LOQ-3.41	1.54	0.797	5.3	0	<LOQ	< LOQ	< LOQ
PFHpA	1.4	3	<LOQ	<LOQ	<LOQ	3.0	0	< LOQ	< LOQ	< LOQ
PFOA	0.80	0	<LOQ	<LOQ	<LOQ	2.2	0	< LOQ	< LOQ	< LOQ
PFNA	0.54	50	<LOQ-4.03	0.742	0.920	0.38	72	<LOQ-7.15	0.966	1.38
PFDA	0.14	75	<LOQ-2.14	0.393	0.330	0.47	76	<LOQ-11.3	1.80	2.47
PFUdA	1.0	3	<LOQ	<LOQ	<LOQ	0.37	80	<LOQ-13.6	1.43	2.63
PFDoDA	0.13	63	<LOQ-0.654	0.186	0.193	0.057	96	<LOQ-30.4	2.11	6.08
PFTrDA	0.027	34	<LOQ	< LOQ	<LOQ	0.066	100	<LOQ-31.5	2.15	6.25
PFTeDA	0.48	3	<LOQ	< LOQ	<LOQ	0.021	76	<LOQ-48.3	2.56	9.61
PFBS	1.9	0	<LOQ	<LOQ	<LOQ	1.3	0	<LOQ	< LOQ	< LOQ
PFHxS	1.8	6	<LOQ	<LOQ	<LOQ	0.73	52	<LOQ-6.24	1.14	1.45
PFOS	2.1	28	<LOQ	<LOQ	<LOQ	2.2	56	<LOQ-16.0	3.30	3.37
FOSA	0.17	0	<LOQ	<LOQ	<LOQ	0.16	0	< LOQ	< LOQ	< LOQ
N-MeFOSA	3.1	0	<LOQ	<LOQ	<LOQ	0.81	0	< LOQ	< LOQ	< LOQ
N-EtFOSA	4.3	0	<LOQ	<LOQ	<LOQ	0.28	0	< LOQ	< LOQ	< LOQ
N-MeFOSE	0.30	0	<LOQ	<LOQ	<LOQ	2.2	0	< LOQ	< LOQ	< LOQ
N-EtFOSE	1.31	0	<LOQ	<LOQ	<LOQ	0.22	0	< LOQ	< LOQ	< LOQ

**LOQ = limit of quantification, Df = detection frequency (%) above LOQ. Range = minimum concentration detected above LOQ to maximum concentration. SD = Standard deviation.*

Table S5. PFASs in blank samples used for method control in the PFAS extraction. Concentrations are given as ng/mL.

	Bank voles Granåsen			**Bank voles Jonsvatnet**			**Earthworms**			**Soil**		
	Blank 1	Blank 2	Blank 3	Blank 1	Blank 2	Blank 3	Blank 1	Blank 2	Blank 3	Blank 1	Blank 2	Blank 3
PFBA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.105	n.d.	0.0500
PFHxA	0.265	0.566	0.539	0.598	0.572	0.653	0.0170	n.d.	n.d.	n.d.	n.d.	n.d.
PFHpA	0.407	0.525	0.327	0.349	0.436	0.373	0.0110	0.0120	0.0090	0.0190	0.0180	0.013
PFOA	0.0580	0.203	0.146	0.308	0.283	0.336	0.0490	0.0420	0.0560	0.0660	0.0540	0.0570
PFNA	0.096	0.0720	0.0770	0.243	0.236	0.209	n.d.	n.d.	n.d.	n.d.	0.0300	n.d.
PFDA	0.0110	0.0230	0.0420	0.0520	0.048	0.043	0.0180	0.0190	0.0140	n.d.	n.d.	n.d.
PFUdA	0.0833	0.107	0.0900	0.0660	0.0420	0.109	0.069	0.0690	0.0680	n.d.	n.d.	n.d.
PFDoDA	0.0150	0.0180	n.d.	0.0060	0.0130	0.0150	0.0080	0.0080	0.0090	n.d.	n.d.	n.d.
PFTrDA	0.0120	0.0120	0.0106	0.0150	0.0160	0.0105	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFTeDA	n.d.	0.0300	0.0310	n.d.	n.d.	0.028	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFBS	0.295	0.318	0.327	0.136	0.0570	0.049	0.0490	0.0560	0.0360	0.0620	0.103	0.126
PFHxS	0.153	0.0730	0.0860	0.04100	0.128	n.d.	0.0860	0.0830	0.104	0.108	0.126	0.147
PFOS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.123	0.0640	0.114
FOSA	0.0170	0.0150	0.0180	0.009	0.0100	n.d.	0.0180	0.0300	0.0150	n.d.	n.d.	n.d.
N-MeFOSA	n.d.	0.0240	0.0630	n.d.	n.d.	n.d.	0.0460	n.d.	0.0500	n.d.	n.d.	n.d.
N-EtFOSA	0.0230	n.d.	0.0360	n.d.	n.d.	n.d.	0.0390	0.0570	0.0450	n.d.	n.d.	n.d.
N-MeFOSE	0.0710	0.0260	0.171	0.0520	0.0700	0.067	0.0360	0.0560	0.0490	n.d.	n.d.	n.d.
N-EtFOSE	0.00700	0.0220	0.0140	0.0740	n.d.	0.0780	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*n.d. = not detected

Additional information for materials and methods

S*ampling of Bank voles (Myodes glareolus)*

The collection of Bank voles was performed in June 2017. All traps were live traps of type "Ugglan" baited with rye bread dipped in sunflower oil and peanut butter (all food products were sold as "ecological food material"). The traps were arranged in stations (16 stations in total) of four traps per station. The trapping stations were spaced out with a minimum of 500 m to avoid population depletion of the species. All traps were controlled every day. Traps with rodents were brought back to the animal laboratory facilities at the Biology Department at the Norwegian University of Science and Technology (NTNU). The animals were anaesthetised with Ketamine (100 mg/kg) and Xylazine (10 mg/kg). The anaesthetics were diluted and given intraperitoneal (IP). The animals were then weighed, measured, sexed and species determined. Blood samples were collected by cardiac puncture using a heparinized syringe, while the animals were under anaesthetics, but still alive (this was used for another part of the study). The animals were then sacrificed by cervical dislocation.

Chemical analysis

0.5g liver or earthworm sample was weighed in Falcon centrifuge tubes (VWR International, LLC Radnor, USA). The samples were then added internal standards (^{13}C-labeled equivalents) to a concentration of 20 ng/mL. The samples were homogenized using ultra thurrax and sonicated for 45 seconds. They were extracted twice using 5 mL of methanol (CH_3OH) (Rathburn chemicals, Walkerburn, Scotland) followed by 30 minutes of mixing in a Vibrax machine (Vibrax VXR, IKA®, MA, USA). The samples were centrifuged at 3000 rpm for 10 minutes (Allegra® X-12R, Beckman Coulter, CA, USA) and the supernatant were collected and evaporated to a volume of 2 mL using Zymark Turbovap® LV Evaporator with water bath (40°C) and a gentle flow of nitrogen

gas (N_2) (Purity: 99.6%, Aga AS, Oslo, Norway). Clean-up was accomplished by adding 0.2–0.3 g active coal (ENVI-Carb™, Sigma-Aldrich, Oslo, Norway) to each sample and mixing well on a vortex machine. The samples were then centrifuged at 3000 rpm for 10 minutes. The supernatant were added to new tubes. 1 mL methanol was then added to the remaining deposits and the previous step was repeated. The supernatant were then evaporated to 0.5 mL and the samples were centrifuged (3000 rpm, 10 minutes) and transferred to vials with plastic inlets (200 µL).

The final extracts were analyzed by separation on a high-performance liquid chromatographer (HPLC) with a Discovery C18 column (15 cm x 2.1 mm x, 5 µm, Supelco, Sigma-Aldrich, Oslo, Norway), connected to a pre-column; Supelguard Discovery C18 column (2 cm x 2.1 mm x, 5 µm, Supelco, Sigma-Aldrich, Oslo, Norway). Detection and quantification was accomplished with a tandem mass spectrometry (MS-MS) system (API 3000, LC/MS/MS System). The injected volume was 5 µL.

External standards were used to produce a standard curve from which the PFAS levels were calculated, using the instrument control and data processing program MassHunter Quantitative analysis Version B.05.02 (Agilent Technologies). The limits of detection (LOQs) were calculated as 3*SD of the procedural blanks, and the limits of quantification were calculated as 10 * LOQ. Where no blank was detected, LOQ was determined as 10 * signal-to-noise ratio (S/N). For the soil and earthworm samples, individual LOQs were determined for each sample, because of matrix effects.

Data treatment

The following contaminants, PFBS, PFHpA, PFOA, PFBA, FOSA, N-MeFOSA, N-EtFOSA, N-MeFOSE and N-EtFOSE had concentrations below LOQ in more than 50% of the Bank vole samples in both Granåsen and Jonsvatnet, and were excluded from statistical analysis. In addition,

PFHxA was below LOQ at Granåsen, and PDUdA, PFTrDA, PFTeDA, PFHxS and PFOS were below LOQ at Jonsvatnet.

For the earthworm samples, the following contaminants, PFHpA, PFBS and PFHxS were below LOQ in more than 50% of the samples in both Granåsen and Jonsvatnet, and were excluded from statistical analysis. In addition, PFOA was below LOQ in Jonsvatnet, and PFBA was below LOQ in Granåsen.

In the soil samples, PFHxS concentration was below LOQ in more than 50% of the samples in both Granåsen and Jonsvatnet, and was excluded from the statistical analysis. In addition, PFDoA, PFTrDA and PFTeDA were below LOQ in Jonsvatnet, and PFBS was below LOQ in Granåsen.

