Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2022:9

Environmental persistent organic pollutants (POPs) – their interactions with behavior and development of colorectal cancer

Persistente organiske miljøgifter – deres påvirkning på adferd og utvikling av tykktarmskreft

Kristine Eraker Aasland Hansen

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1 Abbreviations

ACF	Aberrant crypt foci
ACTH	Adrenocorticotropic hormone
ADHD	Attention-deficit/hyperactivity disorder
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
APC	*Adenomatous polyposis coli*
AR	Androgen receptor
BM	Barnes maze test
BPG axis	Brain-pituitary-gonad axis
CRC	Colorectal cancer
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
EDI	Estimated daily intake
EFSA	European Food Safety Authority
ER	Estrogen receptor
FAP	Familial adenomatous polyposis
GR	Glucocorticoid receptor
HPA axis	The hypothalamic-pituitary-adrenal axis
IARC	International Agency for Research on Cancer
Min	Multiple intestinal neoplasia
MR	Mineralocorticoid receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NOAEL	No observed adverse effect level
NOEC	No observed effect concentration
OCP	Organochlorine pesticide
OECD	Organization for Economic Co-operation and Development
OF	Open field test
POP	Persistent organic pollutant
PXR	Pregnane X receptor
ROS	Reactive oxygen species

SXR	Steroid and xenobiotic receptor
UNEP	United Nations Environment Program
WHO	World Health Organization

Chemicals:

α-HCH	α-Hexachlorocyclohexane
AOM	Azoxymethane
β-HCH	β-Hexachlorocyclohexane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
γ-HCH	γ-Hexachlorocyclohexane
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HE	Hematoxylin eosin
PAS	Periodic acid-Schiff
PBB	Polybrominated biphenyls
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PFAS	Perfluoroalkyl substance
PFC	Perfluorinated chemical
PFDA	Perfluorodecanoic acid
PFHxS	Per- and polyfluoroalkyl substance
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PFUnDA	Perfluoroundecanoic acid

2 List of papers

Paper I

Hansen, K. E. A., A. M. Hudecová, F. Haugen, E. Skjerve, E. Ropstad, K. E. Zimmer.

Comparing two different strains of mice (C57BL/6J and the hybrid B6129SF1/J) using behavioral testing – A small scale study –

Submitted manuscript April 2022 to Laboratory Animal Research.

Paper II

Hudecova, A. M., K. E. A. Hansen, S. Mandal, H.F. Berntsen, A. Khezri, T. L. Bale, T W. F. Fraser, K. E. Zimmer, E. Ropstad.

Human exposure-based mixture of persistent organic pollutants affects the stress response in female mice and their offspring.

Published in Chemosphere 197 (2018) 585-593.

DOI: 10.1016/j.chemosphere.2018.01.085

Paper III

Myhre, O., K. E. Zimmer, A. M. Hudecova, K. E. A. Hansen, A. Khezri, H. F. Berntsen, V. Berg, J. L. Lyche, N. Duale, T. L. Bale, S. Mandal, E. Ropstad.

Maternal exposure to a human-based mixture of persistent organic pollutants (POPs) affects gene expression related to brain function in mice offspring hippocampus.

Published in Chemosphere 276 (2021) 130123.

DOI: 10.1016/j.chemosphere.2021.130123

Paper IV

Hansen, K. E. A., S. M. Johanson, C. Steppeler, M. Sødring, G. C. Østby, H. F. Berntsen, K. E. Zimmer, M. Aleksandersen, J. E. Paulsen, E. Ropstad.

A mixture of Persistent Organic Pollutants (POPs) and Azoxymethane (AOM) show potential synergistic effects on intestinal tumorigenesis in the A/J Min/+ mouse model.

Published in Chemosphere 214 (2019) 534-542.

DOI: 10.1016/j.chemosphere.2018.09.126

3 Abstract

Persistent organic pollutants (POPs) are a class of chemicals formerly widely used by the production industry and as pesticides in agriculture. Some of them are suspected endocrine disruptors and may interfere with hormone homeostasis, while others are carcinogenic.

In order to study the effect of a mixture of these chemicals on cognition, stress responses, and the development of cancer, a Scandinavian diet-based mixture of 29 POPs was designed to add to an experimental diet. Before the exposure studies, a small-scale study was done, to assess the performance of two different mouse strains in the different behavior tests going to be used in the behavior project. Then, two exposure studies were conducted. Firstly, a behavior project, where we investigated if early-life exposure to POPs affected anxiety, using an open field (OF) test, stress responses, using a restraint stress test, as well as spatial learning and memory, using a Barnes maze (BM) test and by analyzing hippocampal gene expression. The aim of the second project was to see if POP exposure affects the development of intestinal cancer, by exposing a different strain of mice, prone to intestinal cancer, to the same experimental diet and analyzing their intestines for cancer lesions.

The results from the behavior tests show that exposure to POPs had no effect on anxiety, but the restraint stress test indicated dysregulation of the hypothalamic-pituitary-adrenal axis and corticosterone secretion by exposure to the highest dose of the POP mixture. Minor group differences in BM performance were probably due to differences in stress susceptibility, rather than a true effect on learning. However, changes in the expression of genes connected to learning and memory were observed. These changes could interfere with behavioral outcomes, either directly or as compensation for POP-induced learning deficits. In addition, the results from the cancer project show that exposure to the mixture of POPs moderately increased the intestinal tumorigenesis in a mouse model of spontaneous intestinal neoplasia.

In conclusion, exposure to this human-relevant mixture of POPs did alter the stress response in mice and changed the expression of genes connected to learning and memory. In addition, exposure to the mixture of POPs increased the risk of colorectal cancer in mice.

4 Norsk sammendrag

Tungt nedbrytbare, organiske miljøgifter (persistent organic pollutants) er en gruppe kjemikalier som tidligere var i utstrakt bruk i industri og som plantevernmidler i landbruket. Noen av dem er mistenkt for å ha hormonforstyrrende effekter som kan endre kroppens hormonbalanse, mens noen også har vist seg å være kreftfremkallende.

For å undersøke effekten av en blanding av disse kjemikaliene på kognisjon, kroppens respons på stress og utvikling av kreft, laget vi en mikstur med 29 miljøgifter, basert på kjente nivåer i den skandinaviske dietten. Før eksponeringsstudiene ble gjennomført, gjorde vi et småskalaforsøk, uten miljøgifteksponering, for å sammenlikne hvordan to ulike muse-typer gjorde det i de forskjellige testene i adferds-forsøket. Dermed ble to eksponeringsstudier gjennomført. Først, et adferds-forsøk hvor vi undersøkte om miljøgifteksponering tidlig i livet påvirket musenes fryktreaksjon, med en open field (OF) test, stressrespons, med en fengslings-stress test, læring og hukommelse, med en Barnes maze (BM) test og genuttrykket i hippocampus, et hjerneavsnitt viktig for læring, ble også målt. I det andre eksponeringsforsøket undersøkte vi om miljøgifter påvirket utviklingen av tarmkreft hos en muse-type som er utsatt for å få tarmkreft, ved å eksponere dem for den samme miksturen og analyserte tarmene deres for kreft-lesjoner.

Resultatene viste at eksponering for denne miksturen av miljøgifter har ingen effekt på angst, men fengslings-stresstesten viste at eksponering for høy dose av POP miksturen førte til dysregulering av HPA-aksen og kortikosteron-sekresjonen. Den lille effekten på læring og hukommelse som ble observert i BM, var trolig et resultat av forskjeller i stressfølsomhet mellom gruppene, heller enn en direkte kognitiv effekt. Videre viste genekspresjonsanalysen at uttrykket av enkelte gener som kan knyttes til læring og hukommelse var endret, noe som kan påvirke atferd direkte eller kompensere for POP-induserte læringsdefekter. I tillegg viste resultatet fra kreftforsøket at eksponering for denne miksturen av miljøgifter kan bidra til en moderat utvikling av tarmkreft.

Konklusjonen er derfor at eksponering for denne blandingen av miljøgifter påvirker stressresponsen i mus og genekspresjon av gener som kan knyttes til læring og hukommelse. I tillegg kan eksponering for den samme blandingen øke risikoen for tarmkreft hos mus.

5 Introduction

5.1 POPs

Persistent organic pollutants (POPs) are chemicals that give great concern for public health globally and are therefore the focus of this thesis. In the introduction to my thesis, I review the history of POPs, their chemistry, how and to what extent humans are exposed to them, and what kind of health threat they pose.

5.1.1 The history of POPs

The history of POPs started as far back as the 1830s when the first chlorinated hydrocarbons were synthesized. Polychlorinated biphenyls (PCBs) were first made in 1881 by Schmidt and Schultz and commercial production started in 1929. The first modern synthetic insecticide dichloro-diphenyl-trichloroethane (DDT) was first synthesized in 1874 by Zeilder and first used in the field in the 1940s (Kodavanti et al. 2008). In the 1960s, the negative effects of POPs on humans and wildlife became a public concern when the book "Silent Spring" was published, conveying the danger of the use of pesticides (Carson 1962). In the 1970s several countries started environmental surveys and monitoring programs, looking for these chemicals in water, sediments, fish, and wildlife, as this was the time when analytical techniques were sufficiently developed to permit the detection of environmental concentrations of these compounds (Wu et al. 2008). The book "Our stolen future", which was published some decades later, pointed at the problems of the endocrine-disrupting effects of these environmental chemicals (Colborn et al. 1997). In 1995 the United Nations Environmental Program (UNEP) started an international working group (UNEP 2015) assessing the effects of 12 POPs, and in 2001 over 100 countries signed the Stockholm Convention banning these 12 POPs (StockholmConvention 2001). Today it contains 21 groups of chemicals and has been put into force in over 180 countries.

During the 2000s, POPs were still making headlines, when the public realized the severity of their persistency, as more research was published, more chemicals were defined as POPs and they were still found in the food chain decades after they were banned. Some publications that contributed to this increased public awareness include the already mentioned book "Our stolen future" from 1997 (Colborn 1997), articles about PCBs detected in polar bears in Nature (Willerroider 2003), and "The dirty dozen" in The Guardian (Doyle 2004).

In 2018, the Lancet Commission published a paper on world pollution and health, where they estimated that chemicals and heavy metals are responsible for 500,000 deaths globally each year (Landrigan et al. 2018). POPs have also been known to accelerate deadly diseases like diabetes, obesity, cancer, and cardiovascular disease (Alharbi et al. 2018).

In Europe today, the use of chemicals is regulated by REACH (the Registration, Evaluation, Authorisation, and Restriction of Chemicals) (REACH 2007). This means that all chemicals must be tested in accordance with guidelines approved by the Organization for Economic Co-operation and Development (OECD) (OECD 1961). For non-genotoxic chemicals, No Observed Adverse Effect Level (NOAEL) is determined by exposure experiments, but for genotoxic chemicals it is a little more complex as also low doses of the chemical could be genotoxic, there would not be a "safe" dose. They must also be tested for their mutagenicity. Therefore, risk assessment of genotoxic chemicals is complex and resource-intensive (ILO 2011, OECD 2015).

5.1.2 POPs - the chemicals

This chapter explains the chemical structure of POPs, physiochemical properties, and usage.

POPs are a class of chemicals earlier widely used by the production industry and as pesticides in agriculture. They are persistent because they are made up of long chains of carbon atoms attached by strong double bonding (C=C) and carbon-halogen bonds (chlorinated, brominated, or fluorinated), which increase the polarity of the structure. For example, PCBs have carbon-chlorine bonding (C-Cl) (figure 1).

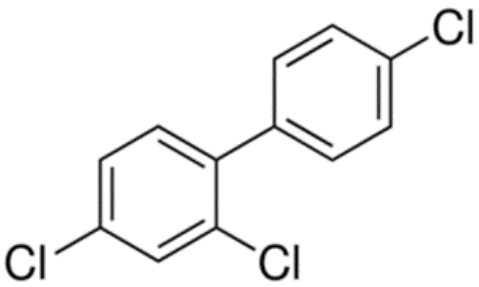


Figure 1: Structural formula of PCB 28 with double carbon bonds and carbon-chlorine bonds (source: SigmaAldrich.com).

This chemical structure makes POPs resistant to degradation and therefore accumulate when released into nature. From water and sediment, they are ingested by plankton, crustaceans, and fish. They are fat-soluble and bioaccumulate in the fatty tissue of these animals. Further, they biomagnify in predatory animals (like carnivores) and humans, when they eat the prey animals containing these chemicals. As POPs are semi-volatile, they can be transported over vast distances in ocean currents, winds, and migratory species. They typically evaporate in warm areas, are transported in the atmosphere and condense, and fall to the ground when entering colder areas. This geochemical process happens again and again, a so-called "grasshopper effect", and is the reason why these chemicals are found at high levels in polar areas and polar animals (Corsolini et al. 2019, Routti et al. 2019).

Because of the omnipresence of POPs, all humans (and animals) are exposed via food, water, and dust every day. Exposure to POPs has been linked to several adverse health effects (Colborn et al. 1993). Some POPs are endocrine disruptors and interfere with hormone homeostasis (Mnif et al. 2011), interrupting the development of, for example, the reproductive system (Gore et al. 2015) and stress responses (Monclus et al. 2018). Some POPs are carcinogenic and are believed to be promoters of cancer by changing gene transcription (Ludewig 2013). Although the POPs in the Stockholm Convention have been banned in many countries, new similar chemicals are produced every day in large quantities, for example, brominated flame retardants in electronic devices like TVs and computers. This might not only set the consumers of these articles in the industrial counties at risk, but also e-waste recyclers in developing countries are exposed to for example polybrominated diphenyl ethers (PBDEs) at considerable levels (Wang et al.

2020). Penta- and octa-BDE were taken off the market in the USA in 2004, but deca-BDE was still being used until it was started to be phased out in 2007. After 2010, companies in the EU and several states in the USA stopped producing Polybrominated biphenyls (PBBs) and the EU started to phase out all polybrominated biphenyls (PBBs) and PBDEs in 2006. Companies are looking to develop safer flame retardants and flame-resistant plastic.

Also, perfluorinated chemicals (PFCs) in water-resistant clothing and fat-repellent food packaging have been used extensively. For example, one study from Korea found small levels of 16 PFCs, including perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS), in frying pans and non-stick baking papers (Choi et al. 2018). Many of these chemicals are found in low concentrations in food, accepted by the authorities, but their health effects are largely unknown. PFOA has now been included in the Stockholm Convention and was banned in Europe by the end of 2020. In Norway, it was banned in July 2020 (NEA 2020) and European Food Safety Authority (EFSA) has set a new safety threshold for perfluoroalkyl substances (PFAS) consumption at 4.4 ng/kg body weight per week in September 2020 (EFSA 2020).

The most well-known POPs belong to one out of three different groups: Chlorinated, brominated, and fluorinated compounds:

Chlorinated compounds

Chlorinated compounds contain organohalogen bonding with chlorine atoms. They include for example DDT (figure 2), dioxins, and PCBs.

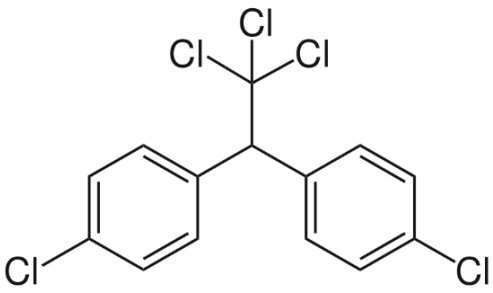


Figure 2: Structural formula of DDT (source: SigmaAldrich.com).

DDT is an agricultural insecticide, first synthesized in 1874 and more than 40,000 tons were used in agriculture annually before its ban (Geisz et al. 2008). It was banned in the USA in 1972 and the EU in 1978, but it has been used in developing countries, long after these bans, to control for example malaria (Mrema et al. 2013). It is tasteless and almost odorless and today, dietary exposure to DDT is most common. DDT and its metabolites, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), have been associated with endocrine-related diseases such as testicular tumors (McGlynn et al. 2008) and breast cancer (Wolff et al. 1993, Safe et al. 1997). Mechanisms of the action of POPs are still being investigated. Review articles summarizing the results of numerous mechanistic *in vitro* experiments conclude that DDT mimics 17β-estradiol and activates the estrogen receptor (ER), promotes the expression of estrogen-dependent genes, but also activates pregnane X receptor (PXR) and steroid and xenobiotic receptor (SXR), which are ER-independent pathways (Mrema et al. 2013, Yoon et al. 2014, Gore et al. 2015).

Dioxins are chemicals produced in incomplete combustion, for example when barbecuing food, burning of waste, coal, or wood, and from automobile emissions. There are 75 different dioxins, of which 7 are considered a concern. They are registered as endocrine disruptors and carcinogens, they bind to ER and aryl hydrocarbon receptor (AhR) (Gore et al. 2015),

PCBs were widely used as dielectric and coolant fluid, for example in transformers, capacitors, and electric motors. They are odorless, tasteless, and mostly colorless, and were mass-produced globally until they were banned. In Norway, they were banned as early as 1980, and in the USA in 1979. They were restricted in the EU in 1985 and banned in 1987. However, as they are persistent in the food chain, we are still exposed to them via food today. Some PCBs are classified as endocrine disruptors, bind to ER, and are associated with the development of breast cancer (Roswall et al. 2018). Some PCBs are called dioxin-like chemicals, causing AhR-mediated toxicity, and also leading to the development of cancer (NTP 2010).

Brominated compounds

Brominated compounds contain organohalogen bonding with bromine atoms (figure 3).

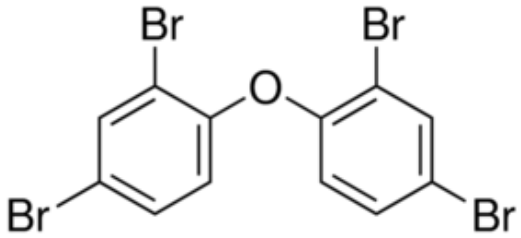


Figure 3: Structural formula of BDE 47 (source: SigmaAldrich.com).

The commercial production of PBDEs began in the late 1970s, just about the time that PCB production was banned. In 2001, approximately 33,000 tons of PBDEs were produced in North America. As I mentioned above, companies in the EU and several states in the USA stopped producing PBBs in 2010 and the EU started to phase out all PBBs and PBDEs in 2006.

Figure 4 shows that PBDEs are still being used today in several places in the world (Sharkey et al. 2020). Unfortunately, we are missing data from several parts of the world, however, we can see that countries that produce large amounts of POPs lack regulation and this will affect global pollution.

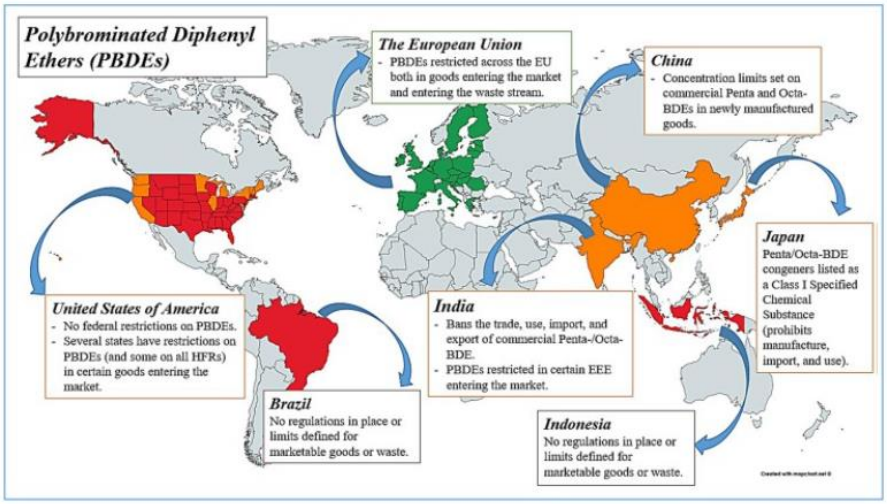


Figure 4: Map of the use of PBDEs all over the world (source: Sharkey et al.2020. Printed with permission: Open access Copyright © 1969, Elsevier)

Brominated flame retardants have an inhibitory effect on the ignition of combustible organic materials and are therefore used as flame retardants in electrical appliances and textiles used for furniture. As they are in our houses, we are exposed via the dust from indoor air and they can also be found in the food chain. A review, looking at human cohort studies, found that PBDE exposure during fetal development was associated with impairments in executive function and poorer attentional control in children and that prenatal and postnatal PBDE exposure adversely impacts behavior like for example hyperactivity and conduct problems (Vuong et al. 2018). Another review found associations between developmental PBDE exposure was linked to reduced IQ (Lam et al. 2017). In 2017, the National Academies of Sciences published a report that stated that there is an inverse association between PBDEs and IQ and an association between PBDEs and Attention Deficit/Hyperactivity Disorder (ADHD) (NAS 2017). Brominated flame retardants, for example, PBB153 have been shown to affect DNA methylation and alter the epigenome in human spermatogenic cells (Greeson et al. 2020).

Fluorinated compounds

Fluorinated compounds, or per- and polyfluorinated alkyl substances (PFASs), contain organohalogen bonding with fluorine atoms (figure 5). They have unique properties to make materials stain-, oil- and water-resistant. They are widely used in the production

industry, like for example, PFOA in non-stick kitchenware and PFOS in water-resistant textiles.

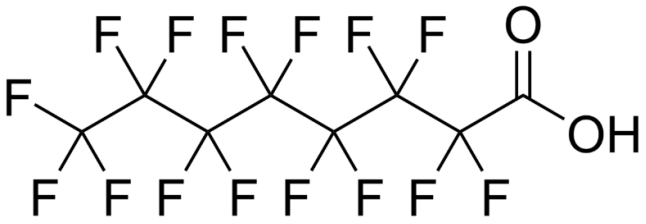


Figure 5: Structural formula of PFOA (source: Wikipedia).

PFOA (figure 5) and PFOS have been phased out in the EU and USA after 2010, but several new PFASs are still used in for example in food packaging (Seltenrich 2020). Therefore, this could be a double risk of exposure, both via the food chain and from the contact with this food packaging. A review paper looking at PFASs, found several studies showing significant associations between PFAS exposure and adverse immune outcomes in children, via suppression of antibody response to vaccination. The majority of the studies also found associations between PFAS exposure and elevated cholesterol (Sunderland et al. 2019). PFASs have also been shown to increase reactive oxygen species (ROS) and are suspected carcinogens (Wielsøe et al. 2015).

Global environmental contamination and human exposure to both chlorinated and fluorinated compounds have decreased in recent years, but as figure 6 shows, exposure to brominated compounds has surged.

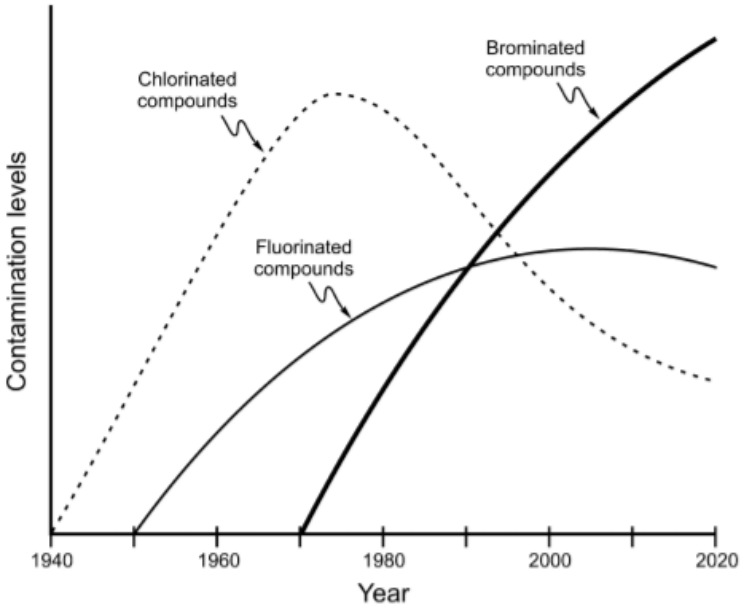


Figure 6: Schematic representation of global environmental contamination trends of organohalogen compounds. (source: Kodavanti et al. 2008. Copyright © 2017 Elsevier: 4954630798683)

5.1.3 Exposure to POPs

The main way of exposure is via our diet (Guo et al. 2019). Both chlorinated compounds (Pruvost-Couvreur et al. 2021) and fluorinated compounds (Estrella et al. 2018) are regularly found in food and fluorinated compounds are used in equipment used for preparing and keeping food. In addition to food, indoor dust is an important exposure source of brominated flame retardants (Wu et al. 2020) exposure to dioxins from the exhaust from vehicle motors (Li et al. 2016). This results in humans being exposed to a mixture of hundreds of chemicals every day. The focus of this thesis will be the mixture of POPs we are exposed to via our diet.