Paper II

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Alteration of neuro-dopamine and steroid hormone homeostasis in wild Bank voles in relation to tissue concentrations of PFAS at a Nordic skiing area

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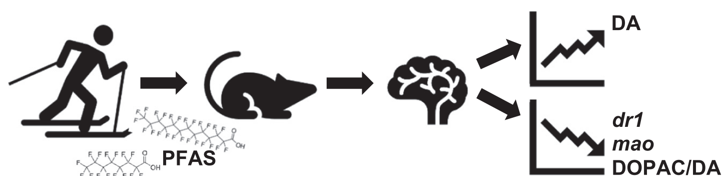
HIGHLIGHTS

- Elevation of DA levels in Bank vole brains from a skiing area parallel PFAS contamination
- A negative association between dopamine receptor 1 (*dr1*) mRNA and several PFAS
- DOPAC/DA ratios and monoamine oxidase (*mao*) mRNA is negatively associated with PFAS.
- Sex-specific negative relationship between some PFAS and T concentrations

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluoroalkyl substances (PFAS) are contaminants that are applied in a wide range of consumer products, including ski products. The present study investigated the neuro-dopamine (DA) and cellular steroid hormone homeostasis of wild Bank voles (*Myodes glareolus*) from a skiing area in Norway (Trondheim), in relation to tissue concentrations of PFAS. We found a positive association between brain DA concentrations and the concentration of several PFAS, while there was a negative association between PFAS and dopamine receptor 1 (*dr1*) mRNA. The ratio between DA and its metabolites (3,4-dihydroxyphenylacetic acid: DOPAC and homovanillic acid: HVA) showed a negative association between DOPAC/DA and several PFAS, suggesting that PFAS altered the metabolism of DA via monoamine oxidase (Mao). This assumption is supported by an observed negative association between *mao* mRNA and PFAS. Previous studies have shown that DA homeostasis can indirectly regulate cellular estrogen (E2) and testosterone (T) biosynthesis. We found no association between DA and steroid hormone levels, while there was a negative association between some PFAS and T concentrations, suggesting that PFAS might affect T through other mechanisms. The results from the current study indicate that PFAS may alter neuro-DA and steroid hormone homeostasis in Bank voles, with potential consequences on reproduction and general health.

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1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic, persistent chemicals that are widespread in the environment (Houde

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et al., 2006; Glüge et al., 2020). They are a group of surface-active compounds that are applied in a wide range of consumer products, such as textiles, carpets, impregnating agents and in some types of ski products, such as ski waxes, gliders and powders (Kissa, 2001). Among the mentioned consumer products, ski products show the highest PFAS concentrations (Kotthoff et al., 2015). Abrasion of waxes from the ski sole results in the deposition of PFAS to nearby environments (Plassmann and Berger, 2013). Since PFAS are very persistent, they can remain in the environment for decades, thus creating PFAS-hotspots at skiing areas (Kissa, 2001; Grønnestad et al., 2019).

Previous studies have shown that some PFAS can cross the blood-brain barrier and accumulate in the brain (Maestri et al., 2006). Studies of large mammals suggest that PFAS can potentially be neurotoxic to exposed individuals. In polar bears (*Ursus maritimus*), brain PFAS levels were found to correlate with neurotransmitter alterations (Pedersen et al., 2015). Further, PFAS in North Atlantic pilot whales (*Globicephala melas*) were found to accumulate in brain, with higher levels only detected in the liver (Dassuncao et al., 2019). Neurotoxicity studies in rodents have shown that PFAS produced neurobehavioral alterations (Johansson et al., 2009; Lee and Viberg, 2013), and developmental and motor deficits (Onishchenko et al., 2011). Due to its important role in both motoric and cognitive functions, the central cholinergic system has received most attention in neurotoxicity studies (Eriksson and Viberg, 2005; Johansson et al., 2009). However, the dopamine (DA) neurotransmitter system plays an equally important role in behavior and cognitive functions, but has received less attention in these types of toxicity studies.

DA 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). Dopamine catabolism occurs through the breakdown of 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (Mao), and then to homovanillic acid (HVA) by catechol-O methyltransferase (Comt) (Ashcroft, 1969). DA controls several brain conditions such as cognition, mood, fear, anxiety, as well as vascular and reproductive functions (Nakajima et al., 2013; Goschke and Bolte, 2014).

Studies have shown that alterations of the DA system can affect a number of signaling cascades in the body (Zohar et al., 2010). The DA neurons can, among others, indirectly regulate estrogen (E2) and testosterone (T) biosynthesis. Biosynthesis of E2 and T is regulated through the hypothalamus–pituitary–gonadal (HPG) axis (Zohar et al., 2010). The hypothalamus produces gonadotropin-releasing hormone (GnRH), and hypothalamic secretion of GnRH stimulates the release of gonadotropins (GtHs): luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the blood. Both LH and FSH controls egg and sperm development, maturation and release, and they induce gonadal hormone synthesis, including E2 and T. DA can block the synthesis and release of GnRH (Yu et al., 1991), modulate gonadotropin levels by increasing and decreasing these hormones based on receptor subtype (Chang et al., 1990), and subsequently lead to decreased levels of E2 and T. Furthermore, DA can also control E2 levels by regulating brain aromatase, the enzyme that converts androgens to estrogens in the brain (Xing et al., 2016).

Neurobehavioral alterations in vertebrates have been examined in several developmental and adult exposure studies, with some behavioral endpoints suggesting that the DA-system is a potential target for environmental contaminants (Hallgren and Viberg, 2016). Northern leopard frogs (*Lithobates pipiens*) exposed to perfluorooctane sulfonate (PFOS) and perfuorooctanoic acid (PFOA) during developmental life-stages showed decreased and increased DA levels and turnover, respectively (Foguth et al., 2019), while the opposite effect was seen in adult rats (*Rattus norvegicus*), where PFOS exposure increased the DA levels (Salgado et al., 2015). These data from experimental studies raise the question of whether the exposure of small mammals to PFAS in the field, such as skiing areas, could lead to neurochemical alterations. In general, there is a paucity of data on PFAS neurotoxicity and especially in relation to environmentally relevant exposure scenarios in nature.

In a previous study, we showed that Bank voles (*Myodes glareolus*) from a Nordic skiing area had significantly higher liver PFAS concentrations, compared to those from a reference area, and that these PFAS were, most likely, derived from skiing products (Grønnestad et al., 2019). However, the reported concentrations were below toxicity threshold levels for laboratory studies on individual PFAS in mice (*Mus musculus*) and rats (Canadian Environmental Protection Act, 1999; Hoff et al., 2004). These results suggest that individual PFAS in ski products may not pose significant risks to biota or the environment. However, we emphasized that the small mammals were exposed to a mixture of PFAS, rather than to individual compounds, thus the issue of mixture toxicity should be considered and addressed in any environmental risk assessment program from skiing areas. Therefore, the aim of the current study was to evaluate the neuro-DA and steroid homeostatic pathways of wild Bank voles, in relation to tissue levels of various PFAS at a Nordic skiing area. Our hypothesis is that PFAS concentrations in Bank voles will affect biological variables in the neuro-DA and steroid hormone metabolic pathways.

2. Materials and methods

2.1. Sampling

Bank voles were sampled in June 2017 in "Granåsen skisenter", located approximately 10 km from the Trondheim city Centre (Norway). Granåsen is the main arena for winter sports in Trondheim and was used as a model contamination site in a skiing area. As a reference site, a natural forest area, not used for ski-sports, was chosen in the vicinity of an ecological farm next to Lake Jonsvatnet. This site is approximately 15 km away from Trondheim city center and 17 km from Granåsen. The sample size (n) was 21 at Granåsen (females (F): $n = 5$, males (M): $n = 16$) and 22 at Jonsvatnet (F: $n = 6$, M: n = 16).

The catching, handling, anesthesia, sampling and euthanizing of the Bank voles were approved by the Norwegian Food Safety Authority (Mattilsynet; references no. 2017/76552) and by the Norwegian Environmental Agency (Miljødirektoratet; reference no. 2017/4061). Permissions for the collection of Bank voles were also given by the landowners. The sampling and handling were performed in accordance with the regulations of the Norwegian Animal Welfare Act and EU legislation; 3Rs (Russell and Burch, 1959). All traps were live traps of type "Ugglan" baited with rye bread dipped in sunflower oil and peanut butter (all food products were sold as "ecological food material"). The Bank voles were sacrificed by cervical dislocation. The animals were weighed, length measured and sexed. The brains (used for DA and transcript analysis), livers (used for PFAS concentrations and steroid metabolism assay) and muscle tissues (used for steroid concentrations) were dissected and snap-frozen in liquid nitrogen and stored at −80 °C. In the current study, muscle tissue was used as a proxy for the free fraction of steroids in blood, since we did not have enough blood sample for this purpose. It has been shown that whole-body homogenate or muscle are suitable tissues for measuring the cellular and circulatory levels of steroid hormones (Arukwe et al., 2008; Preus-Olsen et al., 2014). Despite the limited blood sample size, the interest in measuring steroid hormone levels in muscle or tissue homogenates, rather than in blood plasma is based on the concept that the pattern of steroids release parallels its pattern of secretion into the bloodstream (Sebire et al., 2007; Sebire et al., 2009).

2.2. PFAS analysis

PFAS concentrations were analyzed at the Environmental Toxicology Laboratory, Norwegian University of Life Sciences (NMBU), Oslo, Norway. The analytical procedures were described in (Grønnestad et al., 2016). The samples were analyzed for the following PFAS: 10

perfluoroalkyl carboxylic acids (PFCA): perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA) and perfluorotetradecanoic acid (PFTeDA), three perfluoroalkyl sulfonic acids (PFSA): perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS) and PFOS, and five perfluoroalkane sulfonamide derivatives (FASA): perfluoro-1-octane sulfonamide (FOSA), *N*-Methyl perfluoro-1-octane sulfonamide (N-MeFOSA), *N*-Ethyl perfluoro-1-octane sulfonamide (N-EtFOSA), *2*-(*N*-Methyl perfluoro-1-octane sulfonamido) ethanol (N-MeFOSE) and *2*-(*N*-Ethyl perfluoro-1-octane sulfonamido) ethanol (N-EtFOSE). For results on PFAS levels, patterns and biomagnification potential of PFAS in Bank voles from the two areas, see Grønnestad et al. (2019). For more analytical details, see supporting information (SI).

The limits of detection (LODs) were calculated as 3*SD of the procedural blanks and the limits of quantification (LOQs) were calculated as 10 * LOD. Where no PFAS were detected in blank samples, LOQs were determined as 10 * signal-to-noise ratio (S/N).

Contaminants with concentrations above LOD in more than 50% of samples were included in the statistical analyses, and missing values (i.e. $<$ LOD) were assigned a random value between the LOD and zero.

2.3. Quality assurance

The Environmental Toxicology Laboratory is accredited by the Norwegian Accreditation as a testing laboratory according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137).

For each series of maximum 30 samples, 3 blank samples, one blind and 4 recovery samples were run. Mean of procedural blanks, consisting of internal standards and solvents, were subtracted from each series separately, because of variation between series. The relative recovery in Bank voles ranged from 84 to 128% for PFCA, 78–129% for PFSA and 86–115% for FASA.

2.4. Dopamine analysis

For the measurement of brain concentrations of DA and its metabolites (DOPAC and HVA), samples were prepared using methods based on Tareke et al. (2007) and Bertotto et al. (2018) with slight modifications. Samples were kept on ice during handling and extraction. The samples were homogenized prior to extraction. Approximately 200 mg of homogenized 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. The samples were extracted twice using ice-cold 0.1% formic acid in water. The extraction was then, performed using a pestle tissue homogenizer. The tubes were centrifuged for 5 min at 4000 rpm and 4 °C. The extracts were then subjected to solid-phase extraction (SPE) with Strata X polymeric reverse-phase cartridges (33 mm, 60 mg, 3 mL; Phenomenex), as described in Tareke et al. (2007). The SPE cartridges were conditioned with 1 mL of 0.1% formic acid in acetonitrile (CH_3CN), followed by 1 mL of 0.1% formic acid in methanol, and 1 mL of 0.1% formic acid in water. The extracts were then added to the column, and the analytes were eluted with 3 mL of 0.1% formic acid in acetonitrile/methanol (1:1, v/v). The resulting extracts were evaporated to dryness with nitrogen gas and reconstituted in 0.4 mL of 0.1% formic acid in water. After vortexing, the extracts were filtered 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 dopamine while deuterated HVA (HVA-d5) was used as internal standard for HVA and DOPAC. Relative recoveries varied from 18 to 94% for Da-d4 and 21–66% for HVA-d5 (SI, Table S1). The samples were therefore adjusted for recoveries for each individual sample.

2.5. Steroid hormone analysis

Approximately 200 mg muscle tissue was added to 3× volume of lysis buffer (50 mM Tris base, 0.5% sodium deoxychalate, 1 mM EDTA, 1% triton, 150 mM NaCl, 0.1% SDS) with 10% phenylmethylsulfonyl fluoride (PMSF, to inhibit AChE). This was incubated on ice for 30 min followed by homogenization and centrifuged for 20 min at 15,000*g* and 4 °C. The supernatant was used for steroid extraction.

Steroid hormones were extracted twice using dichloromethane (DCM). DCM was added to a volume of 4× sample volume. The extracts were evaporated to dryness under nitrogen in a water bath at 30 °C and reconstituted with 210 μL of enzyme immunoassay (EIA) buffer. The extracts of muscle tissue were used for the measurement of E2 and T, using EIA kit from Cayman Chemicals (Ann Arbor, MI, USA). All assay solutions were prepared according to kit instructions with deionized water. Absorbance readings were performed on a spectrophotometer (Spectra Max Plus 384, Molecular Devices) at 412 nm. Steroid hormone concentrations were calculated by extrapolating sample absorbance on a linear standard curve, using the analysis tool provided by the kit's manufacturer.

2.6. Microsome extraction

Approximately 100 mg liver tissue was homogenized in 500 μL homogenization buffer (ice-cold 10 mM Tris-HCl buffer with 10% glycerol at pH 7.4). The homogenate was centrifuged for 20 min at 12,000*g* and 4 °C. The supernatant was transferred to new tubes and 1 mL homogenization buffer was added, then centrifuged for 60 min at 38,000 rpm in a vacuum centrifuge at 4 °C. The pellet was then re-suspended in 50 μL microsomal buffer (50 mM Tris-HCl with 0.1 mM EDTA and 20% glycerol, pH 7.4). Total microsomal protein content was determined using the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as standard.

2.7. Steroid hydroxylation assay

For the steroid hydroxylation assay, 0.4 mg of microsomal protein was incubated with 4 μM testosterone and assay buffer (50 mM Tris–HCl, 10 mM MgCl2, pH 7.4) to a final volume of 240 μL. Duplicates were made for each sample in parallel analysis with and without the addition of NADPH (300 μM). These were vortexed and incubated for 60 min at 30 °C. The reactions were stopped by adding 250 μL acetonitrile (ACN) and centrifuged for 10 min at 10,000*g*. The ACN extracts were filtered and transferred to glass vials and run on an LC-2030 (Shimadzu, Kyoto, Japan) with a UV detector. See more detailed information in SI.

Steroid hydroxylase (OHase) activity was measured as the change in testosterone concentration, after addition of NADPH, compared to when no NADPH was added.

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. Thereafter, RNA quantity and quality were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The NanoDrop showed that all samples had high quantity of RNA and good purity (260/280 ratio ranged between 1.97 and 2.0 and 260/230 ratio ranged between 2.0 and 2.1).

Transcripts expression analysis related to the dopaminergic and HPG pathways were performed using 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 1:5 diluted cDNA, 0.5 μM each of the forward and reverse primer pair sequences (SI Table S2) were amplified using Mx3000P real-time PCR machine (Stratagene, La Jolla, CA). See Khan et al. (2019) for detailed protocol for qPCR analysis of gene

expression patterns. Transcripts tested include - dopamine receptor 1 and 2 (*dr1* and *dr2*), monoamine oxidase (*mao*), vesicular monoamine transporter (*vmat*), dopamine active transporter (*dat*), catechol-*O*-methyltransferase (*comt*), tyrosine hydroxylase (*th*), aromatase (*cyp19a*) and gonadotropin releasing hormone (*gnrh*). However, no positive amplifications were observed for *vmat*, *cyp19a* and *gnrh*.

2.9. Statistical analysis

The program R (version 3.6.3, the R project for statistical computing) was used for the statistical analysis. Normal distribution was tested with Shapiro Wilk's test, and homogeneity of variance was tested with Levene's test. Data were log-transformed prior to data analyses to reduce deviation from normality and homogeneity of variance. Two sample Student's *t*-tests were used to test for significant differences between the skiing and reference areas. The significance level was set at 0.05, and all tests were two tailed.

Multivariate analyses (principal component analyses; PCA) were carried out to investigate for possible relationships between the response variables (DA-related variables or steroid-related variables) and the explanatory variables (individual PFAS) in the skiing area (Granåsen). Explanatory variables were entered as passive variables in the PCA plots. Passive variables do not affect the ordination but are projected onto the unconstrained axes, allowing for visualization of correlations among response and explanatory variables. Variables were standardized to unit variance due to different units. Based on the visualization of possible relationships from the PCAs, general linear models (GLM) were used to quantify the amount of variance explained (R^2) by the respective single explanatory variables. Since there were no effects of gender on the PFAS concentrations, or response variables in the skiing area (*t*-test, $p > 0.05$), the dataset was not divided by sex in the multivariate analysis, to increase the sample size (n).

3. Results and discussion

3.1. Dopamine and dopamine metabolite levels

DA concentrations were significantly higher in brain samples in Bank voles from the skiing area with higher PFAS body burden (Granåsen), compared to the reference area (Jonsvatnet) (t-test, M: $p = 0.05$, F: $p = 0.03$, Fig. 1). The DA metabolite concentrations (DOPAC and HVA), were lower in the skiing area, compared to the reference area. The differences were significant in both sexes for DOPAC (t-test,

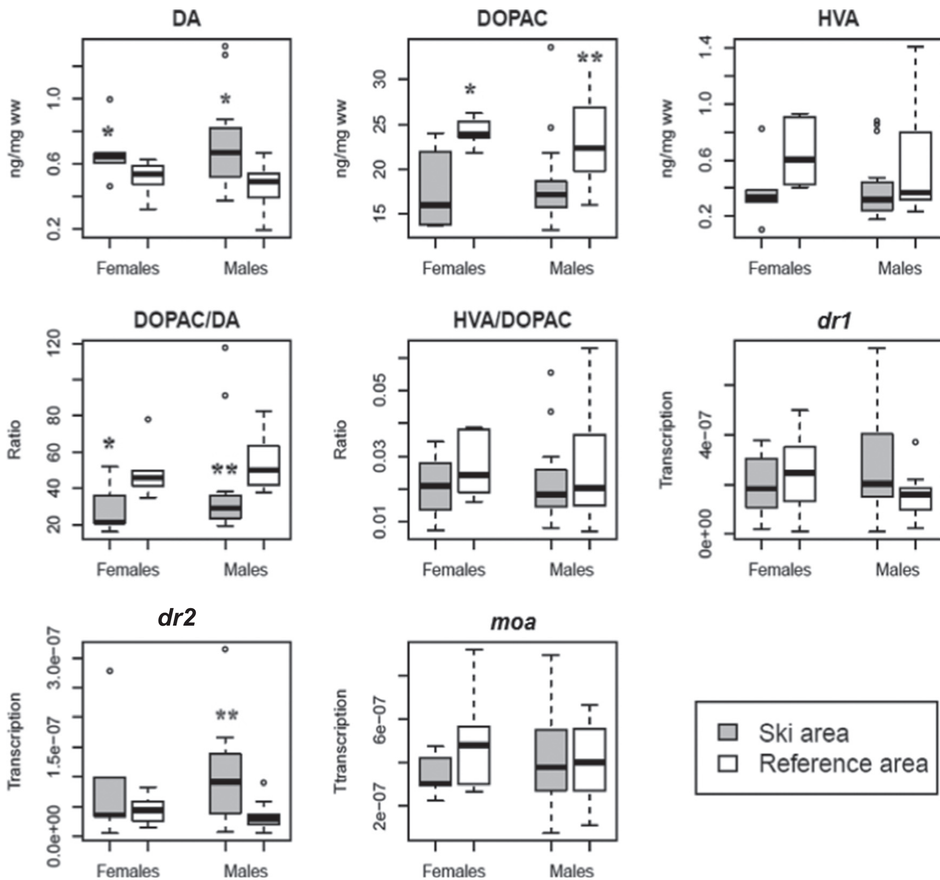

Fig. 1. Boxplots of variables related to the dopamine system in male and female Bank voles from Granåsen skiing area (females: $n = 5$, males: $n = 16$) and Jonsvatnet reference area (females: $n = 6$, males: $n = 16$). Variables are dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), DOPAC/DA ratio, HVA/DOPAC ratio, dopamine receptor 1 (d*r1*), dopamine receptor 2 (*dr2*) and monoamine oxidase (*moa*). Asterisks indicate the significance level: $^*p < 0.05$, $^{**}p < 0.01$.