Health concerns

Exposure to POPs may increase the risk for a large range of health effects. POPs have been connected to among others adverse reproductive function (McCue et al. 2019), and development of cancer (Fernández-Navarro et al. 2017), diabetes, and obesity (Kahn et al. 2020). Many of these effects are suspected to be related to their endocrine disrupting

effects, interfering with reproduction (Rodprasert et al. 2021)and in the development of hormone-related cancer, such as breast cancer (Wan et al. 2021). European Food Safety Authority (EFSA) made a report on endocrine disruptors in 2015 and concluded that more research was needed (EFSA 2015). A lot of research has been done, however, they asked for more research again five years later (EFSA 2020). Several reviews have been conducted to try to get an overview of all the research, see table 1. The collection of these reviews was done over several searches, during the writing of this thesis. The collection is not a total list of all research done in this field, however, it gives an overview.

Table 1: Reviews of health effects of POPs. PubMed was used and several different search words were chosen (for example POPs, review, health, including both animal studies and epidemiological studies).

Health concerns	**Ref**
Child health and neurodevelopment	(Vrijheid et al. 2016)
Epigenetic effects	(Poston et al. 2019)
Obesity, diabetes, reproduction, cancer, thyroid, and neurodevelopment and neuroendocrine systems	(Gore et al. 2015)
Cancer, reproductive defects, neurobehavioral abnormalities, endocrine, and immunological toxicity	(Mrema et al. 2013)
Obesity, diabetes, reproduction, cancer, cognitive deficits, and attention-deficits	(Kahn et al. 2020)
Metabolic syndrome	(González 2022)
Neurodevelopmental disorders	(Simhadri et al. 2020)
Neurotoxicity	(Costa et al. 2008) (Pessah et al. 2019) (Klocke et al. 2020) (Latchney et al. 2021)
Behavior problems	(Vuong et al. 2018) (Lam et al. 2017)
Immune system	(Winans et al. 2011)
Immune responses	(Sunderland et al. 2019)
Reproductive system	(Yoon et al. 2014) (Rodprasert et al. 2021)
Reproduction and cancer	(Bonde et al. 2016)
Cancer	(Fenton et al. 2015) (Mouly et al. 2016) (Ennour-Idrissi et al. 2019) (Han et al. 2019)

Sensitive windows of exposure

Another concern is related to exposure at sensitive stages of life. Exposure early in life is the most damaging to an individual (Landrigan et al. 2003). There are windows of susceptibility when the organism is especially sensitive to negative impact (Verner et al. 2008).

There are several reasons why prenatals are more sensitive to exposure to chemicals. Firstly, the fetal development is in a crucial phase, when alterations in one single cell out of line will have large consequences for the structural development of an organ or a system in the body, because of for example a DNA mutation (Deardorff et al. 2012) or an epigenetic change affecting gene expression following exposure to a hormone (O'Neill 2015). Compared to adult life, when organs are fully developed and systems are mature, the same change in a single cell may not have such large effects on the body.

Secondly, the system for metabolizing and excreting chemicals is not fully matured in a fetus. Chemicals will therefore remain for a longer time and cause more harm than in an adult (Wright 2017). As POPs are lipophilic, they cross cell barriers, both the placenta (Eguchi et al. 2018) and the blood-brain barrier (Rasinger et al. 2014). This results in the fetus being exposed indirectly to what the mother is ingesting. Another problem with these lipophilic chemicals is that they are excreted in breastmilk (Tsygankov et al. 2020). This way the offspring will continue to be exposed to the POPs stored in their mother's fatty tissue, also after birth.

Some of the effects of prenatal exposure to endocrine-disrupting chemicals include problems with growth (Kadawathagedara et al. 2018), obesity (Darbre 2017, Vrijheid et al. 2020), reproductive dysfunction and infertility (Beszterda et al. 2018), and neurodevelopment (Vrijheid et al. 2016, Spratlen et al. 2020, Wang et al. 2021),

In 2016, Bonde *et al*. reviewed epidemiological reports on prenatal and early postnatal exposure to endocrine-disrupting chemicals and their effect on male reproductive disorders. They found only a small increased risk of male reproductive disorders later in life, but also mentioned interest in semen quality and testicular cancer (Bonde et al. 2016). Declining semen quality later in life has been reported in connection to exposure to maternal smoking and endocrine-disrupting chemicals have been discussed (Virtanen et al. 2017). A Swedish research group has found a connection between the rise of incidents of testicular cancer in men born during periods with high concentrations of

POPs compared to men born during a time where the levels of POPs were substantially lower (Hardell et al. 2006).

In humans, the development of the brain starts as early as the first trimester, and the development of bodily functions peaks in the middle of the second trimester, but continues throughout the first three years of life. Thus, during the perinatal period, the brain is particularly sensitive to environmental factors. For example, early-life stress can increase neuroactive steroids, which influence neurodevelopment and can negatively modulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Murgatroyd et al. 2011, Maccari et al. 2014, Brunton 2015).

It has been known for a long time that endocrine-disrupting chemicals has developmental effects in animals and humans (Colborn et al. 1993). Several reviews show studies that indicate that exposure to POPs during critical periods of the development is associated with severe health effects like the development of the immune system (Winans et al. 2011), the endocrine system (Gore et al. 2015), reproduction (Rodprasert et al. 2021), breast cancer (Fenton et al. 2015), neurodevelopmental disorders like autism and ADHD (Heyer et al. 2017), metabolic disorders and neurobehavioral diseases (Simhadri et al. 2020) and can act like developmental neurotoxicants (Pessah et al. 2019, Klocke et al. 2020, Latchney et al. 2021).

Concerns related to exposure to mixtures

Different chemical combinations can affect each other in different ways. These are either additional effects, synergistic effects, or antagonistic effects. Additional effect means that the combined effect of the chemicals is the sum of the effects of each chemical. A synergistic effect means that the combined effect of the chemicals is larger than the sum of the individual chemical effects. Antagonistic effect means that the combined effect of the chemicals is less effective than the sum of the effects of the individual chemicals (Landis et al. 2018). We would assume that POPs have the potential for any of these effects, even in low doses. A study has shown that estrogenic agents are able to act together to produce significant effects when combined at concentrations below their no observed effect concentration (NOEC) and that calculation of additive mixture effects was able to predict this fairly accurately, but

independent action and effect summation led to underestimations (Silva et al. 2002). Actual synergistic effects are difficult to study and also low doses may have a larger effect than first assumed (Kortenkamp 2008). Not many studies have been performed on mixtures of POPs. Some authors have used cell lines to study this phenomenon (Berntsen et al. 2021, Gogola-Mruk et al. 2021, Krawczyk et al. 2021).

How to interpret results in toxicology

When discussing effects in toxicology studies it is important to take into account the different possible dose-response curves (Ramaiah et al. 2016). A dose-response curve shows a line that represents the response/effect dependent on the dose of the chemical that the individual is given/exposed to. A common dose-response curve would be a linear curve (figure 7a) or a sigmoid curve (7e). However, in some cases, you could have a nonmonotonic curve, for example, a u-shaped curve (7f). In this example, some chemicals may give severe health effects in very low concentrations, for example by imitating hormones. At the same time, the same chemicals may also give severe health effects in very high concentrations, like for example acute toxic reactions. Medium concentrations, effects may for example be difficult to assess, and the effects may actually be less severe or not measurable.

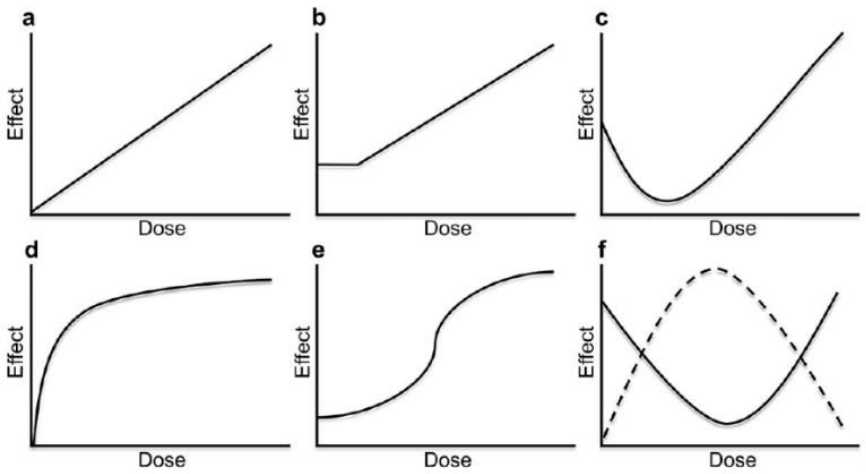


Figure 7: Illustration of common dose-response curves (source: Rimaiah et al. 2016, published 2017. Printed with permission: Copyright © 2017, © SAGE Publications)

Nonmonotonic curves can give problems when conducting a risk assessment for non-genotoxic chemicals. Figure 8 shows an example where the no observed adverse effect level (NOAEL) would be inaccurate in the case of a chemical with a u-shaped curve (Vandenberg et al. 2012). This means that if the NOAEL is determined with very few measurements, there is a risk that even lower concentrations, here defined as "safe doses", actually might cause severe health effects. Without conducting a large experiment, with many concentrations, starting from very low to very high, we cannot know the true dose-effect relationship for a mixture.

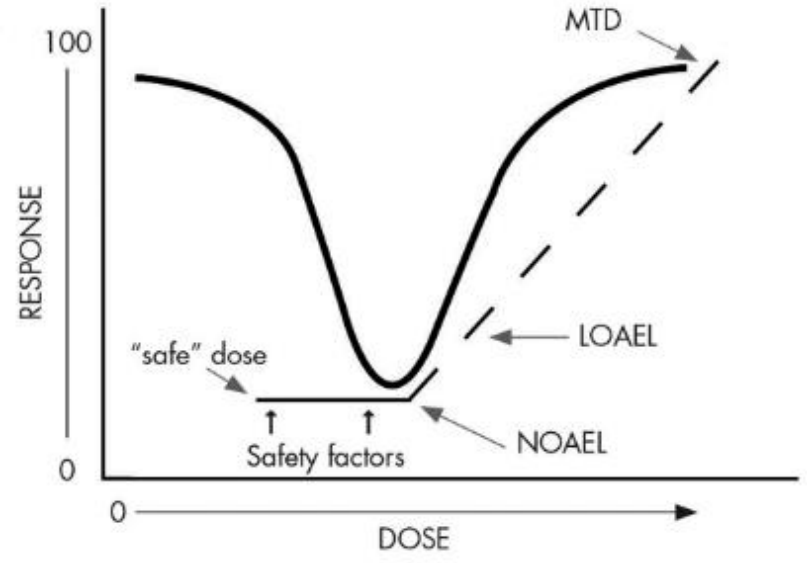


Figure 8: Example of how NOAEL would be inaccurate for a chemical with a u-shaped dose-response curve (source: Vandenberg et al. 2012. Printed with permission: Copyright © 2012, Oxford University Press. License Number 4952511355473).

5.2 Behavior and factors affecting behavior

Finding definitions of different behavioral terms is not always easy, as updated publications seldom define the basic terms. In addition, behaviors are often based on a human description and may be difficult to extrapolate to experimental animals. Behavior

is, defined as the way an animal or person acts in response to its environment (Dictionary 2020). POPs are potential neurotoxicants and could thereby affect behavior. In this chapter, I look further into factors affecting behavior, anxiety, learning, memory, stress, and the basic sciences connected to this. Further, I describe how POP exposure could affect behavior. The causes for different behaviors are many and complicated. Behavior can be affected by for example hormones (Maguire et al. 2016), genetics (Hu et al. 2022), learning and copying others (Evans et al. 2021), disease (Baandrup et al. 2021), intake of chemicals (Gakare et al. 2022), feelings like anxiety and depression (França et al. 2022) and many other components.

5.2.1 Anxiety

Spielberger (1972) defined anxiety as "an unpleasant emotional state of condition which is characterized by subjective feelings of tension, apprehension, and worry, and by activation of arousal of the autonomic nervous system" (Sarason et al. 1990). The current definition of anxiety, according to Oxford Medicine, says: "Anxiety refers to multiple mental and physiological phenomena, including a person's conscious state of worry over a future unwanted event, or fear of an actual situation." (OMO 2021). It is also suggested that anxiety is a reaction to other emotions, like fear or anger, and that biogenic amines, for example adrenaline, noradrenalin, octopamine, and dopamine, released under stressful situations are similar to the ones triggering anxiety (Belzung et al. 2007). This indicates a clear link between anxiety and stress. The fact that exposure to early life stress, activating the HPA axis, can lead to anxiety disorders, also shows such a link (Juruena et al. 2020). Another example is a review suggesting that perinatal exposure to bisphenol A, a different organic endocrine disruptor, dysregulates neurotransmitter systems sensitive to stress, disrupts the HPA axis, and this could exacerbate symptoms of anxiety or depression (Wiersielis et al. 2020).

5.2.2 Stress and the stress reaction

There are many different definitions of stress. The first definition of stress is from Hans Selye in 1936 and says "Stress is the nonspecific response of the body to any demand." (Fink 2010). Since then, many definitions have been suggested, some being physiological, others more psychological in nature. The UK Mental Health Foundation defines it as: "Stress is the feeling of being overwhelmed or unable to cope with mental or emotional pressure." (MHF 2021). Again, this human definition explaining the

feeling of stress is difficult to measure in the experimental animal. What we are able to measure in the animal is for example levels of stress hormones. Whether there is a need for a conscious perception to call it stress has been debated and so has the nonspecific response described by Selye, as different stressors may evoke different biochemical signatures (Fink 2010). Still, there are some basic, common physiological changes in the stress reaction. The stress reaction starts when the eyes or ears send information to the amygdala in the brain and it activates the hypothalamus that activates the "fight- or flight"-response in the sympathetic nervous system and the endocrine system, including the adrenal glands. The adrenal gland is made up of an inner core, the medulla, and an outer layer, the cortex (figure 9a).

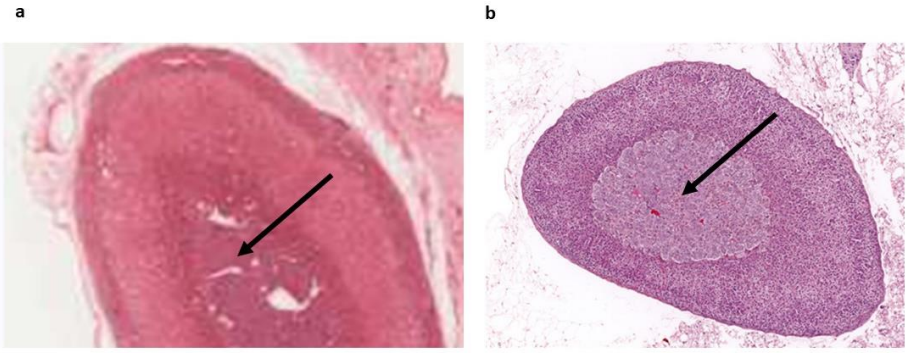


Figure 9: a. Section of a normal human adrenal gland (40X) (source: WebScope by University of Michigan). b. Section of a normal murine adrenal gland (source: Focusontoxpath.com). The arrows in both figures show the medulla.

The medulla produces catecholamines; adrenalin and noradrenalin. In stressful situations, these hormones will contract blood vessels to raise blood pressure and increase the heart output. Noradrenaline makes the brain more focused and vigilant. The cortex is divided into three zones: Zona glomerulosa, zona fasciculata and zona reticularis. Cells of zona fasciculata produce glucocorticoids (cortisol in humans and corticosterone in rodents), zona reticularis androgens and zona glomerulosa mineralocorticoids (the most important being aldosterone). Glucocorticoids have many functions, among them, increasing blood glucose during stressful situations. They also

play a role in the development of the fetus, for example, pulmonary surfactant, maturation of the lungs, and general growth of the fetus (Davies et al. 2021).

In mice, the adrenal gland cortex is composed of superficial zona glomerulosa and deep zona fasciculata (figure 9b). The zona reticularis is not recognizable in mice. At the junction of the cortex and medulla there is the so-called X zone, which represents a specific feature in the mouse adrenal gland. The function of the X zone is not well understood. In males, the zone disappears by the age of puberty, whereas in females it continues to increase in size until the age of about 9 weeks and then regresses rapidly during the first pregnancy. In virgin female mice, however, it regresses slowly and undergoes lipid vacuolization. Female adrenal glands are generally larger than those in males (Hans et al. 2004).

The hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is a complex set of interactions between the hypothalamus, the pituitary gland, and the adrenal glands (figure 10). It is a significant part of the system that controls our reaction to stress, trauma, and injury.

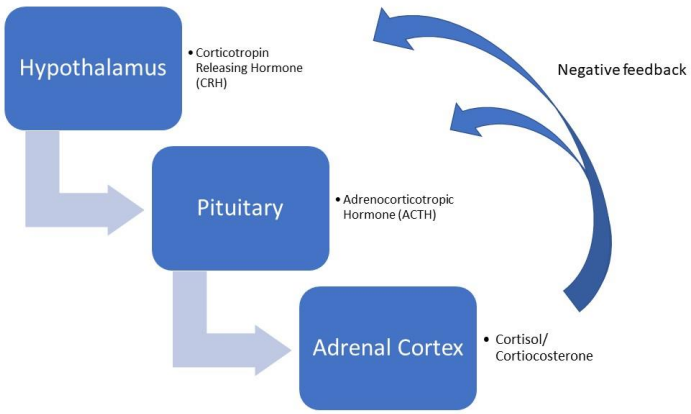


Figure 10: Schematic of the HPA axis (based on Brian M Sweis 2012).

As the stress response is activated, the corticotropin-releasing factor (CRF) / corticotropin-releasing hormone (CRH) is released from the hypothalamus, binds to CRH receptors (CRHr) in the pituitary gland, and adrenocorticotropic hormone (ACTH) secretion is stimulated. When ATCH binds to ATCH receptors (ATCHr) in the adrenal cortex, glucocorticoid synthesis, and secretion increase. Cortisol then attaches to glucocorticoid receptors (GR) in the cell nucleus which stimulates cells in the liver and muscle to increase the blood glucose. This is done by the breakdown of glycogen, gluconeogenesis, and the breakdown of fat in fat cells as an alternative energy source, whereas the glucose is saved for the brain (Liu et al. 2018). The GR is expressed in almost all cells in the body and in the brain. There are two types of receptors, connecting both to cortisol and corticosterone: Type I mineralocorticoid receptor (MR), and type II glucocorticoid receptor (GR) (Vielkind et al. 1990).

5.2.3 Learning and memory

The definition of learning, according to ten Cate *et al*. is "… when the learner acquires knowledge of a topic or subject matter through processing information…". They describe three different components that are critical to the learning process in humans: The cognitive component, which is the mental capacity to acquire knowledge, the affective component, meaning internal motivation for learning, and the metacognitive component, the ability to evaluate the learning progress (ten Cate et al. 2004). According to Baram *et al*., "Memory is a complex biological function incorporating multiple neuronal networks." They define two different categories of memory, declarative and procedural, and describe declarative memory as explicit, like facts and events, while procedural memory is implicit, as for example skills like driving a car or playing the piano (Baram et al. 2019). Memory reflects the ability to learn new information and therefore learning and memory are closely linked. When encountering a new problem, we use our memory to come up with the correct choice on how to solve it (Balkenius et al. 2020). Again, measuring if knowledge is acquired in an animal is impossible, so what you are left with is looking at behavior.

Development in humans can be influenced by environmental factors such as drug exposure during adolescence which affect learning and memory (Mooney-Leber et al. 2018) and exposure to bisphenol A effected learning and memory in rat offspring (Wu et al. 2020). If a person's brain has difficulties sending, receiving, or processing

information, this is called a learning disability, and can be caused by for example genetics, illness, stress, or exposure to toxins like lead (Lyon et al. 2003). Also, PCBs (Panesar et al. 2020) and several insecticides (Brown et al. 2018) have been identified as risk factors for Autism spectrum disorder, which often includes learning difficulties.

5.3 Behavior and POPs

In humans, neurobehavioral disorders are increasing (Bloom et al. 2010) and as this has taken place while environmental pollution has been increasing, there is a concern that there is an association between these two factors (Grandjean et al. 2014).

I started by collecting some of the epidemiological studies I found from around the world that looked at POPs and behavior. One study including 239 mother-child pairs in the USA found that PBDE maternal serum concentration was inversely associated with reading skills and Full Scale IQ (FSIQ) and positively associated with externalizing behavior problems. However, no significant associations were found in associations with PCBs (Zhang et al. 2017). The same was also found in the CHAMACOS cohort, in the USA, where prenatal DDE levels were associated with lower FSIQ and Processing Speed in girls (Gaspar et al. 2015). And a study of 140 toddlers in the CHECK cohort, in Korea, found that maternal blood PCB levels were associated with behavioral problems in their children (Kim et al. 2018). Another study of 161 children from a cohort in the USA showed inverse associations between maternal PBDE serum concentrations and children's reading skills, but they also observed positive associations between PCBs and PFAS and reading skills (Vuong et al. 2020). In a different cohort in the USA, researchers found associations between childhood PFAS concentrations and behavioral and executive function problems (Harris et al. 2021). In addition, a study of 181 mother-infant pairs showed that prenatal background exposure to PCBs affected mental development in the children in the Netherlands (Ruel et al. 2019).

Then, I looked for studies looking at the HPA axis. Many studies show that POPs may affect the secretion of cortisol/corticosterone in for example ruminants (Zimmer et al. 2009, Gutleb et al. 2011, Zimmer et al. 2013) and birds (Tartu et al. 2014). It is known that some of the POPs are adreno-toxic, for example in mice (Lahiri et al. 1991) and rats (Mohammed et al. 1985). Furthermore, some POPs have been shown to interrupt neuronal development in the fetal brain (Coburn et al. 2008, Westerink 2014). All this

indicates that POPs can affect the hormones, the endocrine organs, neurons, and the brain and there is, therefore, a chance that this can affect behavior.

5.4 Cancer

In this chapter I first focus on how cancer develops, the occurrence of colon cancer, the effects of POPs on cancer in general and on colon cancer specifically.

5.4.1 The development of cancer

Cancer occurs when cell growth becomes uncontrollable and abnormal. The process is divided into three stages: Initiation, promotion, and progression (Martínez-Reyes et al. 2021). Initiation can be caused by several factors, and chemicals inducing DNA mutations can be one of them. Promotion is characterized by proliferation, and avoidance of apoptosis, which is the controlled cell death of damaged or aging cells. This can be caused, for example, by decreasing the expression of tumor suppressor genes. In the third stage, progression, the cancer cells become invasive to other tissues, either the surrounding tissues or other organs, or by spreading throughout the body via the circulatory system, and form metastasis (Xiao et al. 2021).

5.4.2 Colorectal cancer

Cancer is the first or second leading cause of premature death (ages 30–69 years) in 134 out of 183 countries in the world, and it ranks third or fourth in an additional 45 countries in the 2020 report published by the International Agency for Research on Cancer (IARC) (IARC 2020). Colorectal cancer (CRC) is the third most common cancer in both sexes worldwide (1.8 million new cases in 2018) and Norway has one of the highest risks of CRC in Europe (IARC 2018).

The Cancer Registry of Norway's report from 2019 shows that colon cancer is the third most common cancer type for males (after prostate and lung/trachea) and the second most common for females (after breast and tied with lung/trachea) (figure 11). The incidence rate of colon cancer has increased steadily, but in the last decade the rate has leveled off for men, but it is still increasing among women (CRN 2019). Figure 11 is not corrected for age and does indicate that as we are living longer now than we did in the

1960s, we also develop more cancer. However, the increase of CRC in females is especially interesting.