M: $p = 0.004$, F: $p = 0.04$), but not for HVA (t-test, M: $p = 0.09$, F: $p = 0.1$).

Multivariate analysis (PCA, Fig. 2) was performed to investigate the possible relationship between the different DA variables and PFAS burden in Bank voles from the skiing area. Most of the PFAS showed positive associations with the DA concentrations, and GLM showed that there was a significant positive relationship between all the long-chain PFAS and DA (GLM, $p < 0.05$). This is in accordance with a previous study in adult male rats exposed to PFOS (Salgado et al., 2015). However, in developing Northern leopard frogs, there were lower DA levels in frogs exposed to PFOS and PFOA (Foguth et al., 2019).

It should be noted that we measured PFAS concentrations in Bank vole livers, and not the brain. Previous studies have shown that PFAS concentrations are usually higher in the liver compared to brain tissues of mammals, and that the accumulation of PFAS is tissue specific (Greaves et al., 2012; Greaves et al., 2013). However, in Polar bears PFAS accumulation in the brain was mostly comprised of long-chained PFAS (Greaves et al., 2013) and this finding is in accordance with the present study showing that the long chained PFAS are those associated with changes in dopaminergic endpoints. Long-chained PFAS were the dominant PFAS found in ski waxes, earth worms and soil samples from the skiing area where the Bank voles were sampled (Grønnestad et al., 2019).

Once released in the synaptic cleft, DA can bind to one of its 2 receptor families: dopamine receptor 1-like (Dr1) or dopamine receptor 2-like (Dr2) (Beaulieu and Gainetdinov, 2011). 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. Dr1, on the other hand, activates cyclic AMP-dependent protein kinase, stimulating the DA neuron (Jaber et al., 1996). Because of the higher DA concentrations in the Bank vole brains, lower concentrations of DA receptors were expected, in order to counteract the high DA levels and to maintain homeostatic balance. In the current study there were significantly higher *dr2* transcript levels in males from the skiing area, compared to the reference area (*t*-test, $p = 0.005$, Fig. 1). However, there was no significant difference in *dr2* in females, or for *dr1* in either sex (t-test, $p > 0.05$). The multivariate analysis showed a negative relationship between most PFAS and *dr1*

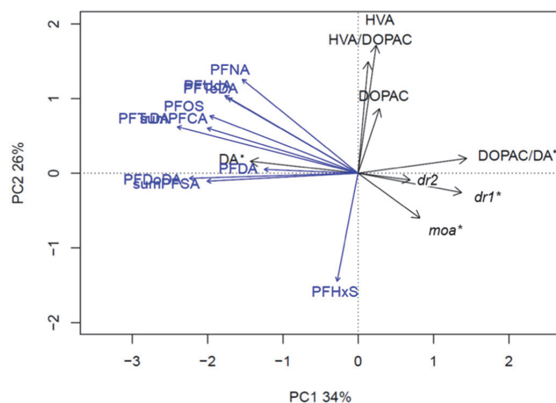

Fig. 2. Biplot of PFAS concentrations and dopamine variables (DA, DOPAC, HVA, DOPAC/DA, HVA/DOPAC, dr1, dr2, moa) in Bank voles from Granåsen skiing area ($n = 21$). Explanatory variables are projected as passive arrows (blue). The % of the total variance explained by each principal component (PCs) is given on each axis. Concentrations of all variables are log transformed and standardized to unit variance. Direction and length of arrows indicate respective strength and increasing variance of loading. Asterisks indicate response variables with significant associations to one or more PFAS.

expression in Bank voles from the skiing area (GLM, ΣPFCA: $p < 0.001$, $R^2 = 0.44$, ΣPFSA: $p = 0.01$, $R^2 = 0.24$, Fig. 2), while no association was observed between *dr2* and PFAS (GLM, $p > 0.5$, Fig. 2). This indicates that the higher *dr2* transcript level observed in males from the skiing area, are most likely not caused by the PFAS. The negative relationship between PFAS and *dr1*, without a corresponding difference between the two study areas, could be explained by other factors not evaluated in our field study, such as other pollutants, human disturbance, predators etc. These un-evaluated factors may be affecting the transcript expression patterns at the reference area. Our results are in accordance with previous findings showing that *dr1* expression was reduced in brain regions of rats exposed to PFOS (Salgado et al., 2016).

A previous study in mice showed that PFAS could modulate the DA system by altering different variables such as synthesis, reuptake, metabolism of DA, transcript and/or protein expression of different receptors (Hallgren and Viberg, 2016). DA is derived from the amino acid tyrosine, which is converted to L-DOPA by the enzyme tyrosine hydroxylase (Th) and further metabolized to DA. Th is considered the rate-limiting step of DA synthesis (Daubner et al., 2011). The plasma membrane DA transporter (Dat) is essential for normal dopamine neurotransmission. Dat terminates the actions of dopamine by rapidly removing DA from the synapse. Inhibition of DA reuptake via Dat thus increases the extracellular and synaptic concentrations and DA lifespan, leading to prolonged stimulation of DA receptors (Shimada et al., 1991). In the current study, *th* and *dat* transcripts did not show consistent positive amplification in the Bank vole brain samples. Overall, only three of 21 samples from Granåsen and two of 22 samples from the reference area showed *th* positive amplification, while only four samples from Granåsen and two samples from the reference area showed *dat* positive amplification (see results in SI Table S3). Other studies have reported that PFOS exposure can disrupt *th* expression in mice, with respective increases and decreases of the neonate and adult mice (Hallgren and Viberg, 2016). However, the authors did not observe any effects on *dat* expression (ibid).

The increased concentrations of DA reported in Bank voles at the Granåsen ski area could lead to alterations in thermoregulation processes (Hasegawa et al., 2000), defense (Sweidan et al., 1991) and aggressive behavior (Ricci et al., 2009), as well as in reproductive pathways (Henderson et al., 2008). DA also plays significant roles in the modulation of fear and anxiety (de la Mora et al., 2010). Consequently, PFAS exposure could alter neurological functions related to these emotional states. In addition, DA is involved in cognitive function, behavioral activation against appetite or aversive events and attention, as well as flexibility responses to stimuli (Seamans and Robbins, 2010). For this reason, the possible PFAS-related changes on the *dr1* and associated signaling pathways might potentially produce neurological disfunctions that may affect individual fitness. Additional studies are needed to confirm these potential linkages.

3.2. Dopamine turnover

The ratios between DA and its metabolites are generally used as a measure of DA turnover (Salgado et al., 2015). The DOPAC/DA ratio is indicative of intra-neuronal metabolism, while HVA/DA provides information on inter-neuronal metabolism of DA in the brain. The DOPAC/DA ratios were significantly lower in Bank voles from the skiing area, compared to the reference area (*t*-test, M: $p = 0.003$, F: $p = 0.05$, Fig. 1). These results are in accordance with previous rodent studies, showing reduced ratios in PFOS exposed rats (Salgado et al., 2015). Thus, PFAS exposure could possibly lead to lower DA turnover. This possibility was supported by the multivariate analysis, showing that there was a negative relationship between DOPAC/DA and several of the long-chained PFAS (Fig. 2), and where GLM confirmed a significant or borderline significant negative relationship (GLM, PFDoDA: $p = 0.05$, $R^2 = 0.15$, PFTrDA: $p = 0.06$, $R^2 = 0.13$). The lower brain metabolism of DA could be caused by a reduction in Mao activity, and thus a build-up of DA in the presynaptic neuron. Mao is responsible for the

metabolism of DA and other amine-containing neurotransmitters (Rutledge and Jonason, 1967). We found a negative association between several PFAS and *mao* mRNA expression (GLM, PFTrDA: $p = 0.04$, $R^2 = 0.17$, PFOS: $p = 0.02$, $R^2 = 0.20$, Fig. 2). There are not many studies that have reported effects of PFAS on Mao activity. However, a study on Polar bears from Greenland reported a positive relationship between Mao activity and PFAS across brain regions (Pedersen et al., 2015). While this finding may contradict our data, it should be noted, that the ΣPFAS concentrations in the Polar bears were almost 100-fold higher than the measured concentration in the Bank voles from the skiing area. Consequently, effects may not only be species-specific, but also dose-dependent. Nevertheless, and regardless of the measured concentration differences, it is possible that Bank voles and Polar bears display possible differences in their sensitivity and mechanisms of action of PFAS on Mao. This uncertainty and possible species-specific differences should be further investigated in rodents and other mammals.

The HVA/DOPAC ratio is a measure of inter-neuronal metabolism in the DA-system. We observed that HVA/DOPAC ratios were lower in the skiing area, compared to the reference area, albeit not significant (*t*-test, M: $p = 0.6$, F: $p = 0.3$, Fig. 1), suggesting that PFAS do not affect the inter-neuronal metabolism of DA from DOPAC to HVA, through the enzymatic actions of Comt. We did not find any correlation between any of the PFAS concentrations in the Bank voles and HVA/DOPAC ratio (Fig. 2). The only exception was PFHxS – although, this relationship was not significant (GLM, $p = 0.08$). These results are in accordance with Salgado et al. (2016), that reported the absence of effects on the inter-neuronal metabolism of DA in PFOS-treated rats.

3.3. Steroid hormone homeostasis

Estrogens and androgens are involved in growth and function of reproductive organs, development of secondary sexual characteristics, and behavioral patterns in vertebrate species. Thus, the balance in various steroid metabolic pathways has been shown to be associated with reproductive health. Consequently, measurement of steroid hormones may help determining the physiological health status of organisms (Gaikwad, 2013). PFAS have been shown to affect several physiological systems, including the endocrine system (López-Doval et al., 2014; Salgado et al., 2015). In the present study, we detected a trend towards lower T concentrations in muscle tissue from male Bank voles in the skiing area, compared to the reference area (t-test $p = 0.06$, Fig. 3), while there were no differences in females (t-test, $p = 0.5$). Specific PFAS associations were observed with a weak negative relationship between T concentrations in muscle tissue and several PFCA (GLM, ΣPFCA = 0.04, $R^2 = 0.17$, Fig. 4). Negative associations between PFAS and T has also been reported in other studies where they found a reduction in T concentrations after PFAS exposure in rats (López-Doval et al., 2014; Zhao et al., 2014). Although some studies have shown that adult male rats exposed to PFOS showed decreased serum E2 levels (Salgado et al., 2015), there was no significant difference in E2 concentrations between the two study areas in either sex in the present study (t-test, M: $p = 0.2$, F: p = 0.6), nor significant correlation between the PFAS and E2 concentrations (GLM, $p > 0.05$). Our results indicate that PFAS could be affecting T synthesis, clearance or cellular distribution (such as to the muscle).

The synthesis of T may be regulated by DA through the HPG-axis (Henderson et al., 2008; Bertotto et al., 2018). DA can affect the HPG-axis by decreasing the release of GnRH from the hypothalamus, leading to reduced secretion of FSH and LH from the pituitary, and consequently to decreases in the production and release of T and E2 from the gonads (Henderson et al., 2008; Zohar et al., 2010). Thus, it is possible that PFAS altered the release of T in the Bank voles by increasing DA levels in the brain. However, we did not observe any associations between DA and T or E2 (GLM, T: $p = 0.9$, E2: $p = 0.8$) in the Bank voles, suggesting that the increase in DA levels in voles from Granåsen may not affect the synthesis and release of T and E2. Other neuromodulators of GnRH synthesis, such as noradrenaline, glutamate and γ-aminobutyric acid (Skorupskaite et al., 2014), which are potentially susceptible to PFAS exposure, but not evaluated in the present study, might alternatively be

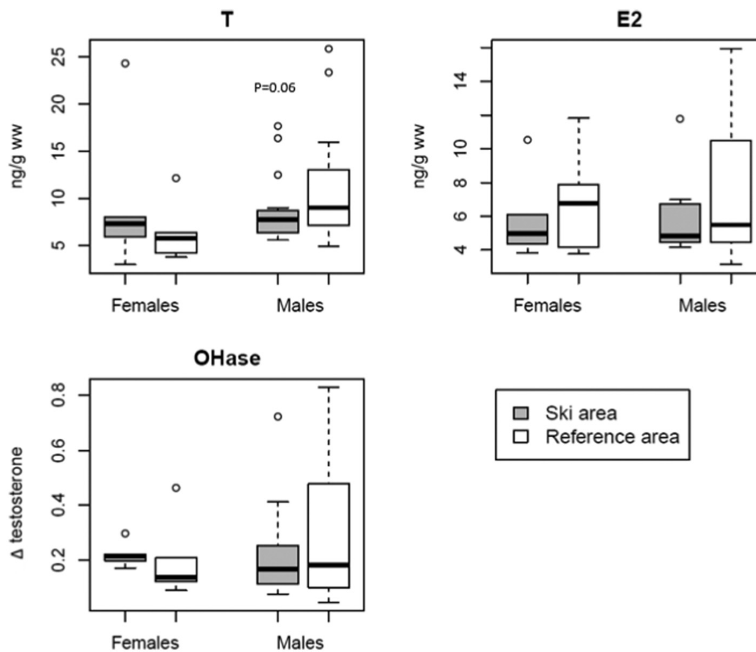

Fig. 3. Boxplots of variables related to the steroid hormone system in male and female Bank voles from Granåsen skiing area (females: n = 5, males: n = 16) and Jonsvatnet reference area (females: n = 6, males: n = 16).

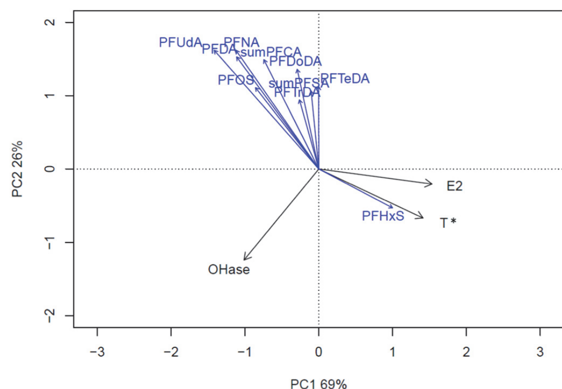

Fig. 4. Biplot of PFAS concentrations and steroid hormone variables (estrogen: E2, testosterone: T and liver steroid hydroxylase activity: OHase) in Bank voles from Granåsen skiing area (n = 21). Explanatory variables are projected as passive arrows (blue). The % of the total variance explained by each principal component (PCs) is given on each axis. Concentrations of all variables are log transformed and standardized to unit variance. Direction and length of arrows indicate respective strength and increasing variance of loading. Asterisks indicate response variables with significant associations to one or more PFAS.

involved in steroid hormone homeostasis through the GnRH signaling pathways (León et al., 2014).

The fact that we found a significant negative association between PFCAs and T (Fig. 4), but not between T and DA, suggests that PFAS could be affecting the synthesis and release of T through other mechanisms. Previously, it has been reported that exposure of male rats to PFOS disrupted the reproductive axis activity through a reduction of T production (Zhao et al., 2014). A study on healthy men also reported a negative association between the concentration of PFOS and T (Joensen et al., 2013). Furthermore, a study by López-Doval et al. (2014) found that oral exposure of male rats to PFOS produced a reduction in *gnrh* mRNA expression and in LH and T secretion. The authors concluded that PFOS exposure disrupted the male reproductive axis at different levels, including the hypothalamus, by decreasing *gnrh* expression and by modifying GnRH release, in the pituitary gland, by inhibiting LH secretion and stimulating FSH release, and, in the testis, by inhibiting T release. A reduction in T concentrations in male Bank voles could eventually affect the reproduction, and thus individual fitness of the Bank voles.

Kang et al. (2016) reported that in vitro exposure to PFOA and PFOS induced and reduced E2 and T levels, respectively, through hepatic CYP-enzyme mediated pathways. They suggested that PFOA and PFOS induced endocrine disruption by affecting the process of steroidogenesis. In the present study, we did not observe significant differences in testosterone OHase activity in Bank vole livers from the skiing area, compared to the reference area (*t*-test, M: $p = 0.9$, F: $p = 0.09$). We did not find any significant association between liver PFAS concentrations and OHase activity (Fig. 4). In addition, no correlation between muscle steroid hormone levels and OHase activity was observed. Steroid hormones serve as endogenous substrates for cytochrome P450 enzymes belonging to the CYP3A subfamily in vertebrate liver microsomes where the major site of hydroxylation is at the 6β-, 16α- and 17α positions and the capacity to hydroxylate steroids is often sex specific (Waxman et al., 1988; Zimniak and Waxman, 1993). In this study, the particular position of testosterone hydroxylation was not determined and as a consequence, our analytical protocol might have omitted the direct effects of PFASs on steroid hydroxylation. Further, it is also possible that PFASs did not directly affect hepatic metabolism of steroids, but rather the synthesis and release of T from the gonads.

4. Conclusion

In the current study, we have addressed the potential effects of PFAS exposure from ski products on the DA and steroid hormone homeostasis in Bank voles inhabiting the environments around a skiing area. We have shown that exposure within the concentration range documented at a Nordic skiing area contaminated by PFAS from ski products showed: a) increased total brain DA level and reduced DA turnover, b) a negative association with *dr1* and *mao* expression, and c) a negative association with cellular T levels in wild male Bank voles. Thus, DA and cellular steroid hormone homeostasis could potentially be altered by environmental PFAS exposure, which could lead to potential consequences on reproduction, general health and fitness of Bank voles from the skiing area.

CRediT authorship contribution statement

Randi Grønnestad: Conceptualization, Methodology, Formal analysis, Visualization, Writing - original draft. **Daniel Schlenk:** Conceptualization, Methodology, Writing - review & editing. **Åse Krøkje:** Conceptualization, Supervision, Writing - review & editing. **Veerle L.B. Jaspers:** Conceptualization, Supervision, Writing - review & editing. **Bjørn Munro Jenssen:** Conceptualization, Supervision, Writing - review & editing. **Scott Coffin:** Methodology, Writing - review & editing. **Luísa Becker Bertotto:** Methodology, Writing - review & editing. **Marissa Giroux:** Methodology, Writing - review & editing. **Jan L. Lyche:** Methodology, Writing - review & editing. **Augustine Arukwe:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

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.scitotenv.2020.143745.

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SUPPLEMENTARY INFORMATION

Alteration of neuro-dopamine and steroid hormone homeostasis in wild Bank voles in relation to tissue concentrations of PFASs at a Nordic skiing area

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Table S2: Primer sequences pairs
Table S3: mRNA transcripts for Dat and Gnrh genes

METHODS

PFAS analysis

PFAS concentrations were analysed at the Environmental Toxicology Laboratory, Norwegian University of Life Sciences (NMBU), Oslo, Norway. The analytical procedure were described in Grønnestad et al. (Grønnestad et al. 2016). The samples were analysed for the following PFASs: ten PFCAs: perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA) and perfluorotetradecanoic acid (PFTeDA), three PFSAs: perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS) and PFOS, and five perfluoroalkane sulfonamide derivatives (FASAs): perfluoro-1-octane sulfonamide (FOSA), *N*-Methyl perfluoro-1-octane sulfonamide (N-MeFOSA), *N*-Ethyl perfluoro-1-octane sulfonamide (N-EtFOSA), *2-(N*-Methyl perfluoro-1-octane sulfonamido) ethanol (N-MeFOSE) and *2-(N*-Ethyl perfluoro-1-octane sulfonamido) ethanol (N-EtFOSE).