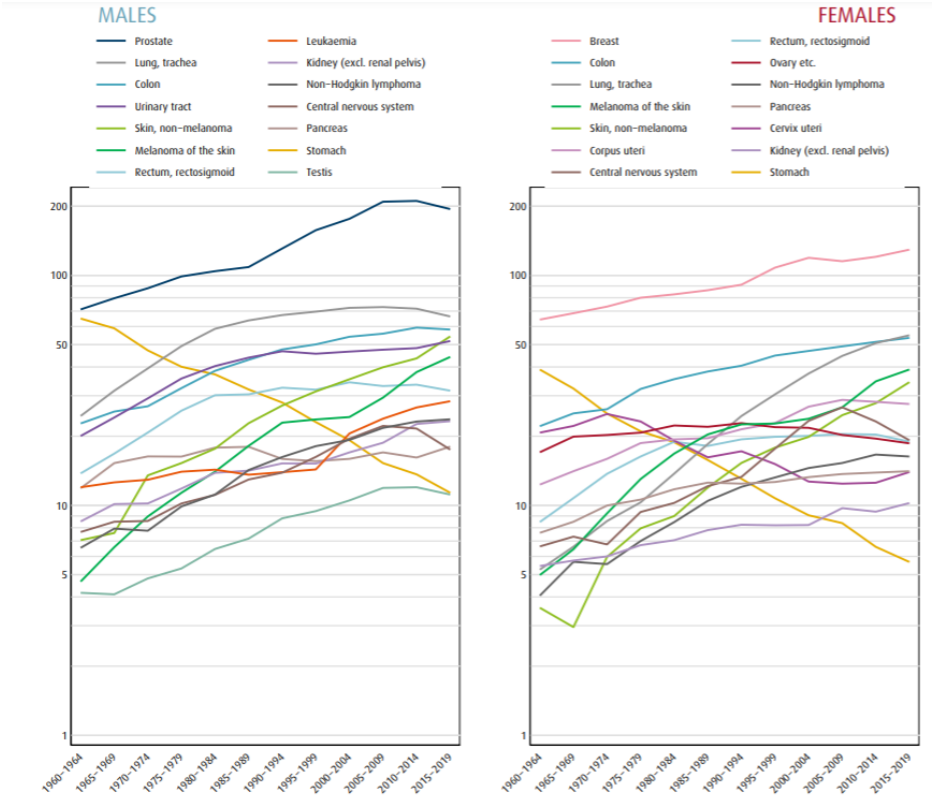


Figure 11: Incidence rates of cancer in Norway from 1960-2019, by gender (source: The Cancer Registry of Norway's Report 2019).

CRC is largely affected by environmental factors, like for example diet. This is called sporadic CRC (CUP 2017). CRC can also be hereditary, and one of the major hereditary forms of CRC is the familial adenomatous polyposis (FAP), which is caused by a germline mutation in the tumor-suppressor gene *adenomatous polyposis coli* (*APC*). Mutations in the *APC* gene are also common in sporadic CRC (Sødring et al. 2016). The development of CRC starts with benign polyps or adenomas from normal epithelium and progresses into malignant invasive and potentially metastatic adenocarcinomas (Sancho et al. 2004).

5.5 Cancer and POPs

POPs have been associated with several types of cancer. For example, chlordane and PCBs serum levels are associated with lung cancer (Park et al. 2020) and blood levels of DDT and HCB are associated with breast cancer (Charlier et al. 2003) in humans. Some POPs are reported to act as tumor promoters, as they can change gene transcription or act like endocrine disruptors. However, certain POPs, for example dioxins, may also induce DNA damage, and thereby initiate cancer development (Ludewig 2013). A study exposing rats to diesel exhaust, which includes dioxins, found DNA-adduct-formation in the rats' lungs and this indicates that POPs can also be involved in inducing pulmonary carcinogenesis (Bond et al. 1990).

It has been suggested that POPs are able to increase methylation, thereby repressing the expression of tumor suppressor genes (Wielsøe et al. 2020). Studies have also shown that PCBs promote cell proliferation via pyruvate kinase M2 (PKM2)-dependent upregulation of glycolysis, which induces reactive oxygen species (ROS) production (Liang et al. 2019, Zhang et al. 2019). PBDEs can induce oxidative stress by downregulating the nicotinamide adenine dinucleotide phosphate (NADPH) generation in the pentose phosphate pathway in the cells (Wei et al. 2018) and organochlorinated pesticides have demonstrated that they can act as agonists on estrogen receptor α (ERα) and/or ERβ and most likely androgenic receptors that may contribute to tumor promotion in hormone-dependent tumors. Pesticides can elicit responses through AhR-dependent and -independent pathways and trigger apoptosis by redox signaling which involves alterations in antioxidant defenses and accumulation of ROS leading to oxidative stress (Mrema et al. 2013).

5.5.1 Colorectal cancer and POPs

As POPs are found in our food chain, our intestines are exposed to these chemicals via the diet. POPs have been associated with the development of CRC (Howsam et al. 2004, Lee et al. 2018, Abolhassani et al. 2019). One of the suggested mechanisms is that POPs increase intracellular ROS in the intestines (Song et al. 2020). An epidemiological study, looking at 277 patients at Kyungpook National University Chilgok Hospital in Korea, showed that high serum concentrations of PCBs were significantly associated with a higher risk of polyp development, and organochlorine pesticides were linked to an increased risk of developing both polyps and CRC (Lee et al. 2018). And a case

cohort with 104 CRC cases and 234 subcohort participants within the Korean National Cancer Center Community showed that serum concentrations of POPs increased the CRC risk (Park et al. 2021).

5.6 Knowledge gaps

Humans are exposed to hundreds of chemicals every day; we are exposed during sensitive windows, and they accumulate in our bodies during our whole lifetime. In the literature review, I have identified research performed on POPs and several POPs have been researched extensively for effects on health and the environment. Since I started my PhD work, a lot of new literature has been published in the field. Still, there are unanswered questions that deserve attention in research.

The majority of studies still describe experiments using only single components or simple mixtures containing a limited number of congeners of one chemical group, like for example PCBs (Karkaba et al. 2017, Liberman et al. 2020). As I mentioned in the introduction, different chemical combinations can affect each other in different ways, and studies using human-relevant complex mixtures better resemble real-life exposure patterns. Some *in vitro* experiments using large mixtures of POPs have been conducted (Sakai et al. 2009, Hendriks et al. 2010, Bittner et al. 2011, Doan et al. 2019) and some have been performed in fish (Walker et al. 2009, Berg et al. 2016, Khezri et al. 2017), looking at effects on for example reproduction. Another study has been conducted on neonatal rats exposed by gavage to a POP mixture based on Canadian breast milk levels, looking at hepatic health (Desaulniers et al. 2005).

I have also identified some research on POPs and cancer and CRC, but again, few studies using large mixtures. As the most important exposure route is via diet and CRC is one of the most common types of cancer, this field of research is of particular significance.

Advanced statistics are being used to predict novel adverse outcome pathways that better reflect the cumulative risk of persisting and emerging pesticides (Cuevas et al. 2018). The World Health Organization (WHO) published guidelines to assess the risk of pesticides in food, where they also discuss how to take into account mixtures (WHO 1990), and the European Food Safety Authority (EFSA) published a guide where they

outly risk assessments for multiple chemicals for both humans and animals (EFSA 2019). The EU also put together a commission to assess the risk of combination effects of chemicals, and they published a report in 2012 (EUCommission 2012). This report also states that more knowledge is needed.

During my work with my PhD, I have identified some publications reporting the effects of mixtures, but the use of constructed, complex, human-relevant mixtures, still seems to be scarce. And although new research on the effects of POPs on stress responses, learning and development of CRC has been published during these years, still no final answer to our initial research questions has been established, this work will contribute to filling important knowledge gaps in this field.

6 Hypothesis, aims, and objectives.

Because of the widespread occurrence in the food chain, along with concerns related to health effects as a consequence of exposure, more research in the field of POPs is warranted. Some literature indicating a connection between exposure to POPs and cognitive dysfunction, raises new questions and hypotheses. So do the indications that POPs may act as carcinogens. I, therefore, have set the following hypothesis:

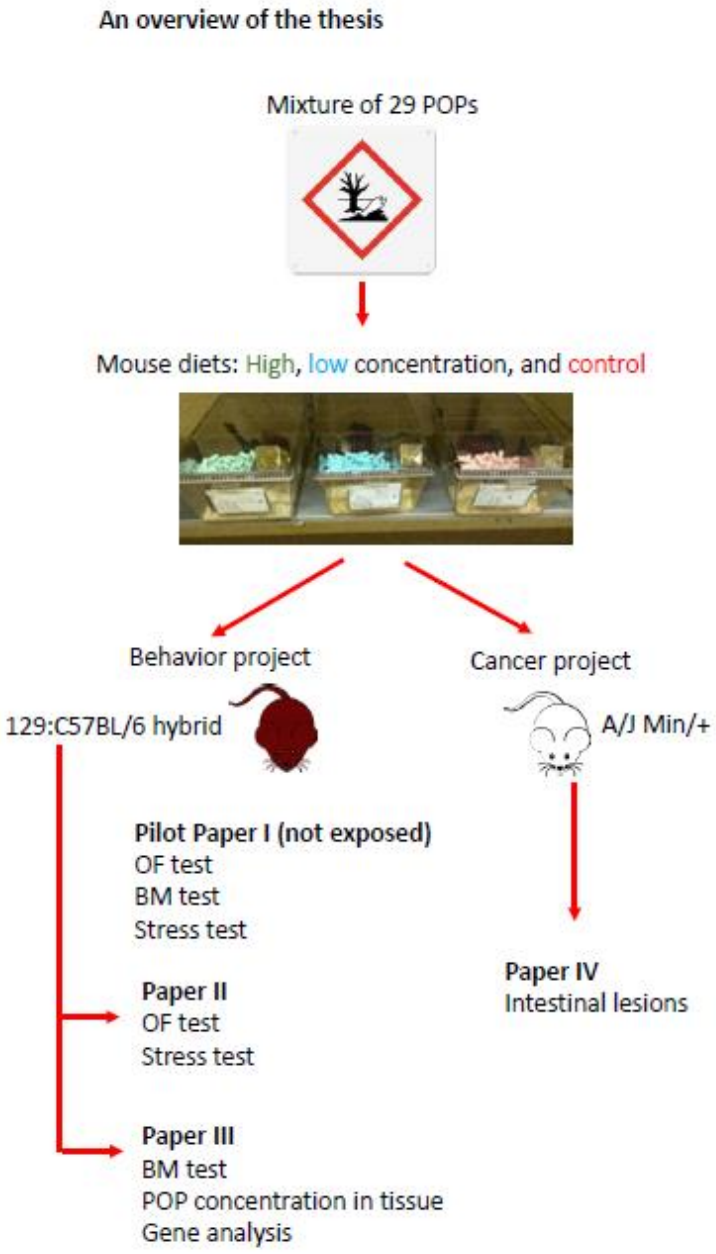
a) POPs influence learning and behavior.
b) POPs contribute to the development of cancer.

The overall aim of this PhD project was to test the hypotheses above and thereby contribute to an improved understanding of how exposure to POPs interferes with behavior and development of cancer in humans. More specifically, the main focus was on whether anxiety, learning, memory, stress responses, and the development of CRC can be affected through dietary and maternal exposure to a human-relevant POP mixture.

To obtain this main aim, the project was divided into the three following sub-parts, each with its own objective:

1. Describe differences in the two mouse strains C57BL/6J and the hybrid B6129SF1/J using behavior testing (OF, BM, and stress test) (Paper I).
2. Describe the effects of exposure to a human-based mixture of POPs on anxiety, learning, memory, and stress response in the 129:C57BL/6 hybrid mouse mothers and their offspring, using behavior testing (OF, BM, and a stress test) and analyzing their hippocampal gene expression (Paper II and III).
3. Describe the effects of the exposure of the human-based mixture of POPs on the intestinal tumorigenesis in the A/J Min/+ mouse (Paper IV).

7 An overview of the thesis



8 Materials and methods

8.1 The mixture of POPs

In order to investigate the aims of my thesis, a mixture of human-relevant POPs was designed and given by feed to mice in two animal experiments. The mixture contained 29 of the POPs most commonly found in food (Haug et al. 2010), human blood (Knutsen et al. 2008), and breastmilk (Polder et al. 2009) in Scandinavia. Dioxins were excluded. Most of the POPs in the mixture were already listed in the Stockholm Convention, except for some of the perfluorinated compounds. The detailed construction of the mixture is described in an earlier published article (Berntsen et al. 2017). The chemicals and the reasons they were chosen are listed in table 2.

Table 2: The chemicals used in the mixture of POPs and the reason for including them. Table made based on Bernsten's work in designing the mixture.

Chemical	Reason for inclusion in the mixture	Ref
Chlorinated compounds		
PCB 28 PCB 52 PCB 101 PCB 118 PCB 138 PCB 153 PCB 180	Found in high levels in human serum and have high estimated daily intakes (EDI) due to the relatively high amount of seafood and wild game in the Norwegian diet.	(Kvalem et al. 2009)
p,p'-DDE	Has a high calculated mean intake in Sweden.	(Tornkvist et al. 2011)
HCB	Has a high EDI in Denmark.	(Fromberg et al. 2011)
α-Chlordane Oxychlordane Trans-Nonachlor	Have high EDIs in Denmark.	(Fromberg et al. 2011)
α-HCH β-HCH γ-HCH	Have high EDIs in Denmark.	(Fromberg et al. 2011)
Dieldrin	Has a high EDI in Denmark. High levels in breast milk in Denmark and Finland.	(Fromberg et al. 2011) (Shen et al. 2008)
Brominated compounds		
PBDE 47 PBDE 99 PBDE 100 PBDE 153 PBDE 154 PBDE 209 HBCD	Have high EDIs in Norway. High levels in breast milk in Norway.	(Knutsen et al. 2008) (Polder et al. 2009)
Fluorinated compounds		
PFHxS PFOS PFOA PFNA PFDA PFUnDA	Have high EDIs in Norway.	(Haug et al. 2010)

Estimates of the daily intake per kilogram body weight were calculated for a 70 kg person and were converted into the daily intake for a mouse of 25 g body weight. The chemicals were dissolved in appropriate solvents and added to corn oil. After solvents were properly evaporated, the mixture, still dissolved in corn oil was blended into an experimental mouse diet which was used in the two different mouse projects. The feed concentration of the mixture was set to provide a mouse consuming 3g feed/day a daily dose of 5,000× and 100,000× the EDI for humans. The two diets were named the low and high concentration diets, respectively. For the control feed, the same corn oil as used for the exposure feeds was added the same solvents as the ones used to dissolve the POPs. After evaporation of these solvents, the corn oil was blended into the feed. For the reference feed, given to the offspring of the exposed mothers, only untreated corn oil was used to exchange the soy oil normally used in the relevant feed. The basis for the mouse diet was AIN-93G, a standard feed used for growth, pregnancy, and lactation, as these were the main life periods of interest in the behavior project, for which the diet was originally made.

8.2 Behavior project

8.2.1 Paper I

A small-scale study was conducted to compare the performance of two mouse strains in the behavior tests relevant to this thesis. 10 male C57BL/6J mice and 10 male hybrid B6129SF1/J mice (both from Jackson Laboratory, Maine, USA) were used. The two mouse strains were compared using three behavioral studies, an OF test, a BM test, and a stress test. The OF testing arena was a white plexiglass box 50 × 50 × 22 cm (Noldus, Wageningen, the Netherlands) with a bright light (Lupoled 1120; approx. 120 lux) placed above. The animals were filmed by an Ikegami ICD-49E B/W infrared camera fixed in the ceiling and tracking was done by the computer program Ethovision XT 9.0 (Noldus, Wageningen, the Netherlands). Each mouse was tracked for 15 minutes. The BM testing arena was a round platform, 100 cm in diameter, with 20 holes, one of which had a black goal box beneath (Noldus, Wageningen, the Netherlands). The animal was filmed by the camera and tracking was done by the computer program Ethovision (Noldus, Wageningen, the Netherlands). Each mouse was tracked until it reached the goal box or for 4 minutes. All animals were trained for 2 sessions every day for 3 days, and a new motivational stimulus was introduced each day:

Day 1: Session 1+2: Bright light over the platform.
Day 2: Session 3+4: Bright light + blowing fan.
Day 3: Session 5+6: Bright light + blowing fan + loud buzzer

During the stress test, the mouse was restrained inside a 50 mL falcon tube for 15 minutes. Blood samples were taken from the tip of the tail at 4 different time points: 0 (start), 15 (before release), 30, and 120 minutes (after release). The mouse was allowed to rest in its home cage between the 15-, 30- and 120-minute sample. Total corticosterone was measured in the plasma using an MP Biomedicals ImmuChem ™ Double Antibody Corticosterone 125 Ria Kit (MP Biomedicals, Santa Ana, CA, USA).

8.2.2 Paper II

For the main animal experiment in the behavior project, the 129:C57BL/6 hybrid was used. 10 male C57BL/6J mice and 20 female 129S1/SvImJ (Jackson Laboratory, Maine, USA) were used for breeding. In total, 110 hybrid pups were born (129:C57BL/6F0, n = 63 males and 47 females). Of these, the 47 females were assigned to one of three groups, the control, low, and high concentration diet groups, and given their respective diets from weaning and through their use as breeders of the F1 generation. F0 hybrid females were mated with F0 hybrid males. In total, the F1 generation consisted of 320 pups (129:C57BL/6F1, n = 163 males and 157 females). At weaning, 18 males and 18 females from each respective group (n = 36/group) were assigned to two different behavioral test groups: One group for the OF test and the stress test, and the other group was used for paper III. Both the mothers and the offspring were tested. The OF test and stress test were conducted as previously described and the results were published in paper II.

8.2.3 Paper III

The mothers and the siblings of the offspring described in paper II were tested in the BM test. The BM test was conducted as previously described for paper I, but the motivator buzzer was excluded and only the bright light and fan were used. After necropsy, the brain, blood, and adipose tissue were analyzed for POP concentrations. Detection of the organochlorine pesticides (OCPs) and PCBs was performed on a gas chromatograph (GC) coupled to Electron Capture Detector (ECD) and low-resolution

mass spectrometry (LRMS). Detection of BDEs and Hexabromocyclododecane (HBCD) was performed on an HRGC–LRMS. Perfluorinated compounds were detected by tandem mass spectrometry (MS-MS). Furthermore, quantitative real-time polymerase chain reaction (qPCR) was used to analyze the transcriptional responses in pooled samples of the hippocampus of the offspring. A panel of 142 genes was investigated. Samples from exposed animals were compared to the controls. From the results of the initial screening, genes with expression level differences of more than ± 2-fold or $p < 0.05$ between POP-mixture exposed and control mice were selected for single sample analysis. Based on these selection criteria, 20 genes were identified.

8.2.4 Unpublished material on adrenal glands

To further investigate the effects of POPs on stress responses, we wanted to see if the size of the exposed mice's adrenal glands was affected. Of special interest is the cortex, which produces corticosterone. Even though these results are not yet published, they add important information and are therefore included. The adrenal glands of both the mothers and their offspring were collected for histology after the behavior project. Histological sections from the paraffin-embedded adrenals were made in the middle part of the organ and stained with hematoxylin eosin (HE) and pictures were taken of 2-4 sections from the center of the organ. The diameter of the gland was measured in all the slides, using ImageJ 1.51g (NIH, USA), and the largest one was assumed to be closest to the middle of the organ and used to measure the area of the total gland and the medulla. The area of the cortex was then calculated.

8.3 Cancer project (Paper IV)

In the second animal experiment, 87 A/J Min/+ mice were used in two substudies. This strain of mice, named the multiple intestinal neoplasia (Min) mouse, is prone to develop CRC, as it has a heterozygous mutated *Apc* gene. Female A/J +/+ mice were mated with male A/J Min/+ mice and their A/J Min/+ offspring were exposed to the diet with the POP mixture from weaning to the age of 13 weeks of age. 66 mice were given the POP diet, randomly divided into the 3 exposure groups (control, low and high). In the other substudy, 21 mice were exposed to the mixture of POPs in the same way, but in addition, these mice were also given one subcutaneous injection of 8.5 mg/kg

azoxymethane (AOM, Sigma-Aldrich, St. Louis, MO, USA) during the second week after birth. At the end of the project, all mice were euthanized and necropsied and the small intestines and colons were collected, fixed, and stained with 0.2% methylene blue dissolved in formalin. The liver was collected and weighed, and all tumors that were found were collected. For surface microscopy and transillumination of the intestines, an inverted light microscope (CKX41, Olympus Inc., Hamburg, Germany) with a digital color camera (DP25, Olympus) was used. In the colon, lesions were identified as either flat aberrant crypt foci (flat ACF;<30 crypts) or tumors (>30 crypts covering more than approximately 0.4 mm2) (figure 12).

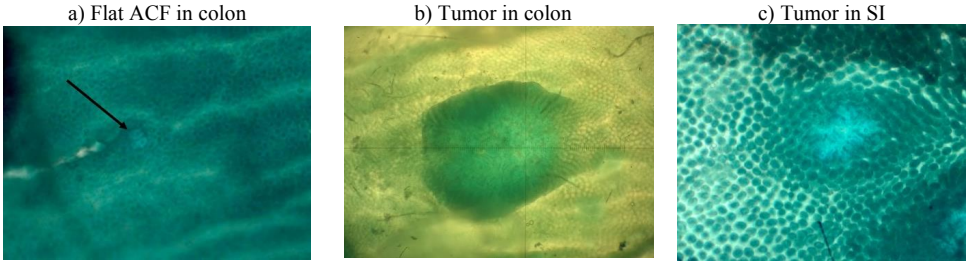


Figure 12: Examples of a) flat ACFs in the colon, b) a tumor in the colon and c) a tumor in the small intestine (SI) of the A/J Min/+ mice exposed to POPs (source: Hansen).

After scoring, the intestines were made into Swiss rolls and embedded in paraffin. 3 mm thick histological sections were cut and stained with hematoxylin eosin (HE) and periodic acid-Schiff (PAS). The examination was conducted in a microscope and lesions were identified, counted, and classified as preneoplastic lesions (hyperplastic and dysplastic cells), adenomas, or carcinomas (figure 13).

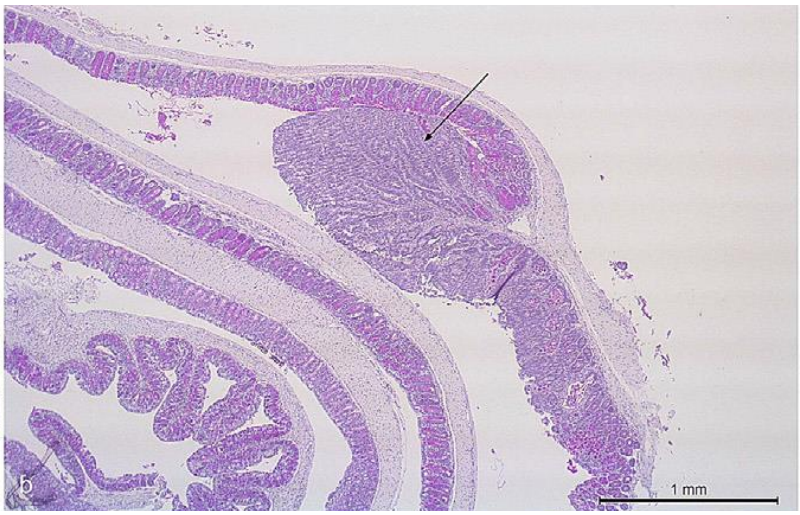


Figure 13: A carcinoma (arrow) in the mucosa infiltrates Muscularis mucosae and submucosa in A/J Min/+ mice exposed to the high concentration of POPs (source: Aleksandersen).

9 Results and summary of papers

9.1 Behavior project

9.1.1. Paper I

Comparing two different strains of mice (C57BL/6J and the hybrid B6129SF1/J) using behavioral testing – A small scale study –

To compare two different mouse strains, C57BL/6J, and the hybrid B6129SF1/J, we conducted a small-scale study. We tested them in three different behavioral tests, an OF test, a BM test, and a stress test.

The study showed that the C57BL/6J were more active during the OF than the hybrid, they were moving more and covering more ground, but only the time moving was significantly different from the hybrid. The time spent and frequency in the center zone, border zones, and the corner zones, respectively, were approximately the same for both mouse strains. Both mouse strains showed progress in learning as the time needed to enter the goal box in the BM decreased for each session, except for the fifth session (also fourth for the hybrid), where the buzzer was introduced. The hybrid was quicker in the three first sessions, but the difference did not reach statistical significance. In the three last sessions, the C57BL/6J was a little quicker, but not significantly quicker. The hybrids covered significantly less ground before they reached the goal box than the C57BL/6J in the first three sessions. The hybrids also covered slightly less ground than the C57BL/6J in all the last three sessions, but not significantly. Both strains had a similar baseline of corticosterone blood levels at the starting point of the stress test. The C57BL/6J did have a slightly lower concentration of corticosterone at all time points after that, however, the only significant time point was at 30 minutes. In addition, the hybrid left significantly more feces in both the OF and the BM.

In conclusion, both strains were found suitable for the behavior studies. The C57BL/6J was suggested to be more robust, while the hybrid appeared more sensitive to stressors as they left more feces and showed a higher corticosterone response to restraint.

9.1.2 Paper II

Human exposure-based mixture of persistent organic pollutants affects the stress response in female mice and their offspring.

This study aimed to investigate whether the mixture of 29 POPs, based on the estimated dietary intake of Scandinavians, could affect the stress response and anxiety in female mice and their offspring. Female mice 129:C57BL/6F0 hybrids were exposed via the diet from weaning, throughout pregnancy, and up until necropsy, in three groups, low and high concentration of POPs and control. Both the mothers and their offspring were tested in the OF and stress test.

The results of the stress test showed that high exposed mothers had significantly higher basal corticosterone levels than controls, whereas the low exposed showed only a trend for higher values than controls. When modeling changes in corticosterone following stress, we found a significant effect of the high exposure between 30 and 120 minutes, as the controls showed a greater level of change during this period compared to the high exposed group, with the low exposed mothers having an intermediate value. There was no effect of exposure on basal corticosterone levels in either male or female pups. But when modeling changes in corticosterone following stress, there was a significant interaction between sex and exposure. Here, high exposed males showed a significantly greater increase in corticosterone between 0 and 30 minutes, but also a greater decrease between 30 and 120 min, than controls, whereas the opposite was observed in females, however not significantly. When modeling the absolute values of corticosterone across the whole experiment, there was a clear dose-response in males with both the low and high exposed groups having significantly greater values than controls, but no such trends were observed in females. There was no effect of exposure on any behavioral endpoint in the OF test, however, there was a significant effect of sex on the bivariate outcome comprised of the frequency of grooming and cumulative duration of grooming. Both the frequency of grooming and cumulative time spent grooming were higher in female mice. In addition, there was also a significant sex effect on the ratio of the duration of grooming against rearing, with male mice spending more time on grooming than rearing compared to female mice.

In conclusion, the human-relevant POP mixture had no effect on anxiety, but affected the stress regulation by dysregulating the HPA axis in mice.

9.1.3 Paper III

Maternal exposure to a human-based mixture of persistent organic pollutants (POPs) affects gene expression related to brain function in mice offspring hippocampus.

After being exposed as previously described, the siblings of the mice tested in the OF and stress test, were subjected to the BM test. After euthanasia, POP concentration was measured in several tissues, including brain, blood, and adipose tissue, and hippocampal gene expression was analyzed.

The study showed that the offspring in the high dose group exhibited performance deficits in the BM when the stressor was introduced. The POP concentrations in the brain tissue in the low exposed offspring were measured and found the organochlorine levels to be comparable to levels in humans. Most compounds detected in the mothers were also found in offspring brain samples, indicating the transfer of these compounds across the placenta as well as the blood-brain barrier. No statistically significant behavioral differences between the sexes were found in the BM test. POP exposure changed gene transcription patterns of hippocampal genes involved in cognitive function (*Adora2a, Auts2, Crlf1, Chrnb2, Gdnf, Gnal, Kcnh3*), neuroinflammation (*Cd47, Il1a*), circadian rhythm (*Per1, Clock*), redox signaling (*Hmox2*) and AhR activation (*Cyp1b1*). Furthermore, *Auts2, Clock, Gdnf, Gnal, Kcnh3,* and *Hmox2* were differentially expressed in males versus females. In addition, the genes most important for passive behavior (not moving in the BM) for the low exposure level were *Hip1* and *Gnal.* And the most important genes for learning deficits (escape latency in the BM) were *Kcnh3, Gnal,* and *Crlf1*.

In conclusion, exposure to the human-relevant POP mixture affected the high exposed mice in the Barnes maze (not moving, escape latency) when combined with moderate stress exposure. Also, exposure altered the expression of genes related to cognitive function, neuroinflammation, circadian rhythm, redox signaling, and AhR activation in the hippocampus.

9.1.4 Unpublished material on adrenal glands

Here follows a summary of the preliminary, unpublished results of the analysis of the adrenal glands: The high exposed mothers had a significantly increased (p=0.0019) total area of the adrenal gland (1.18 (±0.034) μm^2 $x10^{-6}$), compared to the controls (1.0 (±0.034) μm^2 $x10^{-6}$). Also, the area of the adrenal cortex (0.94 (±0.025) μm^2 $x10^{-6}$) was significantly increased (p=0.0005), compared to the controls (0.79 (±0.025) μm^2 $x10^{-6}$). The offspring of the mothers in the high group also had a significantly increased (p=0.0091) size of the adrenal cortex (0.74 (±0.02) μm^2 x 10^{-6}), compared to the offspring of the controls (0.66 (±0.02) μm^2 x 10^{-6}). Figure 14 shows the enlarged glands and the thickened cortex.

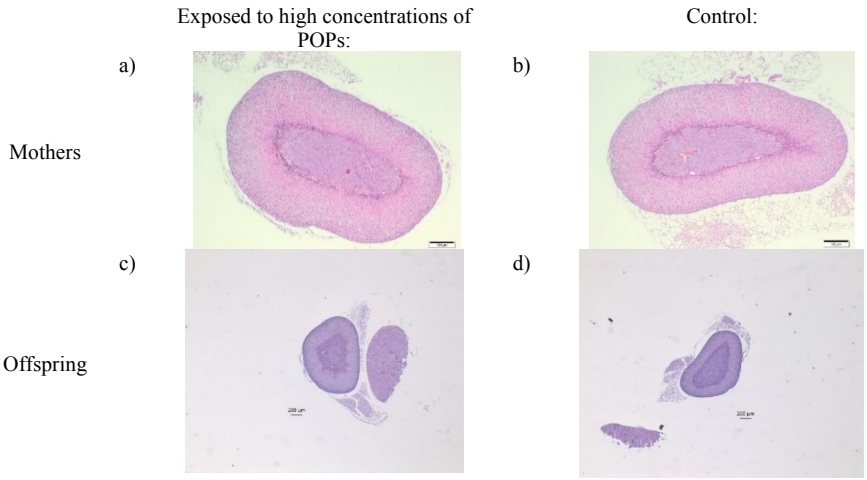


Figure 14: Histology section of adrenal glands. a) Mother exposed to high concentration of POPs. b) Mother in the control group. c) Offspring of mother in the high exposed group. d) Offspring of mother in the control group.

9.2 Cancer project (Paper IV)

A mixture of Persistent Organic Pollutants (POPs) and Azoxymethane (AOM) show potential synergistic effects on intestinal tumorigenesis in the A/J Min/+ mouse model.

This study aimed to investigate whether the mixture of POPs could affect intestinal tumorigenesis in the A/J Min/+ mouse, a model for human CRC. The mice were exposed to the same diet as in the behavior project, for 10 weeks. In a separate experiment, mice also received one subcutaneous injection of AOM to explore whether

this carcinogenic compound influenced the effect of the POPs. Intestinal tumorigenesis was examined by surface microscopy and histopathology.

The results of the surface microscopy showed that high levels of POPs significantly increased the number of flat aberrant crypt foci (flat ACF) in the colon, when compared to the control group. Although not significant, a trend was observed towards increased flat ACF load (total surface covered by lesions) in the high and the low groups. The low mixture concentration increased the number of colonic tumors, compared to the control group. However, the same was not evident for exposure to the high mixture concentration. No other parameters measured in the small intestine and colon were affected by dietary POPs alone. In combination with the AOM injection, POPs significantly increased the number of flat ACF, flat ACF load, number of tumors, average tumor size, and tumor load in the colon of mice in the high group. In addition, the number of tumors in the small intestine was significantly higher in the high group compared to the controls. No significant changes were observed after exposure to the low mixture concentration of POPs in combination with AOM. However, there were clear trends towards increases in several parameters, including a 7x increase in the colonic tumor load. Histology on the tumors collected from non-intestinal tissue showed no metastases originating from the intestinal lesions. Instead, they were either hyperplastic lesions or metastases from the local tissue. The histological examination showed that the total number of lesions in the small intestine was higher than that of the colon. No significant differences were observed between the control group and the exposed groups, but when combined with AOM, the mice fed the high concentration of POPs had significantly more colonic lesions of all types compared to the control group. Preneoplastic changes and adenomas were the most frequent lesions, and only a few animals had carcinomas. A trend was also evident toward increases in the number of small intestinal preneoplastic lesions.

In addition, the results show that the high mixture concentration of POPs significantly decreased the terminal body weight of both the mice who only were exposed to POPs and also the mice that were injected with AOM, compared to the control group. There was a significant increase in liver weight, relative to body weight, in the high group of both experiments. Colon length, relative to body weight, was not affected by AOM or POPs. However, the length of the small intestine, also relative to body weight, was

significantly increased by the high concentration of POPs after the AOM injection. Notably, AOM alone did not change any of the parameters measured.

In conclusion, the human-relevant POP mixture increased carcinogenesis in mice and there was an indication of synergistic carcinogenesis with a known carcinogen (AOM).

10 Discussion

This chapter will start with a discussion of the materials and methods, followed by a more result-oriented discussion. Finally, I will discuss the relevance of the results.

10.1 Discussion of the materials and methods

10.1.1 Reproducibility and validity of laboratory animal experiments

Some research fallacies are linked to an inherent dilemma, which is to improve reproducibility/reliability, to improve internal validity and still keep a high external validity when results are translated to humans. Reproducibility is the ability to reproduce or copy an animal experiment, usually in a different laboratory animal facility, and receive the exact same results. When using laboratory animals in experiments, a long list of variables is introduced: The genetics of the animal, the biology of the animal, the housing conditions, among other things. To control the variables as much as possible we have to standardize all these variables as much as we can. For example, by standardizing the housing conditions. The EU Directive on using laboratory animals in research (EU 2010) states standardized housing regulations that are followed in our study. The most important variable is, however, the animal itself. We try to minimalize the effect of this by using animals with standardized genetics and microbiological status. If you use inbred animals, their genetics are close to identical, you get a high reproducibility, high internal validity and you can use a low number of animals in each group. Still, the use of inbred animals is not necessarily a good model to be translated to a human population, which is mainly outbred. In our behavior project, we used F1 and F2 hybrids from two inbred strains. The genetics of the F1 can only be the product of the mother and father and the genetics of all F1 offspring would be close to identical. This gives the advantage of a higher reproducibility and at the same time a better model for the outbred human population. In our study, we wanted to look at *in utero* exposure, so we exposed the F1 hybrid mothers and tested the F2 hybrid offspring. The F2 hybrid offspring will have more variation in their genetic composition than their parents, but this should still be low, compared to an outcrossed or outbred mating. In the cancer project, we used backcrossed strain, thus, the individual variation is so large, that the effect of the litter is likely to be outplayed.

All of these details improve the validity and reproducibility of our study. As I mentioned, the most important variable is the animal itself and in the next chapter, I will expand on that.

10.1.2 Animal models, breeding, and housing

The results of the small-scale study (paper I) showed that both strains, C57BL/6J and the hybrid B6129SF1/J, learned the BM, but the hybrid covered less ground before they found the goal box during the sessions with the fewest motivators, but appeared more sensitive to stressors in the BM, as they left more feces and showed a higher corticosterone response to restraint.

Results from this study were taken into consideration when deciding which mouse strain to use in the main behavior project. The limitations of the small-scale study were that, for practical reasons and to keep it a small-scale study, we chose to include only young males. This makes it difficult to transfer our findings to the main project where we planned to test both males and females. The animals were group-housed during acclimatization, but changed into single housed during testing, for practical reasons. This change, so close to the testing was not optimal and single housing can produce stress in the animal, but since the project was so short (a few days), it was not considered a large factor. Thus, the results can only be considered in young, males who are single housed. We concluded that both strains are appropriate for the behavior studies we wanted to conduct in the main project, and even that the C57BL/6J strain seemed more robust in stressful situations. Apart from what was tested, we experienced the hybrid to be less aggressive and easier to handle. The strain has been used in several behavioral studies (Bale et al. 2000, Bale et al. 2002, McEuen et al. 2009, Teegarden et al. 2009) and experienced researchers in the field recommended using the hybrid due to their good experiences using it for the same tests, its pleasant nature, and good maternal care abilities. C57BL/6, can be aggressive in some cases and are also prone to infanticide (Gaskill et al. 2017). As we wanted the smallest degree of stress before testing and loss due to deaths from fighting and poor maternal care, the hybrid was chosen for the main behavior study (paper II and III).

In the cancer project (paper IV) we were interested in CRC, as it is one of the most common cancers in humans. As food is the main exposure route for POPs, the intestines are at risk. The most widely used animal model for human CRC is the multiple intestinal neoplasia (Min/+) mouse, which has a heterozygous mutation in the *Apc* gene (Su et al. 1992) and since mutations in *APC* in humans are found in more than 80% of sporadic CRC cases (Fearnhead et al. 2001), the Min/+ mouse is a good model for human CRC. The conventional Min/+ mouse model is bred on a C57BL/6 background, however, it

does not mirror the disease phenotype the way the A/J Min/+ mouse does, as it mainly develops lesions in the small intestines, which rarely develop into invasive adenocarcinomas. In contrast, the A/J Min/+ mouse develops lesions in both the small and large intestines, has an incidence of invasive carcinoma development of a 100% in older animals (Sødring et al. 2016), and is more susceptible to AOM-induced colon carcinogenesis than the C57BL/6 Min/+ (Ølstørn 2009). Still, having a large number of lesions, both in the small and large intestines, must be a significant load for the animals and could be more severe than for humans with CRC. This could explain the effect we have seen on for example reduced body weight.

The reason why we decided to add a group that received an AOM injection in the cancer project (paper IV), was that we expected rather small effects of POP exposure. After the AOM injection, we experienced high mortality in the offspring, which is why further breeding of mice was stopped early for animal welfare reasons. This resulted in a lower number of animals in the POPs + AOM group than originally planned. This was unexpected and discussed at length with colleagues. The concentration had been chosen on basis of recommendations from colleagues and earlier studies (Moen et al. 2016). We conducted a quality control on the injection procedure, without any findings. Then we discussed if the amount of AOM from the production company could be higher than shown on the statement of contents, resulting in the concentration being too high, but the manufacturer stated that the AOM concentration was according to the label. To exclude this theory, measurements of the concentration of AOM in the offspring's tissue would have to be done. At the time of the injections, the mothers and offspring were moved from open cages to individually ventilated cages (IVC), for health and safety reasons. This is a big change for the mothers, which can be stressful and may result in abandonment or infanticide. This is another possible reason for the observed offspring mortality. We could not see clear signs of injury on the dead pups, but abandonment could be a possibility.

10.1.3 The experimental design

The experimental design for the two animal experiments in this thesis is called class comparison experiments, as we are comparing three groups exposed to different concentrations of POPs (high, low, and control). The number of animals in each group for behavioral testing of the offspring (paper II and III) was 18. In the cancer study, it

was about 20 mice in each group (paper IV). And in the substudy, where the mice also got the injection of AOM, we also aimed at 20 animals in each group. However, because of the high mortality after the injection, the breeding had to be stopped, resulting in smaller group sizes (high n=7, low n=6, control n=8). Even though our study ended up with fewer animals in the POPs + AOM group than planned, the effect of dietary POPs was large enough to reach statistical significance. The possibility of ending up in an underpowered study may be a consequence of the Reduction principle, failed breeding, or increased mortality in animal experiments, and in some cases, you could be unable to conclude from the experiment.

There were some limitations in our studies. The mating scheme and breeding were conducted in a standard manner, using 1 male and 2 females. This is not optimal, but the placement of the hybrid mothers into exposure groups and the offspring into testing groups were randomized and as the hybrid offspring are produced by two inbred strains, they are genetically similar, who the parents were would not be a big determining factor on the outcome. For the behavior tests, we used both male and female offspring and, to avoid stress-related effects of single-housing, we chose group housing.

Also, when conducting BM to test learning and memory in paper I, we were advised to add motivators (fan and buzzer) to motivate the mouse to find the goal-box. However, we experienced that the motivators were stressful for the animals, especially the buzzer, so we decided to not use it in the main project. The BM test has several limitations, especially if you use these kinds of motivators. The benefits of using motivators are to motivate the animal to find the goal-box faster each time and avoid habituation to the surroundings. However, a disadvantage could be, that it would act as a stressor resulting in confused animals prone to making mistakes, rather than increased performance (Shanks et al. 1988). Our choice to exclude the buzzer in the main study, made it better, but the fan might also have stressed the animals. The timepoint chosen for testing the animals was, for the BM and stress test, during working hours (between 08:00 and 16:00) for practical reasons, and for the OF, during the animals' dark hour (between 20:00 and 03:00), as this was the most crucial test the animals needed to be alert and in calm surroundings to get the best results. But the timing of the stress test did not take into account any variation in corticosterone levels during the day, which can variate during the day and night in mice (Ishida et al. 2005).

The time of POP exposure in the behavior project (paper II and III) was during pregnancy and nursing, in order to study the effects of perinatal, maternal exposure in the offspring. POPs are known to cross the placenta and indirectly expose the fetus (Vizcaino et al. 2014). Also, the offspring were exposed via their mothers' milk while nursing. Nevertheless, it is common for the offspring to start to eat a little bit of their mothers' diet before weaning. This might have resulted in direct exposure during this time period. There was also a risk of contamination through dust from the other diet groups in the behavior project, as these were housed in open cages. This might result in additional exposure of the offspring and control animals. As mentioned earlier, we have to remember that humans are continuously exposed throughout their whole lifetime, so if we wanted to look at long-time exposure, we would have continued the exposure of the offspring after weaning. In these offspring, we wanted to look at exposure during early life only. However, the exposure time of the mothers, who were exposed all the way to necropsy, for several months, might be a reflection of long-time exposure, although they were not exposed during early life. In the cancer project, animals were housed in IVC racks, so the risk of additional exposure via dust was low. A small risk of contamination from drinking bottles and the cages themselves were also present, as they are made of plastic and may contain polymers, which can be added to the effects of POPs, however, this would apply to the control animals as well.

The observed effects in the offspring, the oversensitive stress response (paper II), the increased size of the adrenal cortex (unpublished), and the changes in genes connected to learning and memory (paper III), show that even though they were mainly indirectly exposed *in utero* and via the milk from the exposed mothers, we cannot point to any specific sensitive time window of exposure. Still, it does show that being exposed in early life is a risk for these problems. In our study, we wanted to see if exposure during these sensitive life stages, could give prolonged effects into young adulthood, even after the exposure stops. We found effects weeks after the end of the exposure. This is comparable to results from a previous study using goats, where the exposed offspring showed an increased cortisol response to stress seven months after exposure to POPs (Zimmer et al. 2009). This indicates that early-life exposure could give long-term effects. Both the offspring in the cancer project and the mothers in the behavior project were exposed from weaning and until necropsy. In the cancer project, exposure lasted

10 weeks, while in the behavior project, it lasted several months during adulthood. Unfortunately, none of our studies had a really long time of exposure, into old age.

We also found corticosterone changes in the exposed mothers themselves. This shows that POPs also can affect adult animals directly. For practical reasons, the offspring were necropsied first, and during the waiting time, the mothers were still on the POPs diet, resulting in the mothers being exposed to POPs for many months. Also, they were directly exposed and obtained a more considerable body burden than the offspring and had considerably higher blood POP levels than the levels in human blood (paper III). This fact must be taken into consideration when interpreting the results. Still, mothers showed only subtle behavioral changes and no effect on the magnitude of the corticosterone response to stress; only their basal levels and adrenal cortex sizes were affected.

The POP exposure time of the A/J Min/+ mice in the cancer project (paper IV) was carefully planned from weaning to 10 weeks of age (which is equivalent to a human in their early 20s). This is the point when the influx rate of flat ACFs is rising in this mouse strain (Sødring et al. 2016). It is assumed that the carcinogenesis in the intestines has progressed enough to be detected at this point. And for animal welfare reasons, the animals should not stay in the experiment longer than this, for the risk of developing symptoms of CRC, for example, rectal bleeding and rectal prolapse. The timing of the AOM injection, during their second week after birth, was chosen because this is the time window in which they are starting to form intestinal lesions (Paulsen et al. 1999).

10.2 Discussion of the results

10.2.1 Discussion of the results from the behavior project

In the behavior project, female 129:C57BL/6F0 hybrid mice were exposed to POPs via the diet from weaning, throughout pregnancy, and up until necropsy, in three groups, low and high concentrations of POPs and control. Both the mothers and their offspring were tested in a BM, OF, and a stress test. The results presented in this thesis show that exposure to the mixture of POPs had no effect on anxiety, but that POPs did alter the serum corticosterone levels, in both the mothers exposed to the high concentration and their male offspring. In addition, exposure to the high dose of POPs did alter the offspring's performance in the BM when a stressor was introduced.

Effect of POPs on behavior, stress, learning, and memory

The results from the behavior project (paper II and III) shows that the mixture of POPs has some effect on the stress reaction. However, the small effect we found on learning and memory can be due to the added stressor in the BM test (fan). If the results we have from the BM truly is a reaction to stress and not a reflection of effects on learning and memory, then this would be in accordance with our results on the effect of the stress reaction. Further, I will discuss the separate results in more detail.

Table 3 shows the results of a literature search on POP exposure, using the same POPs as in our mix, associated with stress, adrenal glands, behavior, behavioral sex differences, learning, and memory in animals and *in vitro*.

Table 3: Overview of references that show effects on stress, learning, and memory in connection to exposure to POPs. PubMed was used and several different search words were chosen (for example POPs, stress, behavior, neurobehavior, learning, memory, neurodevelopmental effects, including both animal studies and in vitro studies).

Chemical	Description	Ref
Effects on glucocorticoid secretion/stress reaction		
PCBs	Cortisol/corticosterone ↑ in fish	(Jorgensen et al. 2002)
	and guinea pigs	(Kato et al. 1981)
	and goats	(Zimmer et al. 2009)
PCBs / DDTs	Corticosterone ↑ in birds	(Monclus et al. 2018) (Tartu et al. 2014)
Organochlorines	Cortisol/corticosterone ↓ in polar bears	(Oskam et al. 2004)
PFOS	Cortisol/corticosterone ↓ in rats	(Pereiro et al. 2014)
Our mix	Enhanced transcriptional activity in the presence of cortisol *in vitro*	(Wilson et al. 2016)
PCBs		(Kraugerud et al. 2010)
POP mix	Alter cortisol synthesis *in vitro*	(Montano et al. 2011)
Our mix		(Ahmed et al. 2019)
PCBs	Oversensitive stress response in goats	(Zimmer et al. 2009)
POP mix	Reproduction changes in fish and Cortisol ↑ *in vitro*	(Berg et al. 2016)
PFOS	Prenatal mortality ↑ in the presence of stress in mice	(Fuentes et al. 2006)
POP mix + PFNA	Corticosterone ↑ in rats	(Hadrup et al. 2016)

Adrenals		
3-methyl-sulphonyl-DDE	Adreno-toxic effects in mice	(Lund et al. 1988) (Jonsson et al. 1995)
HCH		(Lahiri et al. 1991)
PCBs	Adrenal weight ↓ and thinner adrenal cortex in sheep	(Zimmer et al. 2013)
Pesticides mix	Adrenal weight ↑ in rats	(Birkhøj et al. 2004)
Sex differences		
PFOS	Delayed task learning in adult female offspring in mice	(Fuentes et al. 2007)
PCBs	Sex differences in lambs exposed to stress	(Gutleb et al. 2011)
Learning		
PBDEs / PCBs	Developmental neurobehavioral defects / learning in mice	(Eriksson et al. 1991) (Eriksson et al. 1996) (Eriksson et al. 1998) (Eriksson et al. 2001) (Eriksson et al. 2002) (Eriksson et al. 2006) (Viberg et al. 2002) (Viberg et al. 2003) (Viberg 2009)
PCBs	Reduction in spatial learning in birds	(Zahara et al. 2015)
PBDEs	Effects on neurodevelopment in rats	(Coburn et al. 2008)
PFOS / PFOA	Neurobehavioral defects in mice	(Johansson et al. 2008)
In vitro		
Our mix	Developmental neurotoxicity ↑ *in vitro*	(Yadav et al. 2021)
Our mix	Neurodevelopmental effects ↑ *in vitro*	(Berntsen et al. 2021)
Our mix	Proliferation of neural stem cells ↑ and synaptogenesis ↓ *in vitro*	(Davidsen et al. 2021)
PBDEs / PCBs	Neurotoxic effects *in vitro*	(Westerink 2014)
Memory		
PBDEs	Neurobehavior and memory ↓ in fish	(Zheng et al. 2017)

From table 3 we see that several studies show POP exposure being connected to cortisol/corticosterone secretion. Also, several experiments show associations between POPs and learning in mice, which can be compared to our studies, but again, only a few have been using mixtures of POPs. One study exposing rats to PFNA alone and in combination with a mixture of 14 POPs has shown changes in corticosterone levels by PFNA alone, an effect that was normalized by co-exposure with the mixture (Hadrup et

al. 2016). To my knowledge, literature on the effects of mixtures of these POPs on memory specifically is scarce.