The final extracts were analysed by separation on a high-performance liquid chromatography (HPLC) with a Discovery C18 column (15 cm x 2.1 mm x, 5 µm, Supelco, Sigma-Aldrich, Oslo, Norway), connected to a pre-column; Supelguard Discovery C18 column (2 cm x 2.1 mm x, 5 µm, Supelco, Sigma-Aldrich, Oslo, Norway). Detection and quantification were accomplished with a tandem mass spectrometry (MS-MS) system (API 3000, LC/MS/MS System). The injected volume was 5 µL.

External standards were used to produce a standard curve from which the PFAS levels were calculated, using the instrument control and data processing program Mass Hunter Quantitative analysis Version B.05.02 (Agilent Technologies). The limits of detection (LODs) were calculated as 3*SD of the procedural blanks (see SI Grønnestad et al. (2019)), and the limits of quantification

(LOQs) were calculated as 10 * LOD. Where no blanks were detected, LOQs were determined as 10 * signal-to-noise ratio (S/N). For the soil and earthworm samples, individual LOQs were determined for each sample, because of matrix effects.

Contaminants with concentrations above LOQ in more than 50% of samples were included in the statistical analyses, and missing values (i.e. < LOQ) were assigned a random value between the LOQ and zero.

Dopamine analysis

Brain concentrations of DA and its metabolites were measured using sample preparation based on Tareke et al. (2007) and Bertotto et al. (2018) with slight modifications. Samples were kept on ice during handling and extraction. The samples were homogenized using a probe blender prior to extraction. Approximately 200 mg of homogenized brain tissue (one brain half) was placed into a 2 mL centrifuge tube, and internal standards were added to yield 1 ng DA-d4 and 2 ng of HVA-d5 per 1mg of tissue. The samples were extracted twice using ice-cold 0.1% formic acid in water (added to an end volume of 0.3 mL), and the extraction was performed using a probe blender (Omni plastic homogenizer probe, Omni International) by vigorous blending for 1 min. The tubes were centrifuged for 5 min at 4000 rpm and 4°C. The extracts were then subjected to solid-phase extraction (SPE) with Strata X polymeric reverse-phase cartridges (33mm, 60 mg, 3 mL; Phenomenex), as described in Tareke et al. (2007). The SPE cartridges were conditioned with 1mL of 0.1% formic acid in acetonitrile, followed by 1 mL of 0.1% formic acid in methanol, and 1mL of 0.1% formic acid in water. The extracts were then added, and the analytes were eluted with 3 mL of 0.1% formic acid in acetonitrile/methanol (1:1, v/v). The resulting extracts were evaporated to dryness using Turbovap (Zymark), with nitrogen gas, and reconstituted in 0.4 mL of 0.1% formic acid in water. After vortexing for 10 seconds, the extracts were filtered and transferred to

autosampler vial inserts for liquid chromatography–tandem mass spectrometry (LC–MS/ MS) analysis.

A Shimadzu Nexera X2 LC system and QTrap 6500 MS (Sciex) were used. The LC column was XBridgeTM C18, 2.5 µm, 2.1 x 50mm connected to a guard cartridge (Waters). The LC gradient program ran from 90% A (0.1% formic acid in water), 10% B (0.1% formic acid in acetonitrile/methanol, 1:1) to 80% A over the course of 3 min, and was then ramped back to 90% A over 1min, and held at 90% A for 1min of equilibration, resulting in a 5-min run time. The injection volume was 20 mL. Dopamine was detected in positive electrospray ionization mode ESI (+), whereas DOPAC and HVA were detected in negative mode ESI (–). The LC gradient allowed sufficient separation of dopamine detected in ESI (+) from DOPAC and HVA detected in ESI (–) with polarity switching at 2 min. In positive mode, the ion spray potential was 5500 V, and in negative mode, the ion spray potential was –4500 V, with the source temperature at 300°C.

Deuterated dopamine (DA-d4) was used as an internal standard for dopamine while deuterated HVA (HVA-d5) was used as internal standard for HVA and DOPAC. Recoveries varied from 8-94% for Da-d4 and 21-66% for HVA-d5. The samples were therefore adjusted for recoveries for each individual sample.

Steroid hormone analysis

Approximately 200 mg muscle tissue was added to 3x volume of lysis buffer with 10% PMSF (to inhibit AChE). This was incubated on ice for 30 min followed by homogenization and centrifuged for 20 min at 15000g and 4°C. The supernatant was used for steroid extraction.

Steroid hormones were extracted using dichloromethane (DCM). DCM was added to a volume of 4x sample volume and vortexed. This was left on the bench for approximately 15 min to separate layers, and the organic phase was transferred to new tubes. The procedure was repeated

two times and the combined extract was evaporated to dryness under nitrogen in a water bath (30°C) and reconstituted with 210 µL of EIA buffer.

The extracts of muscle tissue were used for the measurement of E2 and T using enzyme immunoassay (EIA) kit from Cayman Chemicals. All assay solutions and buffer were prepared according to kit instructions with deionized water. Absorbance readings were performed on a spectrophotometer (Spectra Max Plus 384, Molecular Devices) at 412 nm. Samples were analyzed in duplicates (max 22% variance), and concentrations were calculated by comparing absorbance of samples with a standard curve, using the analysis tool provided by the kit's manufacturer. Samples were analyzed in duplicate.

Steroid hydroxylase (OHase) activity assay

0.4 mg of microsomal protein was incubated with 4 µM testosterone and buffer (50 mM Tris–HCl, 10 mM MgCl2, pH 7.4) to a final volume of 240 µL. Duplicates were made of each sample for parallel analysis of with and without NADPH. For example, 10 µL of 300 µM NADPH was added to one vial, and 10 µL buffer to the other vial. This was vortexed and incubated for 60 min at 30°C. The reaction was stopped by adding 250 µL acetonitrile (CH_3CN) and centrifuged for 10 min at 10000 g. The final concentration of solvent in all samples was 50% ACN, 50% H_2O. The extracts were filtered (0.5 micron, PTFE) and transferred to glass vials and run on an LC-2030 (Shimadzu, Kyoto, Japan) with a UV detector (D2 lamp operated in positive polarity mode). Optimal UV wavelengths for analytes were determined using a 2D scan with a spectrophotomer (Spectra Max Plus 384, Molecular Devices). Following optimization, 255nm was used for testosterone and methyl-testosterone, and 230nm was used for estradiol. The column was C18, 5µm, 4.6 mm x 150 mm (Beckman, USA), with the oven set to 40˚C. The LC was programmed to run at a low pressure gradient of 2.0 mL/min, with a gradient from 50%H_2O/50%ACN to 5%H_2O/95%ACN starting at

1 minute, and ending at 8 minutes , following by 2 minutes of wash time at 95% ACN, and 2 minute to re-equilibrate to starting conditions. Testosterone eluted at 4.6 minutes, 17-alpha-methyl-testosterone eluted at 5.3 minutes, and estradiol eluted at 7.8 minutes. Blanks (50% H_2O/50% ACN) were analyzed after calibration curves, and between every 4 samples. For each analysis, an 11-point calibration curve (0.25 to 1,000 ng/mL) was run prior to analysis of samples. All calibration curves had linear regression R^2 values>0.999.

The OHase activity was measured as the change in testosterone concentration, after addition of NADPH, compared to no NADPH.

Table S1. Deuterated dopamine (DA-d4) was used as an internal standard for dopamine while deuterated HVA (HVA-d5) was used as internal standard for HVA and DOPAC. The table shows recovery in percentage (%) of Da-d4 and HVA-d5 in individual samples from Granåsen and Jonsvatnet.

Recovery (%)					
Jonsvatnet			**Granåsen**		
Sample ID	**DA-d4**	**HVA-d5**	**Sample ID**	**DA-d4**	**HVA-d5**
C1	18	31	1	36	33
C2	35	29	2	27	27
C4	25	22	3	27	35
C5	33	24	4	39	48
C6	19	30	6	40	52
C7	23	35	7	20	50
C8	28	35	8	15	37
C9	29	52	10	25	56
C10	28	31	11	19	66
C11	24	36	12	11	21
C12	20	29	13	44	42
C13	43	36	15	30	43
C14	30	46	16	27	41
C15	32	49	17	16	47
C16	38	37	18	20	50
C17	94	35	19	38	44
C18	40	34	20	24	38
C19	37	28	21	26	61
C20	21	36	22	32	36
C21	28	35	23	32	52
C22	21	50	24	12	31

Table S2. Primer sequence pairs used for qPCR analysis

Gene	Forward	Reverse
dat (Slc6a3)	CACAGCTACCATGCCCTATG	GATCCACACAGATGCCTCAC
th	TGCAGCCCTACCAAGATCAA	ACATCAATGGCCAGGGTGTA
mao (Maoa-201)	TTGACTGCCAAGATCCACTTTA	ATGCAGCCACAATAGTCCTT
dr1 (Drd1-202)	GACTCTGCCCTACAACGAATAA	CAGCATGAGGGATCAGGTAAA
dr2 (Drd2)	ATCTCTTGCCCACTGCTCTT	GAACGAGACGATGGAGGAG

Table S3. Transcripts of individual Bank vole samples for dopamine active transporter (*dat*) and tyrosine hydroxylase (*th*).