The results from the OF (paper II) showed that the female offspring had a significantly higher frequency and duration of grooming than the male offspring. This is a behavior that prey animals show when they are stressed and have nowhere to escape (McBride 2017). It is believed that they use grooming to self-soothe. This might indicate that the female offspring needs to self-soothe to cope with the OF test, more than the males. Other studies have indicated similar differences between the sexes, for example, that female mice show higher rearing activity after a stressful experience, than males (Faraji et al. 2020). There were no effects of POP exposure on the OF test in our study, but in a different study in rats exposed to a small mixture of POPs in low doses and tested in a Morris water maze, OF and light-dark box, showed an increase in anxious behavior (Lahouel et al. 2016). Interestingly, in the stress test in our study, we found increased basal corticosterone levels in the mothers and increased stress-induced corticosterone levels in the high exposed male offspring and one would expect that these measurements would reflect the OF test, but in our case, it did not. As mentioned before, some animals use grooming to calm themselves when stressed. Perhaps this could work as an alternative stress or anxiety relief. Still, we only observed sex differences in grooming and no exposure-related differences. While the OF tests activity and anxiety in a novel environment, the stress test looks at stress response in a stressful environment. A possible explanation for the presumably deviating results of the two tests might be that the POPs do not affect the mice's basal anxiety level, when faced with a low-leveled strenuous exercise, like the OF test. On the other hand, when faced with a high-level strenuous exercise, like the stress test, which is designed specifically to activate a large stress response, the effect of POPs is more evident. In a study exposing lambs *in utero* to PCBs they observed significant, sexually dimorphic changes in behavior in connection to stressful situations, however, there was no clear pattern that severe stress (presence of a dog) caused more pronounced exposure effects than milder forms of stress (isolation from mother) (Gutleb et al. 2011).

Elevated basal cortisol in connection to PCB exposure has been shown in fish (Arctic char) (Jorgensen et al. 2002) and guinea pigs (Kato et al. 1981). On the other hand, basal cortisol levels were lower in polar bears with high plasma levels of organochlorines (Oskam et al. 2004), as well as in rats when exposed to PFOS (Pereiro et al. 2014).

As we measured total corticosterone levels in the blood, the change in basal corticosterone levels may also be seen in context with the transport protein transcortin (Breuner et al. 2020). In humans, as much as 75% of the cortisol and in rodents, about 78% of the corticosterone in circulation is bound to transcortin and only the free non-bound hormone is biologically active. Transcortin is produced by the liver and its expression is increased by estrogens. If some of the POPs in our mixture have estrogenic actions, which some PCBs (Gallo et al. 2018) and DDT components (Bolt et al. 2002) have shown to have, the levels of transcortin in the mothers may increase. This may increase the total level of corticosterone in the blood. There is an equilibration between the bound and non-bound fractions. If more transcortin is released into the blood, more corticosterone will bind. Then, the free biologically active fraction of corticosterone will decrease, which in turn, will stimulate the pituitary gland to increase secretion of ACTH and thereby stimulate the adrenal cortex to increase cortisol secretion. This is a possible explanation for the increased basal corticosterone levels in the mothers.

Concurrent with our findings, the male offspring of goats exposed to PCBs also showed an oversensitive stress response (Zimmer et al. 2009). The question is, why? Could indirect exposure to the POPs of the offspring *in utero* and via their mothers' milk be enough to cause these changes alone? A Canadian review looking at studies on animals found that POPs were associated with neurochemical changes and alterations in maternal care behaviors (Fong-McMaster et al. 2020). Maternal neglect (separation) could potentially lead to altered HPA axis activity in the offspring (Orso et al. 2020). Alternatively, the elevated basal levels of corticosterone in the mothers may have affected the offspring's ability to regulate their own HPA axis response. Mouse mothers exposed to noise stress during pregnancy gave offspring which had significantly higher corticosterone levels after being tested in a Morris water task (Jafari et al. 2017). Could the POPs the mothers were exposed to represent some kind of stress that would interfere with the offspring's ability to cope with stressful situations? And if that was the case, would their increased basal or chronically elevated corticosterone levels be the causative factor? In a different study, rat mothers given drinking water containing a moderate dose of corticosterone during lactation had offspring who showed improved learning capabilities, reduced fearfulness, lower metabotropic glutamate receptors (mGluR), and higher GR density in the hippocampus (Catalani et al. 2011). This does show that

mothers' altered physiology affects the offspring, but that a stressed mother is trying to prepare her offspring to cope with a stressful world. In a similar study, mouse mothers were given drinking water containing two different doses of corticosterone showing that the offspring from mothers drinking the higher dose of corticosterone had a higher corticosterone response to stress, while mice from mothers drinking the lower dose had no effect (Macrì et al. 2007).

Also, several epidemiological studies in humans find that stress in mothers can affect their children. Researchers in the Netherlands performed a longitudinal study of infant cortisol reactivity to stressful events in 173 mothers. Maternal prenatal fear of bearing a disabled child was a consistent predictor of infant cortisol reactivity. Higher fear was significantly related to higher salivary cortisol reactivity during a bathing session and decreased cortisol reactivity to vaccination and maternal separation (Tollenaar et al. 2011). In a different study, in Germany, researchers evaluated 61 healthy young adult volunteers, of which 31 had mothers who experienced severe stress during their pregnancy. While pre-Trier Social Stress Test (TSST) cortisol levels were lower in subjects of mothers that experienced stress, these subjects exhibited significantly higher ACTH concentrations, as well as a higher increase in cortisol in response to the TSST. And cortisol concentrations following a ACTH1–24 test were significantly lower in the subjects of mothers who experienced stress. These results show an increased pituitary reactivity to stimulation in these subjects, although it appears to exist a counterregulatory adaptation of the adrenal cortex as reflected by lower pre-task cortisol levels and lower cortisol levels in response to stimulation via an ACTH injection (Entringer et al. 2009). All of these studies support the hypothesis that a mother's altered HPA axis activity can affect the offspring. Cortisol can be transferred across the placenta from the mother to the fetus, it is regulated by 11-β-hydroxysteroid dehydrogenase which metabolizes 26.5% of the hormone to be inactive, while 3% passes through the placenta and 70.5% stays in the mothers' bloodstream (Stirrat et al. 2018). Also, in swine, maternal cortisol accounts for almost 50% of fetal cortisone, and the metabolism of maternal cortisol by the placenta is 7.5% at 100 days of gestation (Klemcke 1995). The question is if the corticosterone level elevation of the high exposed mothers in our study was high enough to affect the fetal HPA axes.

The unpublished results from our analysis of the adrenal glands showed that the mothers in the high concentration group had enlarged adrenals and enlarged adrenal cortex. The

offspring of the mothers in the high concentration group also had an enlarged adrenal cortex. In addition, the analysis shows that the female offspring have larger glands than the male offspring, but this is in line with normal biology (Hans et al. 2004), and exposure to POPs had little effect on the sex difference. While our POP mixture seems to stimulate adrenal growth and hormone production, 3-methylsulphonyl-DDE has been shown to exert adrenotoxic effects (Lund et al. 1988, Jonsson et al. 1995). Also, lamb fetuses maternally exposed to PCBs, presented with decreased adrenal weight and a thinner adrenal cortex (Zimmer et al. 2013). Thus, these findings are not in line with our results. The DDT-metabolite 3-methylsulphonyl-DDE was not included in our mixture, while the PCBs in the study of Zimmer *et al*. were. However, the adrenals were collected at a different life stage and in a different species than in our study. This might affect the outcome. In a study, exposing rats to a mixture of five pesticides, they found significantly increased adrenal weights (Birkhøj et al. 2004). Our findings of enlarged adrenal glands and the thickened adrenal cortex support the results from our stress test, where the mothers had increased basal levels of corticosterone and the male offspring had an oversensitive stress response. It also indicates that these effects have lasted over a longer period of time, resulting in increased activity in the HPA axis of the mothers and persistently elevated ACTH secretion which stimulates the growth of the adrenal cortex and corticosterone secretion.

The observed sex difference in our study, with an over sensitized stress response in the male offspring exposed to POPs, is very interesting. There are indications that the HPA axis is affected by ovarian steroids, both during basal and stressful conditions (Roy et al. 1999). Several studies have shown sex differences, but it is not clear which sex handles stress best. Various theories of reasons for these differences have been presented, but the reason may lie in the difference in the sex hormones of the two sexes. A study in mice, exposing offspring to PFOS and maternal stress, showed delayed task learning in adult offspring, but only in the females (Fuentes et al. 2007). Also, studies exposing mothers to stressors during pregnancy showed a different response to stress in male and female rats (Nishio et al. 2001) and in mice (Romeo et al. 2003). Also, Gutleb *et al*. found sex differences in lambs, prenatally exposed to PCBs, and tested in behavioral tests (Gutleb et al. 2011). A review looking at neuro-psychiatric disorders also found sex differences and discussed reasons like for example immaturity at birth in addition to immune activation in the brain, including complex interactions between sex hormones,

brain transcriptome, activation of glial cells, and cytokine production (Ardalan et al. 2019). It has also been suggested that there are sex differences in the vulnerability in the way prenatal stressors affect the offspring's HPA axis reactivity, due to changes in placental glucocorticoid metabolism (Gifford et al. 2017). A possible explanation for sex differences could also be that some of the POPs in our mixture interfere with sex steroid secretion, action, or metabolism and therefore affect the development of males in a different way than females. When looking at the effects of PCBs on male reproduction, inverse associations were found between PCBs and serum testosterone levels (Meeker et al. 2010). And in a different paper, showing the results from an examination of the testicles from our behavioral project, showed that the POP mixture can affect testicular development, sperm production, and sperm chromatin integrity (Khezri et al. 2017). These processes are strongly dependent on testosterone, but hormone levels were not measured in this study. A study done in Greenlandic Inuits found a connection between levels of POPs and ER and AR transactivity (Krüger et al. 2008).

Stress can affect the different sexes in different ways, via the brain-pituitary-gonad (BPG) axis. A study using male fish showed that long-term cortisol treatment inhibited pubertal development by dysregulation of the BPG axis (Consten et al. 2001). Several epidemiological studies show an association between high cortisol and low testosterone (Rosmond et al. 1998, Malan et al. 2014). In addition, DDT and PCB have been shown to feminize males by binding to the ER (Nisbet et al. 1996, Hany et al. 1999, Wolf et al. 1999, Kaya et al. 2002, Lilienthal et al. 2006, Qin et al. 2007, Roy et al. 2009).

It is also interesting to look at the effects of exposure to POPs on learning and memory. As mentioned above, we had indications of changes in learning and memory shown in session 5 in the BM test, but it was unclear if this was affected by the added stress of the fan. A Swedish research group has performed several experiments using PBDEs and PCBs. They exposed mice neonatally to several PBDEs and PCBs, and their results show developmental neurotoxic effects and impaired learning and memory (Eriksson et al. 1991, Eriksson et al. 1996, Eriksson et al. 1996, Eriksson et al. 1998, Eriksson et al. 2001, Eriksson et al. 2001, Eriksson et al. 2002, Viberg et al. 2002, Viberg et al. 2003, Viberg et al. 2003, Eriksson et al. 2006, Viberg 2009). It is clear from these papers that these POPs enter the brain, and alter the hippocampus, a part of the brain which is very important for learning and memory. A different study in birds (European starlings)

exposed to a mixture of PCBs and tested for habituation, learning, cue selection, and memory, showed that exposure resulted in a reduction in spatial learning (Zahara et al. 2015). Adverse neurodevelopmental effects due to POP exposure have also been reported in several epidemiological studies. For example, cognitive deficits have been associated with organochlorine exposure in children with the suggestion that the chemical interacts with the picrotoxin receptor in the nervous system and interferes with the γ-aminobutyric acid neurotransmission system (Kilburn et al. 1995). Also, children prenatally exposed to PBDEs were at higher risk of attention deficit disorders, which may make learning more difficult, showing that circuitous neural pathways may be particularly sensitive to the effects of PBDEs (Cowell et al. 2015).

Whether the effects observed in session 5 of the BM are due to learning and memory deficits or to a stress reaction that interferes with performance remains to be resolved.

Gene expression

The results of the qPCR from the behavioral study (paper III) show differential expression of hippocampal genes related to cognitive function with increased expression of *Crlf1, Chrnb2, Gdnf, Kcnh3*, and decreased expression of *Auts2*. In addition, we found that *Hip1, Gnal,* and exposure to the low dose of POPs were the most important three predictors for not moving in the BM, and *Kcnh3, Gnal, and Crlf1* were most important for the mice using prolonged time to find the goal box in the BM.

The reported neurological function of these genes and these associations between behavioral changes and gene expression add biological plausibility and mechanistic support to our findings (table 4).

Table 4: Overview of genes that were influenced by exposure to the mixture of POPs. Table made based on Myhres work on the gene expression analysis.

Gene	Expression	Function	Ref
Crlf1	↑	Encodes for a protein that supports differentiation and survival of a wide range of neural cell types during embryonic development and in adult neural tissues.	(Rousseau et al. 2006)
Chrnb2	↑	Increased expression may reflect dysfunctional cholinergic signaling on hippocampal neurogenesis and learning performance.	(Kaneko et al. 2006)
Gdnf	↑	Encodes a neurotrophic factor that contributes to normal hippocampal development.	(Irala et al. 2016)
Kcnh3	↑	Cognitive function is changed when this gene is knocked out.	(Miyake et al. 2009)
Auts2	↓	Associated with for example autism, mental retardation, and developmental delay.	(Bedogni et al. 2010)
Gnal	↑	Encodes a stimulatory G protein alpha subunit that couples adenosine A2A receptors (Adora2a), which enhance spatial memory and hippocampal plasticity.	(Laurent et al. 2016)
Hip1	↓	Knockout mice show neurological deficits.	(Metzler et al. 2007)

It has been shown that central learning and memory function is closely related to the expressions of hippocampal genes (Kosarussavadi et al. 2017, Xiong et al. 2021). In our study, the POP exposure changed the transcription of genes involved in cognitive function, neuroinflammation, circadian rhythm, redox signaling, and aryl hydrocarbon receptor (AhR) activation. The change in the gene expression involved in cognitive function and neuroinflammation indicates that learning might be affected by POP exposure. This was not clearly evident phenotypically in the behavior tests of this study. Still, the analyses suggested expression changes to be related to learning and memory processes in the mouse brain, either directly or as compensation for learning deficits. Could the change in the expression of genes involved in the circadian rhythm interfere with HPA axis activity? It is known that the HPA axis and the circadian rhythm system influence each other, and dysregulation of these systems can cause for example mood

disorders (Nicolaides et al. 2014). Redox signaling is the transduction of signals involved in the production of free radicals or reactive oxygen species (ROS). ROS can for example damage DNA and impair mitochondrial function. AhR is a key regulator of the cellular response to xenobiotic exposure and induction of xenobiotic-metabolizing enzymes (Mrema et al. 2013). A study confirming our results shows a changed expression of *Gnal* in rats exposed to PCBs (DasBanerjee et al. 2008). In a different study exposing zebrafish larvae to the same mixture as in our studies, results showed increased swimming speed in the larvae. They also found changes in the expression of genes related to the stress response: *manf, crhb, hrh1, hdc, chrna7, sertb, bdnf,* and *gabra1*, these are involved in GABAergic, dopaminergic, histaminergic, serotoninergic, cholinergic systems and neuronal maintenance (Khezri et al. 2017).

In the introduction chapter, I mentioned that several review articles discussing the mechanisms of POPs, reported that HCB, HCHs, PCBs, as well as DDT can affect the redox balance and that HCB, PCBs, and DDT can affect AhR activation (Mrema et al. 2013). This is in accordance with our results from the genetic analysis. Although our study is not a mechanistic study, these results can help us to better understand the mechanisms of the POPs in the mixture.

10.2.2 Discussion of the results from the cancer project

In the cancer study, A/J Min/+ mouse offspring were exposed to the POP diet for 10 weeks. In a separate experiment, mice also received one subcutaneous injection of AOM. Intestinal tumorigenesis was examined by surface microscopy and histopathology. The results presented in this thesis show that exposure to the mixture of POPs moderately increased the intestinal tumorigenesis in the A/J Min/+ mouse, in both the high and low exposed group. In addition, results show that POPs can interact synergistically with AOM, causing increased intestinal tumorigenesis in mice.

The biggest limitation of the cancer project is that it was separated into two different experiments which had to be analyzed independently. This was again, for practical reasons, as all the breeding for experiment 1 was finished before the breeding was started again to add offspring for experiment 2 in which AOM was given. Also, the control group in experiment 2 was given the control diet (without POPs), for

comparison with the exposed group, but we did not have any "pure" control group, only receiving the control diet, no POPs, and no AOM.

Carcinogenesis

In the cancer project (paper IV) we found moderately increased tumorigenesis after POP exposure. Our project does not include any mechanistic studies, therefore, we cannot give a conclusive answer to how this occurs. However, in the introduction, I mentioned that POPs can act both as initiators and promotors. Our results show that exposure to the highest dose of POPs significantly increased the number of flat ACFs, which may point to a role of POPs in initiation. Our gene expression analysis showed a change in genes connected to redox signaling and the production of ROS. ROS can for example cause DNA damage (Song et al. 2008). Our results also showed that exposure to the low dose of POPs gave an increased number of tumors, suggesting that POPs stimulated the growth of already existing lesions. We know that several of these POPs can act as endocrine disruptors (Calaf et al. 2020), which may indicate that POPs may have a role in promotion.

Ngo *et al*. exposed C57BL/6J Min/+ mice *in utero* to low doses of PFOA and PFOS and saw no increase in intestinal cancer (Ngo et al. 2014). Interestingly, a different study, using the same mixture of POPs in the A/J Min/+ mouse showed a decrease of lesions in the intestines of the offspring of POP exposed mothers (Johanson et al. 2020). In chapter 10.1 I discussed reproducibility in laboratory animal experiments and the large number of reasons for these types of discrepancies. The authors themselves also discuss several different possibilities that could explain the inconsistencies, such as the different routes of exposure between our two studies, the time window of exposure, and the length of exposure. Some of the mothers were also mated several times to produce offspring for their study, which can give uncertain results as the concentration of POPs tend to decrease in the mother during nursing (Schecter et al. 1996, Thomsen et al. 2010, Brantsæter et al. 2013). As I explained in the introduction, without a complete dose-response curve, it is difficult to predict the effect of all the chemicals in the mixture. If a mixture for example has a u-shaped curve, moderate doses could give no effects, whereas low doses and high doses could give adverse effects. In a different study, rats exposed to fish oil contaminated with POPs and AOM, showed increased preneoplastic lesion formation in the colon (Hong et al. 2017). In that study, consumption of fish and

fish liver oil increased the risk of exposure to POPs, despite the documented positive health benefits of fish and fish liver oil, due to high levels of polyunsaturated fatty acids and vitamin D. A study based on the Norwegian Women and Cancer Study (NOWAC) from 2007 showed that fish liver consumption was not associated with increased cancer risk in breast, uterus or colon (Brustad et al. 2007). In 2017, a research group at the University of Bergen warned about giving fish to children because of the high levels of POPs (Bolann et al. 2017). Potentially harmful and beneficial effects need to be weight against each other when it comes to nutritional recommendations.

Effects of AOM

In our study, we gave one set of animals an AOM injection in addition to the POPs (paper IV) to see the effects of POPs together with a known carcinogen. AOM is a known carcinogen and has been used frequently in CRC research. It affects the *Wnt* signaling pathway (Zhang et al. 2021), which is the same pathway that is affected in the Min/+ mouse (Sødring et al. 2016).

AOM causes DNA mutations that prevent the β-catenin from being degraded by GSK-3, and the accumulation of β-catenin leads to cell proliferation. It inhibits Transforming Growth Factor β (TGFβ), which is a pro-apoptotic protein, and this way, cancer starts to develop (Chen et al. 2009). However, a study has found mutations in the beta-catenin gene and the *Apc* gene in tumors induced in C57BL/6J mice by AOM, which may indicate that the differences in mutation status between Min/+ and C57BL/6J mice can have different genetic pathways for developing colon tumors (Suzui et al. 2002).

As mentioned, one of the limitations of our study was that the experiments on the groups of mice that received only POPs and the POPs + AOM groups were done at two different time points, and they are considered two separate experiments and cannot be analyzed together. Also, because of the increased mortality, there were few individuals in each of the POPs + AOM groups, and we were not able to analyze the differences between the sexes. Nevertheless, the results give important information about possible synergistic effects between POPs and AOM. In hindsight, we should have added a "pure" control group, only receiving the control diet, no POPs and no AOM. However, the AOM treatment shows a normal pattern that you could expect from AOM exposure. The control group in the first experiment also had no unexpected findings, and as the two experiments were done the same way under the same conditions, they could at least

be compared to each other. A comparison of the results from both experiments suggests a synergistic effect on lesion formation between AOM treatment and POP exposure, especially in the colon. As I explained in the introduction, a synergetic effect means a combined effect that cannot be explained as an additive effect. Additive or synergistic effects of, for example pesticides, have been suspected for a long time (Thompson 1996). The cause of synergy is usually one or a combination of processes that are important for the toxicity of a chemical in an organism: Bioavailability, uptake, internal transportation, metabolization, binding at the target site, and excretion (Cedergreen 2014). It would be difficult to pinpoint which one or ones of these that caused the potential synergy between the chemicals in this study. Administered in these ways, both the AOM (by injection) and the mixture of POPs (via the diet) have high bioavailability, they are both easily transported in the blood.

AOM affects several signaling pathways, for example K-ras, Src/PI3K/Akt, β-catenin, TGFβ, and p53 (Chen et al. 2009). As there are several different POPs in the mixture, many different signaling pathways can be involved in the mechanisms of effects, like for example PCBs using the *Wnt7a* signaling pathway (Ma et al. 2006) and *p,p'*-DDE using estrogen and androgen signaling pathways (Aubé et al. 2008). It is known that for example PCBs can act like promotors for a special type of liver tumor (Strathmann et al. 2006), this can mean that chemicals could change signaling pathways that make other chemicals even more carcinogenic. In the Min/+ mouse, it is the *Wnt* pathway that is changed by the mutation in the *Apc* gene, and AOM affects the β-catenin, which is central in this pathway.

It may be that POPs and AOM act together in a synergistic way, AOM as an initiator and the POPs as promotors, or that they both alter different mechanisms leading to a synergistic effect. Previous studies have shown similar effects between POPs and other carcinogens. For example, PCBs promoted carcinogenesis in lung and liver tissues when the tumors were initiated by N-nitrosodimethylamine (Anderson et al. 1994, Strathmann et al. 2006) and PCBs had also a promotional effect when given together with 1-Nitropropane to induce lung tumorigenesis (Nakanishi et al. 2001).

Another idea is that the metabolism of one chemical can be changed by another chemical. Another example of two toxins working synergistically together this way is nicotine and ethanol. If taken together, they can cause increased CYP2B1 protein,

mRNA, and CYP2B1-mediated nicotine metabolism. CYP2E1 protein and activity were induced by nicotine, but no changes were seen in levels of CYP2E1 mRNA. This shows that these two toxins taken together can result in metabolic cross-tolerance, indicating that nicotine use may increase the elimination of ethanol, and ethanol use may increase the elimination of nicotine (Schoedel et al. 2003).

Whether POPs and AOM exert such a collaboration in developing cancer remains to be elucidated in future studies.

Health, body weight, and liver weight

The mice were subject to daily health checks, looking at general health and normal behavior in the home cages and we did not observe any clinical signs, such as signs of discomfort or effects of POPs on general health parameters in the animals during any of our studies. However, in the cancer study, we found effects on the body weight at euthanasia: Our results show that the high concentration of POPs significantly decreased the body weight and increased the liver weight of both the mice who were only exposed to POPs and also the mice that got POPs and AOM, compared to the control group. However, there was no significant effect of the POPs on body weight in the behavior study. This can suggest that there is a strain difference in sensitivity to the POPs, but it can also suggest that living with cancer in addition to POPs exposure is an extra burden on the body. In addition, the high number of large intestinal lesions may have caused a decreased absorption of nutrients, and this could also have reduced the body weight. The increased liver weight is in accordance with other studies where animals have been exposed to perfluorinated compounds (Seacat et al. 2003, Tan et al. 2013). Hypertrophy in the liver is often seen in toxicological studies in rodents and the cause could be either an adaptive effect when the hepatocytes increase their metabolic capacity in reaction to a xenobiotic compound or an adverse effect when the liver develops functional impairment (WHO 2015).

10.3 Relevance of the findings

This project is important for adding to the knowledge about the effect of POPs on behavior and cancer. As we have seen, both neurobehavior problems and cancer are serious health concerns for people and we know we are exposed to these chemicals, so

what kind of effect they have on us is important to know. Our findings show changes in the adrenals, corticosterone levels, and HPA axis activity and indicate stress. Our findings on learning and memory also seem to be linked with stress rather than learning capability as such, but our gene expression analyses indicated that POPs might have the potential to affect learning. The results from the cancer study show effects on carcinogenesis. Due to limitations and the nature of experimental studies, the question is how relevant these findings are to humans.

10.3.1 The POP mixture and dose levels

As discussed earlier, it is important to research mixtures, and not only individual compounds, to get closer to describing the real-life exposure scenario (Kortenkamp et al. 2018). For the design of the mixture of POPs for this project, we decided to use the "bottom-up"-approach, where you start with the human intake of the chemicals and produce a mixture reflecting a real-life exposure scenario. As we were interested in its effects on humans, we wanted to make a mixture based on human consumption. A different way to do it would have been to use the "top-down"-approach, where you chose doses based on their effect and mode of action and design a mixture to test hypotheses on mathematical predictions on effects (Beketov et al. 2012). This is the more traditional approach, but would not have given us the same insight into the real-life scenario.

Although not a member of the EU, Norway has included the bans on POPs in its legislation, according to the European Economic Area Agreement (EEA). Thousands of Norwegian mothers and children are continuously evaluated in a health screening called the Norwegian Mother and Child Cohort Study (MoBa) conducted by the Norwegian Institute of Public Health (NIPH). The study looks at the health of mothers during pregnancy and their children after birth, they screen for dioxins and PCBs in pregnant women (Caspersen et al. 2013), for POPs in men (Nøst et al. 2013), and for PFASs in mothers (Poothong et al. 2017). They all show that Norwegians do have POPs in their bodies. As our studies was performed in Norway, it was a goal to look at the Scandinavian eating habits and Scandinavian levels of POPs. The levels of POPs in the mixture for the mouse experiments were based on POP levels in food in Scandinavia (Berntsen et al. 2017). Thus, the mice feed was designed to represent the POP mixture found in a typical diet from Scandinavia. Again, it needs to be taken into consideration that the Norwegian diet tends to include more fish and wild game than the rest of

Europe and the world average (FAO 2018). Especially fish tends to contain high levels of for example PCBs, and according to Shaw *et al*., Norwegian fish in 2006 had among the highest levels of PCBs in the world (Shaw et al. 2006). This contributes to the concentration of PCB being high in our mixture. Native people in Canada also eat a lot of wild game and as a result have high levels and health problems from POPs (Valera et al. 2013). The USA has historically had high concentrations of PBDEs in their fish, although concentrations have been decreasing during the late 2000s. On the other hand, levels of hexabromocyclododecane (HBCD), have increased from 13 ng/g in 2002 to 4,640 ng/g fish in 2007 (Chen et al. 2011), as the use of PBDEs was banned and many producers changed to HBCD. Levels of HBCD were measured in Norwegian fish in 2012, it was much lower, at 13.9 ng/g fish (Bustnes et al. 2012). The EDI for HBCD in America in 2010 was 0.50 ng/kg/day, where the main source is meat (Schecter et al. 2010), compared to 0.33 ng/kg/day in 2008 in Norway, mostly from oily fish (Knutsen et al. 2008). We believe that our experimental mouse diet is relevant to the Scandinavian diet, without taking into account local and individual variations, and it should not be viewed as a model for the global population. The Norwegian cod liver oil companies remove all PCBs and dioxins from their oil (Thonhaugen et al. 2014) and the same POPs are removed from fish feed used in Norwegian salmon farming (Haugan 2014). This may decrease the exposure of the public. The 2014 report from the Norwegian Scientific Committee for Food and Environment states that recommended daily intake of fish and fish liver oil should not exceed the recommended TDIs of POPs and that they find the health benefits of consuming fish and fish liver oil trump these risks. Although recommending an upper limit to the consumption of fish, they still encourage people to eat fish within the recommended amount for health reasons (NSCFE 2014).

The concentration of POPs in the "high" experimental diet was set to 100,000x higher and in the "low" concentration diet 5,000x higher than in the human diet (paper II, III, and IV). Therefore, we need to be careful when extrapolating these results from mice to humans. During the planning of the project, we discussed having a third diet, with concentrations that would reflect human EDIs, however, due to lacking resources, it was not possible. There were several reasons why we decided to use these high concentrations: Firstly, we had to follow the laboratory animal golden rule to reduce the number of animals to a minimum (Russell et al. 1959). If we had used very low concentrations and there were small differences between the groups, we would have had

to use a large number of animals to find the differences. Secondly, mice have much shorter intestines (even relative to body weight) and a quicker intestinal transit time than humans (Shen et al. 2011, Padmanabhan et al. 2013). The exposure time in the intestines is, therefore, shorter in mice than in humans. Several physiological factors are different between the gastrointestinal tract of rodents and humans, for example, stomach pH, bile composition, and microbial content. Despite greater resemblance between humans and species like pigs, mice are more frequently used for practical reasons (Kararli 1995). In addition, as the surface relative to body volume is larger for mice, compared to humans, mice require a higher metabolic rate to keep a stable body temperature (Terpstra 2001). Furthermore, there are differences in xenobiotic metabolism in mice and humans (Martignoni et al. 2006). Thirdly, we suspected that the oil used to produce the experimental diet may contain low levels of POPs. Also, the other ingredients in the mouse feed could contain low levels of POPs and could vary from batch to batch, which could interfere with the results of the experiment (Schecter et al. 1996). By using higher concentrations of POPs in the experimental diet, we were aiming to eliminate this "background exposure". Fourthly, in a laboratory animal experiment, we anticipate some individual variability in the animals and maybe subtle effects from the exposure of POPs, especially on behavior as this is dependent on many different factors. Therefore, we wanted to have a group exposed to a high POP concentration that would more likely show the effects of the exposure. In addition, in real life, humans will be exposed to many more chemicals throughout their whole life, so the true accumulated burden may be more similar than first assumed. Finally, we considered the standard safety factor used in toxicology. It is usually 10x considering individual variation and another 10x considering extrapolation from an animal species to humans. Therefore, 100x difference is the standard (Dorne et al. 2005). All these elements are important reasons to why we decided to use these high concentrations.

Nevertheless, analyzing POP levels (paper III) shows that the concentrations of organochlorines in the offspring and the mothers' brains of the low dose group are similar to concentrations measured in autopsy tissue from Greenland (Dewailly et al. 1999). As discussed earlier, the mothers were exposed continuously until necropsy, for several months, so this might be comparable to humans who are exposed during their whole lifetime. However, the exposure of the offspring was stopped after weaning and as there were several weeks until necropsy, we should assume that the tissue

concentration in the offspring, during exposure, was higher than when it was measured at necropsy. Paper III also compared the POP levels in the mice plasma to the average blood levels of POPs from the Scandinavian population, published in (Berntsen et al. 2017). The comparison showed that for some compounds, plasma levels in the low dose offspring group are human-relevant. *p,p'*-DDE in humans is in the same range and even higher than the levels measured in the low group. Furthermore, the levels of most of the PCBs are only 10x higher than in humans. The BDEs, on the other hand, were mostly not detected in the offspring, whereas blood levels were up to 450x higher in the low dose exposed mothers than in human blood. PFOS levels were similar to human blood levels in the low dose offspring. PFHxS and PFOA were 5-6x higher and the remaining three PFAAs were 10-60x higher. The POP levels in the experimental diet have also been analyzed (Berntsen et al. 2017), showing that measured concentrations did deviate up to 30% from added levels in both directions. Contamination was detected for HCB, at 0.5, 8.04, and 2.24 ng/g in the three different feed types (normal mouse diet (RM1), reference diet including only the oil, and control diet from the study including both the oil and the solvents), respectively. But as these levels were low when compared to the actual levels (low dose 37.4 ng/g and high dose 588 ng/g feed), we consider the deviation to be of low significance. However, it is important to take into account the timing of measuring the concentration of the POPs, which was done after necropsy. Necropsy was done fairly shortly after the testing of the offspring, but for the mothers, this was months after giving birth and nursing the offspring, we, therefore, do not have an accurate measurement of the exact level the offspring were exposed to during pregnancy and nursing. Some of the POPs might have decreased in the mothers because of the nursing, but some of the POPs might have increased as they were further exposed during the last months before testing and necropsy. It is also difficult to say what the levels were in the offspring at the time of exposure, as we did not expose the offspring any further between weening and necropsy.

Although most of our statistically significant differences were found in the high exposed group, for both stress response (paper II) and learning (paper III), we did see the same trends in the corticosterone response in the low exposed group. In the cancer project (paper IV) we did see statistically significant differences in the colon in both the high group (number of flat ACFs) and the low group (number of tumors).

POP concentrations were also measured in the earlier mentioned study, done by Johanson *et al.,* and it showed that lipid-adjusted values of PCBs, OCPs, and BFRs were 2-35x higher than the average human blood levels (ng/g lipid). The levels of PFOS and PFOA were only 2x higher in the offspring's livers than in human blood and PFHxS levels were lower than in humans (Johanson et al. 2020).

It has to be taken into account that the exposure protocol in our studies were not optimal, because the mice were only exposed for a short period of time, and not during their whole lifetime, as humans are. Long-term effects of early-life exposure could not have been pinpointed if we let the mice stay on the POP diet. For the measures that we have, at least for some of these chemicals, the blood concentrations of maternally exposed offspring are human-relevant.

10.3.2 Relevance to humans

Are experiments done on mice relevant for humans? Mice share up to 99% of our DNA, have the same organs and bodily functions, and mechanistic similarities. This is one of the reasons why mice are so frequently used in studies to describe human disease and are often used as genetically modified models. It is also a practical choice, as they are small, cheap, easy to house, and readily available in many different strains. Evolutionally, only primates, and pigs are closer to us (Hewitt 2015). However, using primates in research has both ethical and practical challenges. In the introduction, we looked at some epidemiological studies indicating links between POPs levels and changes in neurobehavior issues. The difference between the epidemiological studies in humans and the experimental studies in mice is that the epidemiological studies in humans are observational studies and cannot prove a causal connection the way an experimental study in mice can. However, it is interesting to look at the epidemiological studies related to POPs and see how our study can substantiate what they have found.

Traditionally, chemical toxicology has focused on single components or technical mixtures. Regulations regarding the use of POPs are mainly based on risk assessments from single-component research, due to the great complexity of mixture toxicology. In real life, humans are exposed to hundreds of chemicals every day and we know very little about the possible interactions between them. Therefore, we decided to look at this mixture of POPs in this project. Our main aim of this project was to model a human-

relevant scenario in order to see how POPs can affect human health, and so the question arises whether the results of our animal experiments are transferable to humans.

As we have discussed, conducting an animal study to extrapolate to humans is a difficult thing and has many challenges and limitations. We had to do some practical compromises and had some unforeseen challenges like for example increased mortality as well as limitations on time and resources. When extrapolating our findings to humans you must consider the limitations of the projects, like for example that the BM was not optimal and that the cancer project was divided into two separate experiments. Although these limitations might count against extrapolating our results to humans, we wanted to see if any epidemiological studies in humans have similar findings as our studies.

POPs and change in behavior in humans

Our findings indicate that POPs affect both corticosterone levels and gene expression connected to learning and memory.

The European Commission estimates that as much as 5% of school-age children in Europe are affected by ADHD, which is often seen in connection to learning disabilities and changes in behavior. This is an emerging disorder that was not recognized as a disorder by the American Psychiatric Association (APA) until 1968. There is a rising concern over the increase of mental health issues in young adults over the last decades (Twenge et al. 2019) and Kramer even foresaw it as a pandemic several decades ago (Kramer 1980). In Norway, the number of diagnoses of ADHD has increased. During 2008-2013 4.3 % of boys and 1.7 % of girls (age 6 to 17) were diagnosed with ADHD and the use of stimulant drugs increased during 2005-2010 and then stabilized (Ørstavik R 2016). The number of people dying of dementia in Norway has also increased from 596 in 1990 to 2,909 in 2018 (NCDR 2018). Is the timing of these findings a coincidence or is it possible that POP exposure contributes to the observed increases in neurological disorders? Even though the production and use of some POPs stopped in the 1970s, exposure will still continue for decades due to their persistence. After the bans, similar compounds have been produced and all this results in the fact that we still see them in blood samples in the population today (García-Villarino et al. 2020). Furthermore, POPs have the ability to cause trans-generational effects, both via DNA damage and epigenetic effects, which means that we may see the effects of past exposure on future generations. A review of studies between 2005 to 2015, looking at

associations between PCBs, PBDEs and DDE and developmental neurotoxicity in children found inconclusive results. While some studies reported negative effects, others showed inverse associations, and yet others found no effects. They did, however, find evidence for an association between increased prenatal PCB levels and declining motor maturity, exposure to PBDEs and lower mental development, psychomotor development, IQ, and poorer attention. Also, exposure to DDE had an effect on psychomotor development, on attention, and ADHD (Berghuis et al. 2015). As I mentioned in the introduction, there are several epidemiological studies researching POPs and their effect on behavior in human cohorts. Table 5 present a collection of these studies looking at the same POPs as in our mixture.

Table 5: Overview of references showing neurobehavioral changes in connection to exposure to POPs in epidemiological studies. PubMed was used and several different search words was chosen (for example POPs, epidemiological, cognition, stress, behavior, neurobehavior, learning, memory, neurodevelopmental effects).

Chemical	Description	Ref
Organochlorines	Cognitive deficits	(Kilburn et al. 1995)
PBDEs	Reading skills ↓ FSIQ ↓ behavior problems ↑	(Zhang et al. 2017)
PFAS	IQ ↓	(Liew et al. 2018)
PBDEs		(Lam et al. 2017)
DDE	FSIQ ↓ Processing Speed ↓	(Gaspar et al. 2015)
PCBs	Behavior problems ↑	(Kim et al. 2018)
PBDEs		(Vuong et al. 2018)
p,p'-DDT		(Forns et al. 2016)
PFAS		(Harris et al. 2021)
PBDEs	Reading skills ↓	(Zhang et al. 2017)
PCBs / PFAS	Reading skills ↑	(Vuong et al. 2020)
Insecticides	ADHD ↑	(Brown et al. 2018)
PBDEs		(Cowell et al. 2015)
PCBs		(Panesar et al. 2020)
β-HCH / PFOS		(Lenters et al. 2019)
p,p'-DDT	ADHD ↓	
PCBs	Language skills ↓	(Caspersen et al. 2016)

PBDEs PFAS β-HCH	Neurodevelopmental problems	(Vrijheid et al. 2016) (Spratlen et al. 2020) (Wang et al. 2021)
HCB / β-HCH / p,p'-DDE	Head circumference ↓	(Wang et al. 2021)
PCBs / PFAS	Mental development problems ↑ Behavior problems ↑	(Ruel et al. 2019) (Harris et al. 2021)

The Scandinavian studies looking at POPs and their effect on behavior are especially interesting to our study. For example, a study into 1,592 pregnancies enrolled in the Danish National Birth Cohort showed prenatal exposure to PFASs was associated with IQ scores (Liew et al. 2018). The Norwegian Human Milk Study (HUMIS), including 612 mothers, found that *p,p'*-DDT in breastmilk samples were associated with behavior problems in the children (Forns et al. 2016). In another study from HUMIS, looking at 1,199 mother-child pairs, the researchers found increased odds of ADHD in children with high β-hexachlorocyclohexane (β-HCH) and PFOS and lower odds of an ADHD diagnosis with increased *p,p'*-DDT in breastmilk (Lenters et al. 2019). In a different study from Norway, including 44,092 children in the Norwegian Mother and Child Cohort Study (MoBa), the researchers found that girls born by mothers with high dietary exposure to PCB153 may have an increased risk of language delay and negative associations for both boys and girls having incomplete grammar (Caspersen et al. 2016).

As we can see in table 5 and especially in these Scandinavian studies, there are several epidemiological studies done in human cohorts that show evidence of POPs affecting neurobehavior problems and things like IQ and reading skills. This does indicate that POPs can affect learning and memory, which is in line with our gene expression findings. Although, there are studies that also show no link to for example autism spectrum disorder (Hamra et al. 2019), a positive association of PCB and PFAS with reading skills (Vuong et al. 2020), and a decreased connection to ADHD (Lenters et al. 2019). The reason for these discrepancies can be for example that different chemicals have different effects. It is also important to remember that epidemiological studies in humans usually have their origin in large screenings of the population without really knowing what you are looking for. And the data is often used in several different projects much later. The advantage is that you have a large number of samples, but the

risk is that you find even small connections, that might not really have an impact, and cause an effect are harder to prove because of all the other variables that influence the results. Compared to a laboratory animal study, where the disadvantage is that you have a smaller number of samples, but the advantage is that you can set your hypothesis beforehand, you expose the animals to the very thing that you want to investigate, you standardize all the other variables, and if you then find an effect, you are much closer to proving the cause and effect that you are looking for.

POPs and cancer in humans

Our results show that POP exposure increases the risk of developing CRC in mice (paper IV). Can we extrapolate this to humans?

Regarding mouse models for CRC, also the *Apc* gene, which harbors the critical mutation in the A/J Min/+ mouse is very similar between humans and mice, with 86% of the nucleotides and 90% of the amino acid sequence being identical (Su et al. 1992). other CRC models, that mainly get lesions in the SI, are not good models for cancer in the colon. The A/J Min/+ mouse is more relevant, as they do get lesions in their colon, but they do get lesions in the SI as well.

As I explained in my introduction, several POPs have been associated with several types of cancer. And cancer in humans has been increasing in parallel with the increase of exposure to pollutants in the environment. Is this a coincidence? It has been reported that several countries have a substantial increase in the incidence of CRC in people younger than 50 years (Araghi et al. 2019). Another study also discusses the increase in testicular cancer. They found that blood POP levels in mothers were indicative of the sons' testicular cancer risk. And that the sons born during the 60s and 70s, when the body burden of POPs was very high, had an increased testicular cancer risk, compared to the started decrease we have now that the body burden of POPs has started to decrease (Hardell et al. 2006).

A review, looking at breast cancer studies, found that POPs measured in breast adipose tissue were associated with a higher risk of breast cancer development and worse breast cancer prognosis (Ennour-Idrissi et al. 2019). A scan of cancer patients in Korea in 2018 showed that chronic exposure to low-dose POPs could be associated with an increased

risk of colorectal polyps and cancer (Lee et al. 2018). Again, epidemiological studies show that POPs can affect the development of cancer in humans, and this is in line with results from our experimental research. Once again, this indicates relevance to humans.

11 Conclusions

The hypotheses for this PhD project were:

a) POPs have the ability to influence learning and behavior.
b) POPs contribute to the development of cancer.

The results from our studies cannot fully support the first hypothesis, although the expression of hippocampal genes related to learning and memory was altered. More research must be performed in order to assess whether the hypothesis related to phenotypic change can be supported. However, our results indicate that POPs could make us more sensitive to stress, which is also an important finding. We do have results that can support the second hypothesis, so this hypothesis is confirmed. Still, the relevance of this result is a question of dose.

To sum up, the main conclusions drawn from this PhD project are as follows:

- Different mouse strains perform differently in behavioral tests. The 129:C57BL/6 hybrid mouse seemed to be more sensitive to stress than C57BL/6
- Exposure to a human-relevant mixture of POPs had no effect on anxiety in the 129:C57BL/6 hybrid mouse in the OF.
- Exposure to a human-relevant mixture of POPs altered the stress response in the 129:C57BL/6 hybrid mouse, by increasing the basal corticosterone level in the mothers and increasing the stress-induced corticosterone level in high exposed male offspring. In addition, the exposure to the high dose gave enlarged adrenals and enlarged adrenal cortices in the mothers and their offspring.
- Mouse offspring exposed to a human-relevant mixture of a high dose of POPs performed poorer in the BM when a stressor was introduced.
- Exposure to a human-relevant mixture of POPs did make changes in hippocampal gene expression related to cognitive function.
- Exposure to a human-relevant mixture of POPs did increase the intestinal tumorigenesis in the A/J Min/+ mouse, in both the high and low exposed group, and indicated synergistic effects when combined with AOM.

- Even though the POP concentrations in the experimental diet were much higher than in human diets, several of the measured concentrations in the offspring mice were human-relevant.
- As the experimental diet was based on the Scandinavian diet and some of the measured levels were relevant to measurements done in the Scandinavian population, the results indicate that POPs can lead to altered stress responses and the development of cancer.

12 Future perspectives

In this PhD project we looked at the effects of exposure to a mixture of POPs on the stress response, learning, memory, and development of CRC. When analyzing the results and writing the thesis several new ideas appeared, and in addition, there are several remaining questions that the project could not answer that would need further research.

12.1 Behavior

The effect on the stress response and enlarged adrenal glands we found in our studies elicits a need to further study the effects of POPs on the HPA axis. It would be interesting to analyze gene expression in the pituitary gland, which is the master gland in endocrinology and thus plays an important role in the HPA axis. For example, qPCR on the pituitary gland tissue could be performed, looking at the expression of the GR gene (*NR3C1*) encoding GR, to look for poorer negative feedback as a possible explanation for the increased stress response in the male offspring. Further qPCR on the CRHr gene could also be interesting to form a picture of the signal from the hypothalamus to the pituitary gland. It would also be interesting to study the expression of the ACTH receptor gene on the adrenal gland tissue, as ACTH is important both for its trophic effect on the adrenal gland and for adrenal steroidogenesis.

We would also like to look closer at the sex difference we found in the offspring. As I discussed earlier, the answer to this might lie in the different sex hormones. Measurement of hormone levels, for example, estrogen and testosterone, levels of the estrogen-induced transcortin expression, and expression of ERs and ARs in different exposure groups, would contribute to a better understanding.

It would also be interesting to do more behavioral studies to look closer at learning abilities and to conclude if the changes were due to an altered response to stress or an altered ability to learn. We could for example do BM without motivators or Intelligence Place Learning tests. Other combinations of POPs in the mixture and lower levels, closer to the levels in human diet, as well as smaller mixtures containing only one group of POPs, for example, the brominated compounds alone would also be relevant. It would also be interesting to compare the effects of our mixture with other mixtures that

are designed, such as mixtures based on human exposures in different parts of the world, and mixtures present in environmental compartments, extracted from for example cod liver.

To test mixtures with different compositions would have added knowledge to the complete picture of human exposure to POPs. For example, adding dioxins might give different effects. And also, adding less persistent chemicals, such as Bisphenol A with its estrogenic effects, would be interesting.

In general, more studies using human-relevant complex mixtures of chemicals should be conducted. And as humans are exposed during their whole lifetime, more long-term studies would have benefitted the overall knowledge of environmental toxicology. This is important for the protection of both human and animal health and in combination with what we know about the single components, it would improve the accuracy of risk assessments. As we exposed the offspring during early life, but not into adulthood, and we exposed the mothers long into adulthood, but not from early life, it would be interesting to conduct an experiment covering both these exposure plans, even into old age. That would be even closer to the way humans are exposed throughout their whole lifetime.

12.2 Cancer

Our results show that POPs affected the development of CRC. For a deeper understanding of how the POPs affect the carcinogenesis and what the synergistic effect was based upon, several investigations must be conducted on a mechanistic level. We would have to investigate each individual POP to look for potential effects on gene regulation and what types of genes/proteins they affect. In addition, also different types of mixtures and in lower doses should be included.

It would also be interesting to add dioxins like TCDD and dioxin-like PCBs to the mixture, as these are known carcinogens, to see if these have effects on CRC.