ID	Th	Dat
Granåsen 1	3,11E-08	2,95E-08
Granåsen 2	3,45E-08	6,13E-08
Granåsen 3	n.d.	1,9E-09
Granåsen 8	1,47E-08	1,08E-08
Control 5	3,47E-09	1,67E-09
Control 12	4,73E-09	n.d.
Control 20	n.d.	1,45E-09

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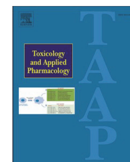

Effects of an environmentally relevant PFAS mixture on dopamine and steroid hormone levels in exposed mice

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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),

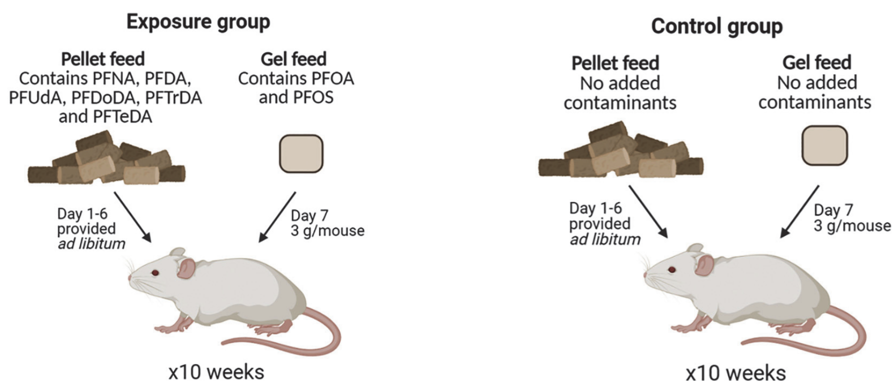

Fig. 1. Illustration of the exposure regime, showing the different types of feed (pellet and gel), when they were provided to the mice (from day 1 to 7 of each week), and the different types of PFAS they contain (only the exposure group). Illustration created in biorender.com.

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 $\times g$ 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 $\times g$ 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 ($\pm$ 2.1) g and 26.0 ($\pm$ 2.2) g for PFAS-exposed females ($n = 10$) and males ($n = 8$), respectively, and 21.5 ($\pm$ 1.8) g and 27.8 ($\pm$ 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

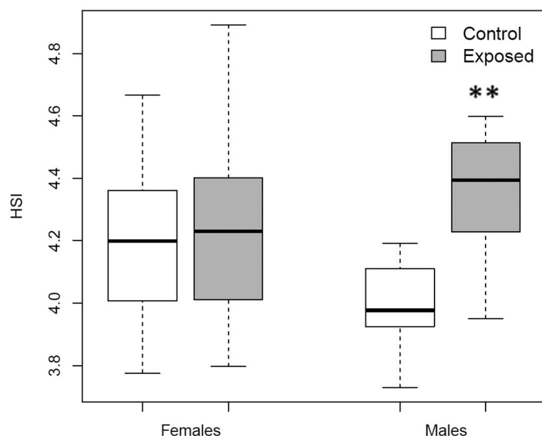

Fig. 2. Boxplot of hepatosomatic index (HSI) in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice.

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

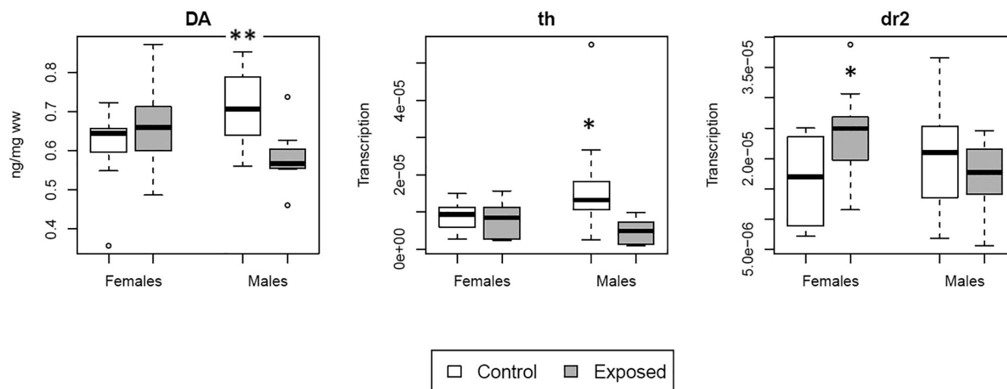

Fig. 3. Boxplot of variables related to the dopaminergic system in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice. Asterisks indicate significant difference between exposure groups (*p < 0.05, **p < 0.01).

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-amino-butyric 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

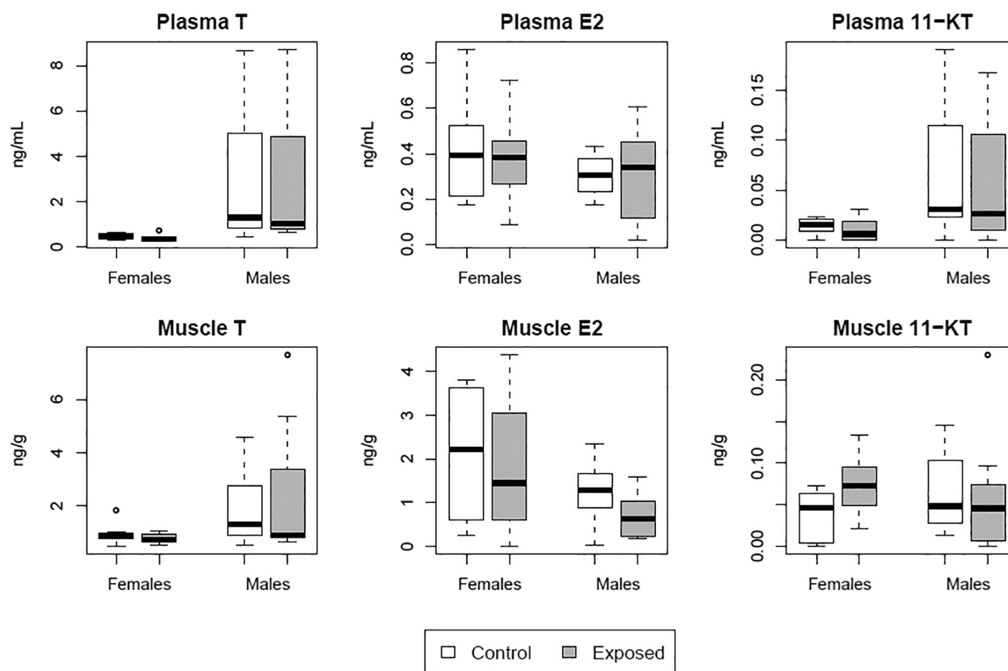

Fig. 4. Boxplot of sex steroid concentrations measured in plasma (ng/mL) and muscle tissue (ng/g) in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice.

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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SUPPLEMENTARY INFORMATION

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

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METHODS: Additional methodological information

Table S1. Internal standards of PFASs used for quantification. All standards were added to the concentration of 20 ng/mL, Wellington Laboratories, USA.

Internal standard	**Compounds**
Perfluoro-n-(1,2-$^{13}C_2$) undecanoic acid	PFUdA
Perfluoro-n-(1,2-$^{13}C_2$) dodecanoic acid	PFDoDA, PFTrDA and PFTeDA
Perfluoro-n-(1,2-$^{13}C_2$) decanoic acid	PFDA
Perfluoro-n-(1,2,3,4,5-$^{13}C_5$) nonanoic acid	PFNA
Perfluoro-n-(1,2,3,4-$^{13}C_4$)octanoic acid	PFOA
Sodium perfluoro-1-(1,2,3,4,-$^{13}C_4$) octanesulfonate	L-PFOS

Table S2. Limit of detection (LOD) and limit of quantification (LOQ) for the different PFAS analyzed.

PFAS	**LOD**	**LOQ**
PFOA	0.107	1.073
PFNA	0.013	0.045
PFDA	0.026	0.258
PFUdA	0.123	1.225
PFDoA	0.014	0.046
PFTrDA	0.007	0.070
PFTeDA	0.010	0.033
L-PFOS	0.052	0.173

Table S3. Primer pair sequences for the genes tested in the qPRC analyses.

Primers	Sequence	Accession#	Common name	Bp
slc6a3 (f)	GTGCTGATTGCCTTCTCCAG	AF109072.1	*dat*	163
slc6a3 (r)	CATCCCTGATGGGCACATTG	-		
comt-202 (f)	ATTGAGGCCAGAGATGCAGT	NM_001111062	comt	82
comt-202 (r)	TGTTCTTGGGTGCAGAGGAT	-		
th-202 (f)	TGCAGCCCTACCAAGATCAA	NM_009377	th	148
th-202 (r)	ACATCAATGGCCAGGGTGTA	-		
maob-201 (f)	AGGCCTGTGATCCACATTGA	NM_172778	maob	186
maob-201 (r)	AACTGAACCCAAAGGCACAC	-		
slc18a1-202 (f)	TGTTCCAGGTTGTTCTGGGT	AY779336	vmat	148
slc18a1-202 (r)	TACAGGAAGGTGGGCACAAT	-		
cyp19a1-201 (f)	GCCAGGATGTGTCTGAAACC	NM_001348173	cyp19a	92
cyp19a1-201 (r)	GCACACTTAAGGCTGGATGG	-		
gnrhr-201 (f)	ATCCGAGTGACCGTGACTTT	NM_010323	gnrhr	174
gnrhr-201 (r)	GACGATCAGAGTCTCCAGCA	-		
maoa-201 (f)	AGCCTACTTCCCTCCTGGTA	NM_173740	maoa	149
maoa-201 (r)	CTCTAGCTGCTCGTTCTCCA	-		
drd1 (f)	TACAGGATTGCCCAGAAGCA	NM_010076	dr1	110
drd1 (r)	GAGCATTCGACAGGGTTTCC	-		
drd2 (f)	ATCTCTTGCCCACTGCTCTT	NM_010077	dr2	99
drd2 (r)	GAACGAGACGATGGAGGAGT	-		
esr2 (f)	CCAGAACCTCCAGTCACAGT	NM_010157	esr2	89
esr2 (r)	CCCACGATGCTAGGGTACAT	-		
esr1 (f)	AGATGACTTGGAAGGCCGAA	NM_001302533	esr1	117
esr1 (r)	AAGGACAAGGCAGGGCTATT	-		