More long-term studies should be done, looking at transgenerational effects on cancer and the effect on health in old age.

13 References

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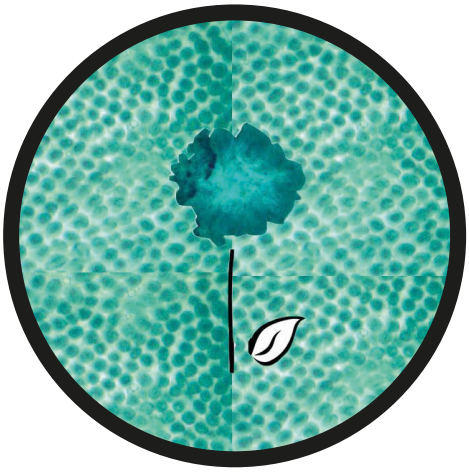
Paper I



Comparison of young male mice of two different strains (C57BL/6J and the hybrid B6129SF1/J) in selected behavior tests – A small scale study –

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Abstract

In this study, we compared young males of two mouse strains, C57BL/6J and the hybrid B6129SF1/J, and gained knowledge on their performance in three different behavioral tests; open field (OF) test, Barnes maze (BM) test and a restraint stress test. We found that the young males of the C57BL/6J strain spent more time moving in the OF. In the BM, the hybrid covered less ground before reaching the goal box during the first three sessions, than the C57BL/6J. The hybrid left more fecal pellets than C57BL/6J both in OF and BM. During the stress test, the C57BL/6J had a lower corticosterone response than the hybrid. Our findings indicate that the C57BL/6J has a presumably higher locomotor activity and/or explorative behavior than the hybrid, while the hybrid appeared more sensitive to stress.

Keywords

Mouse strain, C57BL/6J, hybrid B6129SF1/J, behavior test, open field test, Barnes maze test, stress test.

Introduction

Differences in behavior between mouse strains are frequently observed in animal facilities, and certain strains are well known, and often chosen, for a certain type of behavior. Also, in behavior tests differences between strains are well documented (Owen et al., 1997). Thus, the strain used for behavioral tests should be carefully considered. For future behavior experiments, we wanted to test two possible strains of mice, to see which one would be more beneficial for our purposes. The ideal strain would be a good learner, robust in stressful situations, gentle mothers, easy to breed, group-house and handle. The C57BL/6J mouse is the most widely used inbred strain in laboratory animal research and is used in a widespread of research fields, including cancer research, diabetes/obesity research and behavioral/learning research. The breeders`, Jackson Laboratory, webpage "Mouse Phenome Database" (https://phenome.jax.org/) lists 282 studies using this strain. The hybrid B6129SF1/J is described as having hybrid vigor and is used in for example tissue transplantation research. It has also been used in behavior studies for several years (Bale et al., 2002). The database shows 9 studies using the hybrid. When asking the database to compare the two strains, there were no datasets found and several searches in several other search tools showed that research comparing these two strains in behavioral tests is scarce. In the experiment described, only the two first beneficial traits listed were tested; learning ability and stress-responsiveness. The two mouse strains were tested in three different behavioral tests, an open field (OF) test, a Barnes maze (BM) test and a restraint stress test. Previous reports conclude that C57BL/6J is a locomotory active strain

in the OF, while the hybrid would be in between C57BL/6J and 129 (Bolivar et al., 2000). Chan *et al.* found the C57BL/6 to have a larger corticosterone output than the similar hybrid in response to acute stress (Chan et al., 2017). However, still, little is known about differences between these two in the BM and corticosterone response to stress. The aim of this article is to add to the knowledge about the behavior of these two mouse strains in the OF, BM and a restraint stress test.

Materials and methods

Ethics statement

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The animal facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (https://www.aaalac.org/). The animal experiment was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) and the Food Safety Authority (application ID: FOTS 4247, 2013/39783) and executed in compliance with the local and national regulations associated with laboratory animal experiments. The rodent and rabbit section of the facility is a Specific Pathogen Free (SPF) unit. It follows a health monitoring program recommended by the Federation of European Laboratory Animal Science Associations/FELASA (http://www.felasa.eu/). The care of the animals was carried out by two veterinary nurses with FELASA B certification and three researchers with FELASA C certification performed the experiments.

Animal models

10 male C57BL/6J mice and 10 male hybrid B6129SF1/J mice (both from Jackson Laboratory, Maine, USA) were used. The mice were 5 weeks old at arrival and were acclimated to the unit for one week before testing started. The age and sex of animals were chosen to avoid female reproductive cyclic variation and as males tend to track the females in behavior tests, which could interfere with results in such a small-scale study.

Housing and husbandry

The animals were housed in open type III cages (Tecniplast, Buguggiate, Italy) in groups of 5 during the acclimatization time and single housed from the day prior to the first test and during the testing period. The reason for the single housing during the testing period was to avoid stress due to fighting during the testing. The cages contained standard aspen bedding (Scanbur BK, Nittedal, Norway), cellulose nesting material and a Bio-Serv igloo as a hide (Bio-Serv, Frenchtown, NJ, USA).

The animals were given a standard maintenance diet (RM1 from SDS, Witham, UK) and tap water ad libitum. The animal room was on a 12:12 light-dark cycle, from 08.00 in the morning and 20.00 in the evening, with a room temperature of 21 ± 2 ^{0}C with 20 air changes per hour and 45 ± 5 % relative humidity. The cages and bedding were changed twice a week for group-housed mice and once a week for single housed mice, and the water was changed daily. The animals were not disturbed 24 hours before testing. All mice were tested in all 3 behavior tests: First OF, then BM and then the stress test, with a couple of days of rest between the tests. The OF and BM were done in a procedure room next to the housing room, but the stress test was done inside the housing room, to avoid stress by transporting right before the test. After behavioral testing, all animals were euthanized using cervical dislocation.

Open field (OF) test

The OF testing was done in the animals` light cycle, after working hours (16.00-20.00), for calm testing conditions. The OF testing arena was a white plexiglass box 50 × 50 × 22 cm (Noldus, Wageningen, the Netherlands) with a bright light (Lupoled 1120; approx. 120 lux) placed above. The animal was lifted by the tail, carried on the arm and gently placed inside a disposable nontransparent cardboard cylinder (guinea pig play tunnel from Scanbur BK, Nittedal, Norway) in the center of the arena and left there for 3 seconds. When released from the cylinder, each mouse was tracked for 15 minutes. The animals were filmed by an Ikegami ICD-49E B/W infrared camera fixed in the ceiling and tracking was done by the computer program Ethovision XT 9.0 (Noldus, Wageningen, the Netherlands). Urine puddles and fecal pellets left in the arena were recorded manually. During the analysis, the floor in the box was divided into 3 zones: Center zone, corner zones and border zones alongside the walls of the box. The three researchers performing the experiments had fixed tasks, one handling the animals, one performing the tracking and one preparing the testing arena between each test.

Barnes Maze (BM) test

The BM testing was done in the animals` light cycle during working hours (08.00-16.00). The BM testing arena was a round platform, 100 cm in diameter, with 20 holes, one of which had a black goal box beneath (Noldus, Wageningen, the Netherlands). There were spatial room cues placed on the walls, to help the mice navigate. The animal was handled the same way and filmed by the same camera and tracking was done by the same computer program, as for OF. Each mouse was tracked until it reached the goal box or for 4 minutes. If the animal had not located the goal box by 4 minutes, it was gently guided to the box. All animals were trained for 2 sessions every day, morning

and afternoon with four hours in between the sessions, for 3 days. To account for the risk of reduced motivation that occurs in repeated tasks a new motivator was added every day as described by Müller and Bale (2007):

Day 1: Session 1+2: Bright light over the platform (Lupoled 1120; approx. 120 lux)

Day 2: Session 3+4: Bright light + blowing fan.

Day 3: Session 5+6: Bright light + blowing fan + high sounding buzzer.

Urine puddles and fecal pellets left on the platform were recorded manually. The three researchers performing the experiments had fixed tasks, one handling the animals, one performing the tracking and one preparing the testing arena between each test.

Stress test

The stress test was performed in the animals' light cycle during working hours (08.00-16.00). The mouse was restrained inside a 50 mL falcon tube for 15 minutes, on a table next to the home cage. Blood samples were taken from the tip of the tail at 4 different time points: 0 (start), 15 (before release), 30 and 120 minutes (after release). The mouse was allowed to rest in its home cage in between the 15, 30 and 120 minute samples. All samples were taken with a 20 µL Minivette POCT capillary collecting tube coated with EDTA (Sarstedt, Nümbrecht, Germany) and transferred to an Eppendorf tube on ice. The blood samples were spun at 5000 rpm at 4 ^{0}C for 10 minutes to obtain the plasma, which was stored at -80 ^{0}C until further analyses. Corticosterone was measured in the plasma using an MP Biomedicals ImmuChem™ Double Antibody Corticosterone 125 Ria Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions.

Statistical analyses

The results of the data were analyzed in JMP Pro 13 ® (SAS, Cary, NC, USA) comparing the two groups using the Student's t-test. Corticosterone levels were analyzed across time, using a Student's t-test for each time Bar graphs and line charts were made in Excel ® (Microsoft, USA) presenting the data in means. We chose not to include the data from the total distance moved in the OF figure, not to misalign the axis. P-values ≤ 0.05 were considered statistically significant.

Results

Open field (OF) test

The C57BL/6J were moving more and covering more ground during the OF than the hybrid, but only the time spent moving was significantly different from the hybrid (p=0.025). Consequently, the time spent not moving was also significantly different between the strains (p=0.025, figure 1a). The

number of fecal pellets left by the hybrid was significantly higher ($p<0.004$) than by the C57BL/6J, and also, the number of urine puddles had the same trend for the hybrid, but was not significantly higher (table 1).

Barnes maze (BM) test

Both mouse strains showed progress in learning as the time used before entering the goal box decreased for each session, except for the fourth and fifth sessions for the hybrid and fifth session for C57BL/6J (figure 2a). The hybrid was slightly, but not significantly quicker than C57BL/6J in sessions two and three. In the three last sessions, the C57BL/6J was slightly quicker, also not significantly. The hybrids covered significantly less ground before they reached the goal box in the three first sessions, than the C57BL/6J ($p<0.001$, $p<0.001$, and $p=0.003$, respectively) (figure 2b). The number of fecal pellets left by the hybrid was significantly higher ($p<0.001$) than by the C57BL/6J, and also, the number of urine puddles had the same trend for the hybrid, however this was not significant (table 1).

Stress test

Both strains had a similar baseline of corticosteroid blood levels at the starting point of the stress test. The C57BL/6J did have a slightly lower concentration of corticosterone at all time points after that, resulting in the response curve of the C57BL/6J being flatter than the hybrid's (figure 3). However, the only significant effect of mouse strain was at 30 minutes ($p<0.001$) which was the time-point of peak concentration in both strains.

Discussion

In the present study, we compared two different types of mouse strains (C57BL/6J and hybrid B6129SF1/J) using three different behavior tests (OF, BM and stress test). We found that the C57BL/6J were more locomotory active during the OF by spending more time moving than the hybrid. Both strains learned the BM, but the hybrid covered less ground before they found the goal box during sessions 1, 2 and 3. During the stress test, the C57BL/6J had a lower stress-induced corticosterone response than the hybrid.

As this small scale study only includes young males, more studies should be done to fully understand the differences between these two mouse strains. As mentioned, the reason for choosing the sex and age of the animals was done to avoid sexual behavior and female reproductive cyclic variation disturbing the results. The mice were single housed from the day prior to test start, to avoid fighting during the testing period. This change in housing close to test-start is not optimal as

it can cause distress that might affect the results. On the other hand, the alternative, group- or pair-housing, could lead to fighting and building of hierarchies that would also affect the results. Nevertheless, the change should ideally have been performed with more time to acclimatization to the facility and to the new situation of being single-housed.

Open field (OF) test

The OF measures locomotor activity and anxiety-like behavior in mice (Kraeuter et al., 2019). Under normal conditions, mice will do explorative behavior and use the whole box and also venture out into the center zone of the box. Mice with anxiety will spend more time in the corners, trying to find a place to hide. As these behavioral differences between groups of mice can be confounded by differences in exploratory and/or locomotor activity, it is suggested that more tests should be performed in order to conclude from an OF study. Furthermore, a good alternative for measuring locomotor activity would be to track the mice in their home cage during the night, when the mice are active (Stanford, 2007).

In the present study, both mouse strains spent the same amount of time in both the center zone, the border zones and the corner zones, but the C57BL/6J did spend significantly more time moving, suggesting this mouse strain to be more explorative and active. Although there was a trend, distance moved in the OF was not different between the two strains. Other studies also found a higher activity level of the C57BL/6J than the hybrid in the OF. Bolivar *et al.* compared inbred strains and F1 hybrids of 129S3/SvImJ, A/J, BALB/ cByJ, C3H/HeJ, CBA/J, DBA/2J, FVB/NJ, (B6 × 129)F1/J and (B6 × C3H) F1/J. This study confirmed that the genetic differences in the different strains affect the intersession habituation to the OF. In their results they ranked the strains by total distance traveled in the OF and the C57BL/6J ranked higher than the B6129F1 hybrid, for both males and females (Bolivar et al., 2000). Logue *et al.* also compared twelve different strains of inbred mice and seven F1 hybrids in several behavioral tests including OF, and they also found the C57BL/6J to be more active, although they used a 129B6F1 hybrid (Logue et al., 1997).

The lack of strain differences in time spent in the center zone indicates no strain differences in anxiety, however, a significantly higher frequency of defecation by the hybrid could suggest this strain to be more anxious. The release of urine and feces is the reaction to the fight-or-flight response that comes with the stress response, in many species (Jiang and Damaser, 2011), also mice. Originally, OF was designed to record defecation as a measure of emotionality (Stanford, 2007).

Barnes maze (BM) test

There are several different versions of BM tests and protocols. The protocol we chose has been validated and frequently used for learning performance (Mueller and Bale, 2007, Fuentes et al., 2012). The BM measures spatial learning and memory in mice (Pitts, 2018). It is an alternative to the Morris Water maze and offers the advantage of being free from the potentially confounding influence of swimming behavior. The BM will measure learning impairments, as shown by a study in which induced traumatic brain injury in C57BL/6 mice resulted in a significantly longer time to learn the BM (Lee et al., 2019). Under normal conditions, mice will use the spatial cues to remember where their goal box is, and adding motivators to succeeding sessions will motivate them to perform quicker for each session. O´Leary and Brown tested the C57Bl/6J in different BM scenarios and found that mice do not use the visuospatial cues to locate the escape hole on the small-diameter maze (69 cm) with a wall and intra-maze visual cues, but they do use the visuospatial cues on small or large diameter mazes (122 cm) with no wall (O'Leary and Brown, 2012). In a different study, this research group tested 13 inbred strains in the BM, and found that the use of visuospatial cues is dependent on the strain and their visual ability, as some stains have reduced sight (O'Leary et al., 2011). There is a theory, put forth by Illouz *et al.*, that mice use different strategies to find the goal box, some use the spatial cues actively and go straight for the goal box, and others randomly search the maze until they find it, using speed as their strategy (Illouz et al., 2016). The fact that the hybrid of the current study covered less ground to find the goal box during sessions 1, 2 and 3 may again indicate that this strain is less explorative and active than C57BL/6, however, it may also mean they used different search strategies. A cued strategy will require less ground than a serial or random strategy. Some mice have a strong preference for using the room cues in their strategy (Harrison et al., 2006). Still, the hybrid was not significantly faster than C57BL/6 in finding the goal box, therefore, C57BL/6J more likely used speed and exploration in its search. O'leary *et al.* also discuss that mice from the 129 sub-strains show lower levels of exploration than the C57BL/6. This fits well with our observations of the hybrid containing this strain.

In the present study, motivators were used to avoid habituation of the repeated session. In the first two sessions, the motivator was a bright light, and in the third and fourth sessions, a fan was added. In the fifth session, the third motivator, a buzzer, was added. The motivators used in the current study seemed to affect the hybrid more severely than C57BL/6 as their progress was reversed during sessions 4 and 5, while the progress of C57BL/6 was only slightly reversed during session 5. Using stressful motivators can combine testing of learning and stress handling (Inman-Wood et al., 2000), and the motivators may distract the mice in their learning (Gawel et al., 2019). In this study, the motivator added in session 5 seemed to distract both strains and did not serve its

purpose. Using positive motivators could be a solution if motivation is needed. Youn *et al.* showed that DBA/2J mice, that originally performed poorer than the C57BL6J mice in the BM, first trained with no motivators and then with a fan as a motivator, actually outperformed them when including a positive motivator (almond chips) (Youn et al., 2012)

Stress test

There are many different tests measuring stress response in mice, for example, the tail suspension test and chronic restraint stress tests. Choosing the correct test would have to be a compromise between good animal welfare and comprehensive results. Taking these into account, we chose the test restraining the mice in falcon tubes for 15 minutes (Zimprich et al., 2014). This test is often called a hypothalamus-pituitary-adrenal axis responsivity test, as it indicates a reaction in this endocrine axis. As mice are prey animals, being restrained in a transparent tube, without the ability to flee or hide, naturally creates a stress response. After the 15 minute restraint, the mouse is allowed to calm down in its home cage, and the levels of corticosterone will slowly go back to normal levels. This stress hormone response is critical for the survival of all species, but chronic high levels of stress hormones have adverse effects on health (McEwen, 2008) and may result in mental problems (Söder et al., 2020, Pal et al., 2019). Therefore, the best way for the body to handle stress is to elicit an appropriate stress hormone response to a stressor followed by efficiently lowering the levels when the threat subsides.

In the present study, the baseline concentration was almost similar for the two strains. Expectedly, both mouse strains responded with elevated corticosterone concentrations following the restraint. However, the hybrid had significantly higher levels after 30 minutes than the C57BL/6J. This shows that the C57BL/6J has a lower corticosterone response to stress, while the hybrid, reacts more severely in terms of corticosterone response to the same acute stressor. Chan *et al.* also performed a similar study. They used the stress test to compare the C57BL/6, the 129, and the C57BL/6:129 hybrids. The F1 hybrid (BL/6 mother and 129 father) showed similar corticosterone levels (150 ng/ml), as observed in the current study (162,8 ng/ml), however, interestingly, the C57BL/6 mice had the highest stress-responsive levels. It rose to over 200ng/ml after 15 minutes of restraint (Chan et al., 2017).

Conclusion

Taken together, the adolescent males of the two strains compared behaved differently in the behavior tests and reacted differently to restraint stress in terms of corticosterone levels. C57BL6/J spent more time moving in the OF and moved a longer distance to reach the goal box in the three

first sessions of the BM than the hybrid. This may indicate a higher locomotor activity and/or explorative behavior in C57BL6/J, although search strategy in the BM also may play a role. This presumably more physically active, explorative strain reacted with a lower corticosterone increase than the hybrid to restraint stress. The hybrid appears more sensitive to stressors as they left more feces in both the OF and the BM, and showed a higher corticosterone response to restraint. If only performance in these behavior tests were taken into account, the C57BL6/J strain would be the most robust. However, there might be many other traits of interest when choosing a model strain. Again, it must be emphasized that these results are only valid for adolescent males of the two strains as no females or mice at other ages were tested.

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Table 1

Summary of results from open field test and fecal pellets/urine puddles (left in open field and Barnes maze) from two different mouse strains (C57BL/6J and hybrid B6129SF1/J). Results are presented as means. Differences between the strains were assessed with Student´s t-test and indicated in bold when significant ($p \leq 0.05$).

Strain	**Mean distance moved in total (cm)**	**Mean time spent moving (sec)**	**Mean time spent not moving (sec)**	**Mean time spent in center zone (sec)**	**Mean time spent in corner zones (sec)**	**Mean number of fecal pellets OF**	**Mean number of urine puddles OF**	**Mean number of fecal pellets BM**	**Mean number of urine puddles BM**
C57BL/6J	7088	**713**	187	233	304	0,30	0,20	0,33	0,08
Hybrid B6129SF1/J	6239	657	**243**	237	313	**0,90**	0,40	**0,70**	0,20

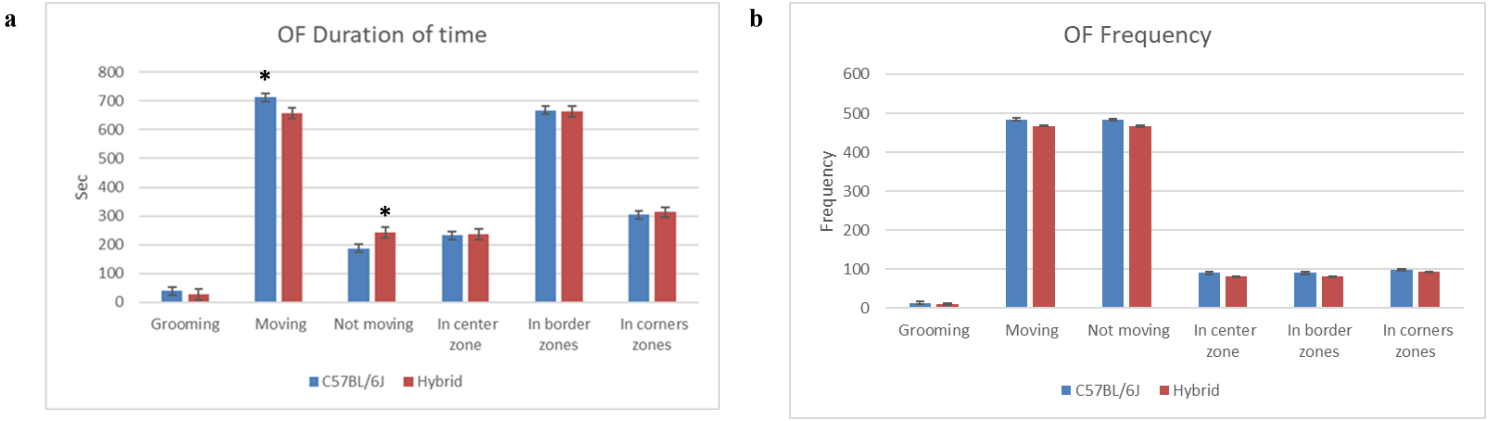
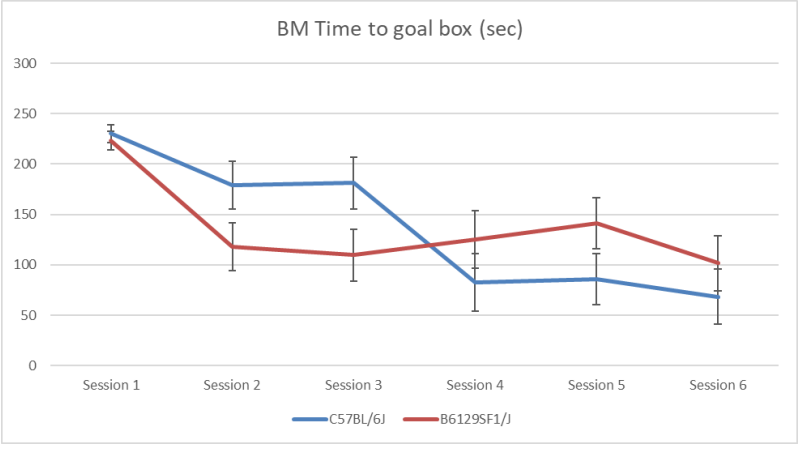


Figure 1

Summary of the results from the open field test of two different mouse strains (C57BL/6J and hybrid B6129SF1/J). Results are presented as means (±SE) and indicated with * when significant ($p \leq 0.05$). Total distance moved is not included. **a** shows the duration of time spent grooming, moving, not moving and in the different zones, presented in seconds. **b** shows the frequency of grooming, moving, not moving and in the different zones.

a



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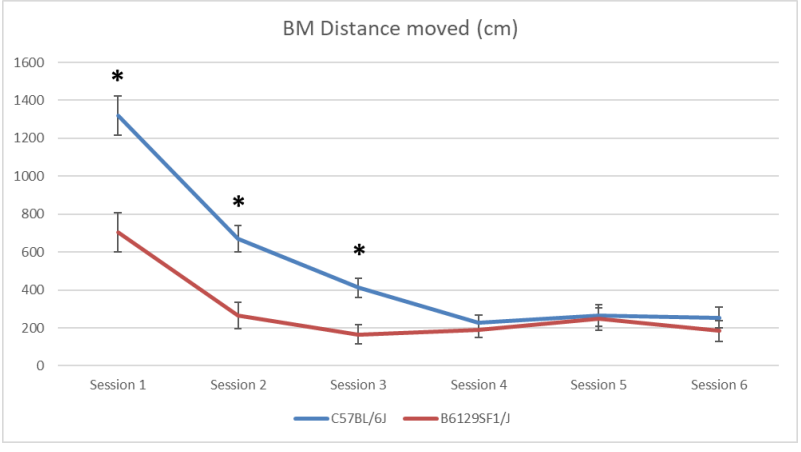


Figure 2

Summary of the results from the Barnes maze test of two different mouse strains (C57BL/6J and hybrid B6129SF1/J). Results are presented as means (±SE) and indicated with * when significant ($p \leq 0.05$). **a** shows time spent to find the goal box, presented in seconds. **b** shows distance moved before the mouse entered the goal box, presented in centimetres.