Table S4. Statistics from the mixed effect ANOVA models. Asterisks indicate significance levels (*p<0.05, **p<0.01, ***p<0.001). Model in R: y = x ~ exposure group*gender + (1|mother id)

	Two-way anova						one way anova			
	Exposure group		Gender		Exposure group:Gender		Males		Females	
x	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value
DA					0.0074**	8.26	0.0076**	10.3	0.32	1.04
th					0.0256*	5.51	0.049*	4.79	0.635	0.23
dat	0.976	0.00089	0.321	1.01						
vmat	0.289	1.16	1.51	0.228						
comt	0.690	0.162	0.362	0.857						
maoa	0.727	0.123	0.452	0.578						
maob	0.075	3.38	0.126	2.47						
dr1	0.741	0.111	0.866	0.0290						
dr2					0.05*	3.92	0.410	0.726	0.037*	5.31
T plasma	0.717	0.133	<.0001***	33.4						
E2 plasma	0.898	0.0166	0.0901	3.07						
11-KT plasma	0.804	0.0626	0.0032**	10.3						
T muscle	0.404	0.713	0.0506*	4.13						
E2 muscle	0.060	3.74	0.848	0.0371						
11-KT muscle	0.364	0.849	0.898	0.0164						
cyp19	0.325	0.999	0.402	0.720						
gnrh	0.326	0.996	0.563	0.342						

Table S5. Statistics from Pearson's correlation tests. Asterisks indicate significance levels (*p<0.05, **p<0.01, ***p<0.001).

	Variables	T muscle	E2 muscle	11-KT muscle	*cyp19*
Males	**T plasma**	r= 0.93, p<0.001***			
	E2 plasma		r=0.54, p=0.02*		
	11-KT plasma			r=0.043, p=0.86	
	esr1				r=0.4, p=0.09
	esr2				r=0.57, p=0.015*
Females	**T plasma**	r=0.24, p=0.3			
	E2 plasma		r=0.046, p=0.84		
	11-KT plasma			r=-0.1, p=0.67	
	esr1				r=0.25, p=0.28
	esr2				r=0.48, p=0.034*

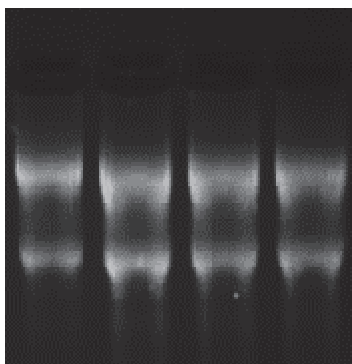

Figure S1. Formaldehyde agarose gel electrophoresis of RNA samples. One sample of each gender in each exposure group were tested.

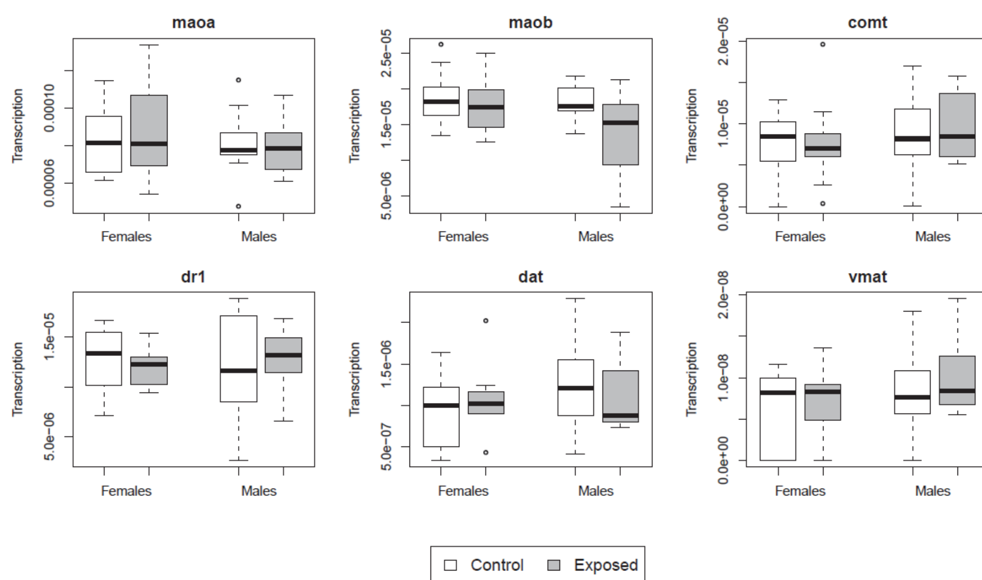

Figure S2. Boxplot of gene transcription related to the DA-system in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice.

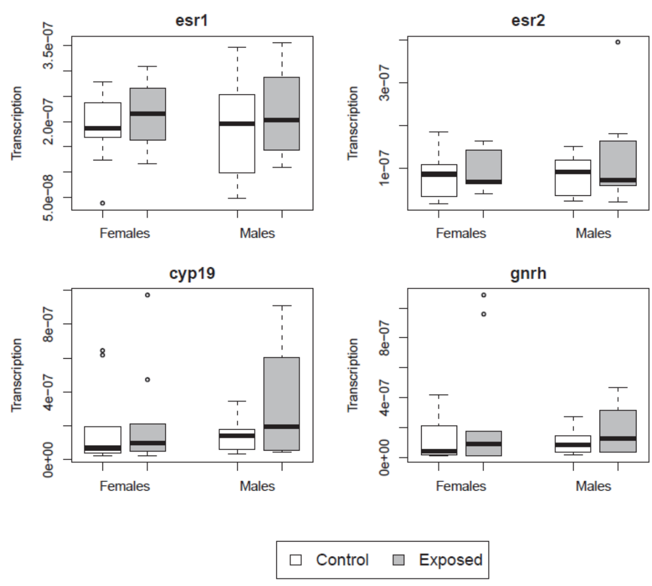

Figure S3. Boxplot of gene transcription related to the sex-steroid system in control (n = 10 females, 10 males) and exposed (n = 10 females, 8 males) A/J mice.

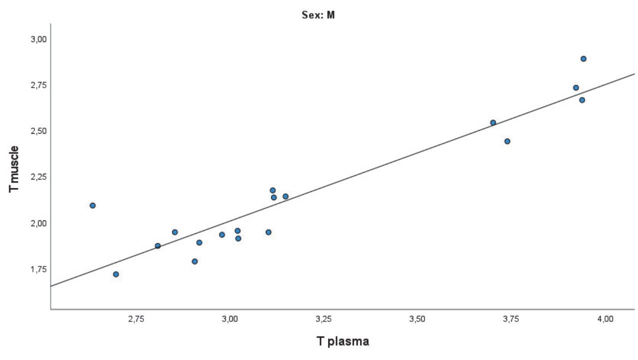

Figure S4. Scatterplot of the significant correlation between T levels in plasma and muscle tissue of male mice

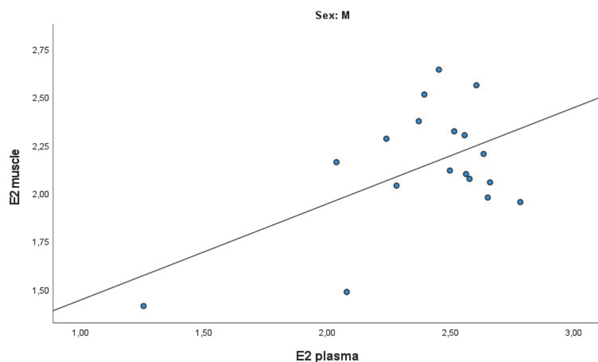

Figure S5. Scatterplot of the significant correlation between E2 levels in plasma and muscle tissue of male mice

METHODS – Additional information

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 M/F) and 18 mice (8/10 M/F) within the two groups, respectively. Litters were separated by gender and, thus, each litter was divided into two cages. These cages were filled up to 6 mice per cage with animals from other litters. The cages thus contained animals from mostly 2, but one time 3, litters. AIN-93G control or exposed pellet diets were provided *ad libitum*. The control and exposed gel diets were given to the mice once per week (3 g/mouse) during the entire 10-week experimental period (see overview of exposure regime in Figure S1 in SI).

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%).

Five male mice (assigned to the exposed group) died 1-2 days after waning. Since they died before they were able to eat solid food, this event is likely related to adaptational challenges postweaning, rather than the PFAS exposure. We therefore included one more litter of 3 males, giving a total of 18 males (8 PFAS exposed) used for the further analyses.

Quality assurance for qPCR analysis

RNA quantity and quality were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA gel. The NanoDrop showed that the samples had high quantity of RNA, and good purity: 260/280 ranged from 1.95 to 2.06, 260/230 ranged from 1.95 to 2.24. The RNA gel (Figure S2) showed two clear bands (28S and 18S ribosomal subunits).

Instrumental parameters for the dopamine analysis

The dopamine (DA) analysis was performed on a Waters ACQUITY UPLC™ system coupled with Waters Micromass triple quadrupole (TQD) mass spectrometer equipped with an electrospray ionization source (Milford, MA, USA). An ACQUITY UPLC HSS T3 Column (2.1 mm × 100 mm × 1.8 μm particle size) was used for the separation of the analytes. Mobile phase A was 0.1% formic acid in water/methanol (95/5, v/v) and mobile phase B was 0.1% formic acid in acetonitrile/methanol (1/1, v/v). The gradient program was as follows: 0-0.5 min, 95% A; 0.5-1 min, 95-40% A; 1–2 min, 40 to 10% A; 2–3.5 min, 10–0% A; 3.5–4 min, 0–10% A; 4–4.5 min, 10–95% A. The flow rate was 0.3 mL/min, and injection volume was 5 μL. The optimized mass spectrometry (MS) parameters were as follows: source temperature 150 °C, desolvation temperature 500 °C, capillary voltage 2.5 kV, cone voltage 14 V. The collision gas (Argon, 99.999%) flow in the collision cell was kept at 0.25 mL/min. Quantitative analysis was performed in multiple reaction monitoring (MRM) mode.