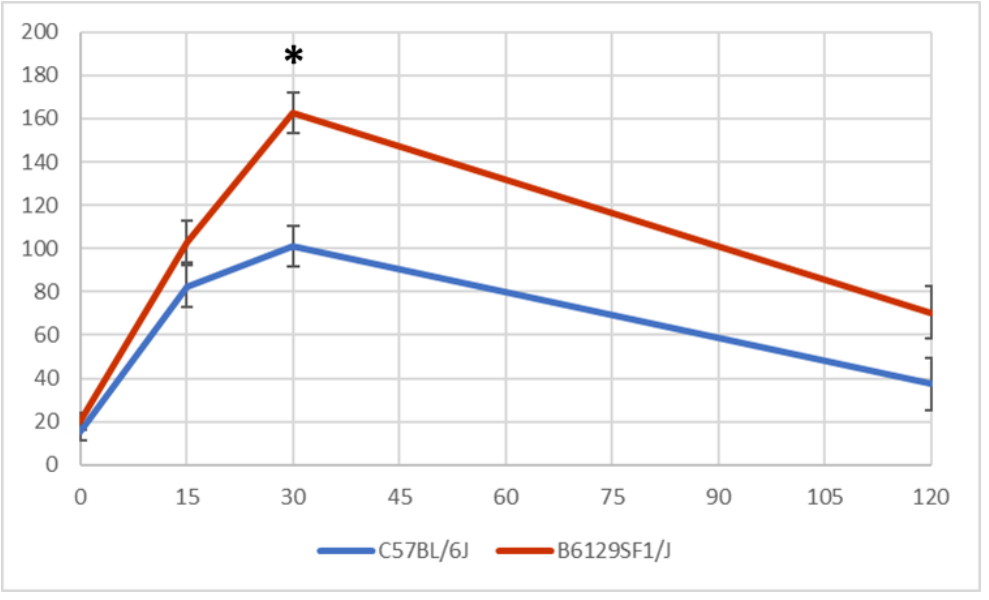
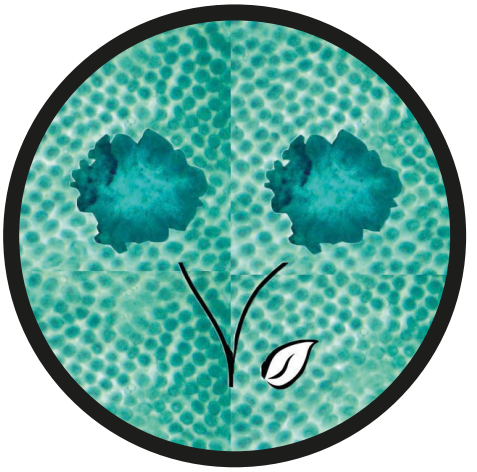


Figure 3

Summary of the results from the stress test of two different mouse strains (C57BL/6J and hybrid B6129SF1/J). Results are presented as means (±SE) of blood concentration of corticosterone (ng/ml) at each different timepoint (minutes) and indicated with * when significant ($p \leq 0.05$).

Paper II



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A human exposure based mixture of persistent organic pollutants affects the stress response in female mice and their offspring

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HIGHLIGHTS

- Increased basal cortisol in mothers exposed to POPs.
- A prolonged stress response in mothers exposed to POPs.
- An over-sensitised stress response following POP exposure in male offspring.
- No effect of POPs in female offspring.

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ABSTRACT

Persistent organic pollutants (POPs) are found in the food chain of both humans and animals and exert a wide spectrum of potentially adverse effects. The present experiment aimed to investigate whether a defined mixture of 29 POPs, based on the dietary intake of Scandinavians, could affect the stress response in female mice exposed through ingestion, and in their offspring. Female mice 129:C57BL/6F0 hybrids were exposed from weaning, throughout pregnancy, and up until necropsy, to either 5000 × or 100 000 × the estimated daily intake for Scandinavians. The offspring were fed a reference diet containing no POPs. Both the mothers and their offspring were tested for basal and stress responsive corticosterone levels, and in an open field test to measure locomotor activity and anxiety-like behaviours. We found mothers to have elevated basal corticosterone levels, as well as a prolonged stress response following POP exposure. In the offspring, there was no effect of POPs on the stress response in females, but the exposed males had an over-sensitised stress response. There was no effect on behaviour in either the mothers or the offspring. In conclusion, we found a human relevant POP mixture can lead to subtle dysregulation of the hypothalamus-pituitary-adrenal axis in mice. As HPA axis dysregulation is commonly associated with neurological disorders, further studies should explore the relevance of this outcome for humans.

1. Introduction

Persistent organic pollutants (POPs) are organic compounds that are resistant to degradation and so remain within the environment for extended periods of time. These compounds were produced for use in industry and agriculture, but were subsequently found to have wide-ranging toxic effects in both humans and wildlife (for a review, see Jones and de Voogt, 1999; Carpenter, 2006). This has led to many POPs being banned from production via the Stockholm Convention, but some are still in use due to a lack of alternative compounds or strict regulation. Due to their resistance to

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degradation, POPs have been transported around the globe, primarily via wind and water, and continue to persist within the environment (Law et al., 2014). Furthermore, POPs bioaccumulate within living organisms and biomagnify up the food chain, even in locations previously considered pristine (e.g. Oskam et al., 2004). As such, POPs continue to be a considerable human and environmental issue.

To date, around 30 POPs are listed in the Stockholm Convention, but these may have multiple congeners. For instance, there are 209 possible congeners of polychlorinated biphenyls (PCBs). Due to the substantial number of compounds and their ubiquitous nature within the environment, vast cocktails of POPs are typically found in both humans and animals at any given time (i.e. Costopoulou et al., 2006). As such, there is a need to understand how such a body burden can influence an individual's health. The traditional approach has been to study single compounds at relatively high doses, but subsequent research would indicate that toxicants can have biphasic response curves, and/or additive, synergistic, or antagonistic effects on biological endpoints (Altenburger et al., 2013). Therefore, effects can be difficult to predict from modelling the results of relatively high doses of individual compounds.

Some POPs are endocrine disruptors, compounds that can result in alterations in hormone synthesis or metabolism, or receptor target modulation via mimicking, antagonising, or altering endogenous hormone levels (reviewed in Frye et al., 2012). Due to the central role of the endocrine system in developing, organising, and maintaining the central nervous system, there is concern over the ability of POPs to impair early brain development. This is especially true within the concept of foetal programming, whereby rapidly developing biological systems appear to be particularly vulnerable to disorganising influences that may then have chronic implications (Gluckman and Hanson, 2004). For instance, POPs are known to cross the placenta (Needham et al., 2011), and have been associated with neural tube defects at birth (Ren et al., 2011), as well as neurodevelopmental outcomes in children such as poor attention and low IQ (for a review, see Berghuis et al., 2015).

Glucocorticoids are essential for maintaining a normal physiological response to stressful situations and are therefore an essential aspect of lifetime fitness. The hypothalamic-pituitary-adrenal (HPA) axis coordinates the response to stress via corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and cortisol (corticosterone in mice). These hormones play a key role in directing energy away from non-essential life processes towards immediate survival. During development, cortisol is also essential for normal foetal development (Liggins, 1994), including development of the brain (Harris and Seckl, 2011). For example, prenatal stress or glucocorticoid exposure can reprogram the HPA axis in offspring leading to long-term dysregulation and alterations in anxiety-like behaviour (Glover et al., 2010; Davis et al., 2011). Of concern, ecotoxicological studies have found POPs to be associated with alterations in cortisol dynamics in polar bears (Oskam et al., 2004), arctic birds (Verboven et al., 2010; Tartu et al., 2014), and fish (Hontela et al., 1992) whilst experimental studies have confirmed that POPs can lead to alterations in basal cortisol/corticosterone in rodents (Pereiro et al., 2014), goats (Zimmer et al., 2009), sheep foetuses (Zimmer et al., 2013), and fish (Jørgensen et al., 2002), as well as impairing the stress response (Jørgensen et al., 2002; Zimmer et al., 2009).

In the current study, we explore whether a human based POP mixture affects basal corticosterone and the stress response in female mice and their offspring. We hypothesise that those mice exposed to POPs will show HPA axis dysregulation. Therefore, we measured corticosterone levels in unstressed and stressed mice, and anxiety-like behaviour and locomotor activity in an open field test.

2. Materials and methods

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The unit is licensed by the Norwegian Animal Research Authority (NARA) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC; FOTS: 5583) and NARA (2013/39783).

2.1. Chemicals and feed

A thorough description of the design and preparation of the POP mixture can be found in Berntsen et al. (2017). In brief, the mixture reflects the levels of POPs found in a Scandinavian food basket as the experiment was designed in order to be of interest to a human exposure. A list of the individual compounds and the concentrations of each within the feed can be found in Table 1. A literature review identified the most relevant POPs and the estimated daily intake (EDI) levels of these compounds for a human of 70 kg. Based on the human EDI, corresponding EDIs of the different compounds for a 25 g mouse were calculated (Table 1). Due to the possibility of background exposure via the mice feed and the higher drug metabolism of mice than humans (Walton et al., 2001), the feed concentration of the mixture was set to provide a mouse consuming 3 g feed/day a daily dose of 5000 × and 100 000 × the EDI for humans (see Berntsen et al., 2017 for further discussion). This resulted in between 24 and 25 of the 29 compounds being detected in the plasma of the mothers depending on the exposure group, and between 18 and 20 were found in the pups (Table 2). In brief, most compounds were found in higher levels in the mothers than the pups and a dose dependent increase (low vs high dose) was found for all compounds. Most (26/29, low and high dose group, respectively) of the compounds were detected in the brains of mothers, as well as the pups brain (20/29 and 22/29 in the low and high dose, respectively) (Berntsen et al. in prep). When comparing blood values between the experimental mice and the Scandinavian population, those mothers exposed to the high dose had values ranging from 100 up to 5000 × the levels of individuals compounds found in Scandinavians (Table 2). For the low exposed mothers, these values ranged from 8–500 × the average Scandinavian (Table 2). Therefore, the tissue concentrations were more similar to the human scenario than the EDI values, as expected based on the higher drug metabolism in mice.

All polybrominated diphenyl ethers (PBDEs), PCBs and other organochlorines were originally purchased from Chiron As (Trondheim, Norway). All perfluorinated compounds and hexabromocyclododecane (HBCD) were obtained from Sigma-Aldrich (St. Louis, MO, USA), with the exception of perfluorohexane sulfonic acid (PFHxS) potassium salt which was from Santa Cruz (Dallas, US). All chemicals were dissolved in an appropriate solvent and added to corn oil (Jasmin, fully refined, Yonca Gida San A.S., Manisa, Turkey) intended for human consumption. All solvents were thoroughly evaporated under N_2-flow before the oil containing the POPs was sent to the feed company (TestDiets, St. Louis, MO) to be incorporated in the mouse feed. Four different diets were made, three exposure diets for pregnant mice, control (non-exposed), low dose (5000 × EDI) and high dose (100 000 × EDI), and a reference diet for males and pups after weaning. For the control feed, the corn oil included the solvents at identical levels to those found in the two exposure diets, whereas for the reference feed only untreated corn oil was used. In all diets, all soybean oil in the original feed recipe was exchanged with corn oil intended for human consumption, in order to reduce background POP exposure,

Table 1
A mixture of persistent organic pollutants (POPs) based on a literature review on estimated daily intake (EDI) values in the Scandinavian population (Berntsen et al., 2017). Average EDI values for a 70 kg human and corresponding values for a 25 g mouse are shown. EDI values for a 25 g mouse consuming 3 g of feed designed to provide daily doses of POPs corresponding to the low (5000× human EDI) and high (100,000× human EDI) doses are shown in grey, and are based on measured feed concentrations. The table is adapted from Berntsen et al. (2017).

Compound	Average EDI [a] 70 kg person ng/day	Daily intake human ng/kg/day	EDI [b] 25 g mouse pg/day	EDI [c] 25 g mouse 5000× ng/day	EDI [d] 25 g mouse 100,000× ng/day	Feed measured [e] 5000× ng/g feed	Feed measured [f] 100,000× ng/g feed	EDI [g] 25 g mouse 5000× ng/day	EDI [h] 25 g mouse 100,000× ng/day
Chlorinated									
PCB 28	10	0.14	3.5	18	350	3.1	46	9	138
PCB 52	23	0.33	8.3	41	825	15.0	182	45	546
PCB 101	39	0.56	14.0	70	1400	25.4	377	76	1131
PCB 118	68	0.97	24.3	121	2425	37.2	612	112	1836
PCB 138	97	1.38	34.5	173	3450	53.8	957	161	2871
PCB 153	97	1.38	34.5	173	3450	61.4	981	184	2943
PCB 180	26	0.37	9.3	46	925	17.4	263	52	789
∑ PCBs	360	5.13	128.4	642	12,825	213.3	3418	640	10,254
p,p'-DDE	201	2.87	71.8	359	7175	136.0	2390	408	7170
HCB	84	1.20	30.0	150	3000	37.4	588	112	1764
α-Chlordane	63	0.90	22.5	113	2250	45.0	723	135	2169
Oxychlordane	21	0.30	7.5	38	750	9.8	297	29	891
trans-Nonachlor	21	0.30	7.5	38	750	14.9	264	45	792
α-HCH	36	0.52	13.0	65	1300	21.2	421	64	1263
β-HCH	29	0.42	10.5	53	1050	22.3	398	67	1194
γ-HCH (Lindane)	40	0.57	14.3	71	1425	31.4	435	94	1305
Dieldrin	126	1.80	45.0	225	4500	70.4	1470	211	4410
∑ OCPs	621	8.88	222.1	1112	22,200	388.4	6986	1165	20,958
∑ PCBs + OCPs	981	14.01	350.5	1754	35,025	601.7	10,404	1805	31,212
Brominated									
PBDE 47	68	0.97	24.3	121	2425	39.7	642	119	1926
PBDE 99	13	0.19	4.8	24	475	8.6	126	26	378
PBDE 100	11	0.15	3.8	19	375	5.6	91	17	272
PBDE 153	2	0.03	0.8	4	75	1.5	22	5	67
PBDE 154	4	0.06	1.5	8	150	2.8	38	8	114
PBDE 209	105	1.50	37.5	188	3750	64.8	1141	194	3423
HBCD	21	0.30	7.5	38	750	9.9	203	30	609
∑ BFRs	224	3.2	80.2	402	8000	132.9	2263	399	6789
Perfluorinated									
PFHxS	1.2	0.017	0.4	2	43	1.7	42	5	125
PFOS	18	0.26	6.5	33	650	3.2	74	10	222
PFOA	31	0.44	11.0	55	1100	6.0	121	18	363
PFNA	9.5	0.14	3.5	18	350	2.1	42	6	127
PFDA	13	0.19	4.8	24	475	3.1	57	9	172
PFUnDA	6.7	0.096	2.4	12	240	1.6	28	5	84
∑ PFAAs	79.4	1.14	28.6	144	2858	17.7	364	53	1094

Abbreviations: PCBs (polychlorinated biphenyls); OCPs (organochlorine pesticides); BFRs (brominated flame retardants); PFAAs (perfluoroalkyl acids).
[a] Average EDI (Estimated daily intake) values of POPs for a 70 kg human – based on a literature review of Scandinavian EDI values (Berntsen et al., 2017).
[b] EDI values for a 25 g mouse corresponding to human EDI values.
[c] EDI values for a 25 g mouse corresponding to human EDI values * 5000
[d] EDI values for a 25 g mouse corresponding to human EDI values * 100,000.
[e] Measured concentrations of the various compounds in the 5000× feed.
[f] Measured concentrations of the various compounds in the 100,000× feed.
[g] EDI values for a 25 g mouse consuming 3 g of the 5000× feed/day – based on concentrations measured in the feed of the current project.
[h] EDI values for a 25 g mouse consuming 3 g of the 100,000× feed/day – based on concentrations measured in the feed of the current project.

Table 2
Plasma levels of persistent organic pollutants in ng/g wet weight (ng/g ww) measured in mothers and pups of the control, low, and high exposed groups (white columns). Measured levels expressed relative to average human blood levels (ng/g ww) in the Scandinavian population (Berntsen et al., 2017) are also included (grey columns). Adapted from Berntsen et al. (in prep).

Compound	Human ng/g ww	Control				Low				High			
		Mother ng/g ww	×Human levels	Pup ng/g ww	×Human levels	Mother ng/g ww	×Human levels	Pup ng/g ww	×Human levels	Mother ng/g ww	×Human levels	Pup ng/g ww	×Human levels
Chlorinated													
PCB 28	0.013	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A
PCB 52	0.010	n.d.	N/A	n.d.	N/A	1.1	108	0.12	12	4.7	472	2.8	281
PCB 101	0.008	n.d.	N/A	n.d.	N/A	1.2	147	n.d.	N/A	2.4	295	n.d.	N/A
PCB 118	0.064	0.07	1.1	0.3	4.4	2.5	39	0.4	6	42	658	11	185
PCB 138	0.222	0.08	0.4	1.2	5.2	12	54	4.0	18	91	409	50	224
PCB 153	0.362	0.13	0.3	1.2	3.2	7.4	21	2.9	8	87	241	49	135
PCB 180	0.194	0.03	0.2	0.3	1.7	2.7	14	0.8	4	24	126	14	72
p,p'-DDE	0.502	n.d.	N/A	n.d.	N/A	5.3	11	0.3	0.6	51	102	1.7	3
HCB	0.117	0.09	0.8	0.3	2.3	2.9	25	0.8	6	41	347	9.5	81
α-Chlordane	0.011	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A
Oxychlordane	0.022	n.d.	N/A	n.d.	N/A	1.6	72	1.3	58	22	978	14	621
trans-Nonachlor	0.041	n.d.	N/A	n.d.	N/A	1.1	26	0.4	9	9.8	240	6.2	152
α-HCH	0.006	n.d.	N/A	n.d.	N/A	0.2	35	n.d.	N/A	n.d.	N/A	n.d.	N/A
β-HCH	0.053	n.d.	N/A	n.d.	N/A	1.5	29	0.15	3	14	272	10	189
γ-HCH (Lindane)	0.006	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A
Dieldrin	0.024	n.d.	N/A	n.d.	N/A	13	537	1.4	56	50	2100	17	701
Brominated													
BDE 47	0.009	n.d.	N/A	n.d.	N/A	1.2	137	n.d.	N/A	11	1196	n.d.	N/A
BDE 99	0.004	n.d.	N/A	n.d.	N/A	0.5	124	n.d.	N/A	3.8	952	n.d.	N/A
BDE 100	0.002	n.d.	N/A	n.d.	N/A	0.5	263	0.09	45	3.8	1903	0.6	305
BDE 153	0.010	n.d.	N/A	n.d.	N/A	0.18	18	n.d.	N/A	2.5	249	1.1	110
BDE 154	0.002	n.d.	N/A	n.d.	N/A	0.3	132	n.d.	N/A	0.9	460	2.0	976
BDE 209	0.011	n.d.	N/A	n.d.	N/A	4.9	444	n.d.	N/A	26	2374	n.d.	N/A
HBCD	0.025	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A	n.d.	N/A
Perfluorinated													
PFHxS	3.45	n.d.	N/A	6.4	1.9	161	47	16	5	3381	994	317	93
PFOS	29.43	0.89	0.03	16	0.5	223	8	29	1	3403	116	635	22
PFOA	4.52	0.53	0.12	12	2.5	345	76	26	6	6980	1543	598	132
PFNA	0.80	0.32	0.4	13	16	176	220	24	29	2531	3164	470	588
PFDA	0.50	0.25	0.5	14	29	246	497	28	56	2580	5212	503	1016
PFUnDA	0.56	0.16	0.3	3.2	5.6	63	113	6.2	11	724	1293	114	204

n.d. = Not detected.
N/A = Not applicable.

and to limit the amount of phytoestrogens, suspected to interfere with hormone homeostasis, and known to be present in soy-based food (Berntsen et al., 2017).

2.2. Animal model

In total, 10 male C57BL/6J mice and 20 female 129S1/SvImJ (Jackson Laboratory, Maine, USA) were used for breeding. The mice were newly sexually mature and were acclimated to the unit for 1 week before mating started. In total, 110 hybrid pups were born (129:C57BL/6F0, $n = 63$ males and 47 females). Of these, the 47 females were assigned to one of three groups, the control, a low dose (5000 × EDI POP mixture), or a high dose (100 000 × EDI POP mixture), and given their respective diet from weaning prior to their use as breeders of the F1 generation. In total, the F1 generation consisted of 320 pups (129:C57BL/6F1, $n = 163$ males and 157 females). At weaning, 18 males and 18 females from each respective group ($n = 36$/group) were assigned to the open field test. The remaining pups were assigned to a later project. Throughout, the assignment of animals to housing, exposure groups, and testing groups was done randomly, either by simple lottery or by computer random numbering.

2.3. Housing and husbandry

All animals were housed in open type III cages (Tecniplast, Buguggiate, Italy) in group housing. All cages contained standard aspen bedding (Scanbur BK, Nittedal, Norway) and cellulose nesting material. The animals had free access to their assigned feed. Tap water was available from standard drinking bottles (Tecniplast, Buguggiate, Italy). The animal room was on a 12:12 light–dark cycle, with a room temperature of 21 ± 2 °C with 20 air changes per hour and 45 ± 5% relative humidity. The cages, bedding, nesting material, and water bottles were changed once a week, when animals were not tested. At weaning, the F0 females were randomly assigned to one of three exposure groups: High ($n = 16$ females), low ($n = 16$ females), and control ($n = 15$ females). All females were given their assigned feed from weaning and throughout the project (approx. 6 months). The F0 generation females were housed in groups of four from weaning until sexual maturity (marked with simple ear punch holes from 1 to 4), then caged with the F0 generation males (non-brothers) in triplets (1 male and 2 females) for one week. One week before predicted birth, they were single housed until their pups were weaned. At weaning, the F1 pups were randomly assigned to a same sex and exposure group cage/housing group of two, where one was marked with a microtransponder. The F1 generation were fed the reference diet after weaning. Feed intake was recorded for one week prior to necropsy and not found to be affected by exposure (data not shown). Similarly, body weights were assessed at weaning and necropsy and not found to be significantly affected by exposure in either the mothers or pups. No mice died or showed clinical signs of disease during the experiment, but 7 males (3 from the control group and 4 from the high exposure) were euthanized due to wounds attained through fighting. These 7 euthanized males occurred at weaning and were replaced with naïve males from their respective groups (the

mothers had undergone exactly the same exposure protocol, but had been housed in a different room).

2.4. Open field test

All testing (open field and HPA axis responsivity test) was done by 3 researchers with FELASA C credentials. Each person had assigned specific responsibilities during the testing and this was kept constant throughout the testing period to ensure consistency and elimination of deviations between experiments.

Both the F0 mothers and their offspring were tested. The open field test was performed during the animals' dark cycle, between 20:00 and 03:00. Four testing boxes (each 50 × 50 × 22 cm) (Noldus, Wageningen, the Netherlands) were used. Each box was divided into three zones, a center zone, corners, and a border zone. Four mice were tracked at the same time using EthoVision 9 (Noldus). The animal was placed inside a disposable non-transparent cardboard cylinder (guinea pig play tunnel from Scanbur BK, Nittedal, Norway) in the center of the arena and left there for 3 s. When released from the cylinder, each mouse was tracked for 15 min. Different endpoints were evaluated: the time spent within the different zones, the total distance moved, and velocity. Rearing (exploratory behaviour) and grooming were tracked manually. Following the behavioural test, the number of urine puddles and faecal pellets was recorded. The testing arena boxes were thoroughly washed with water and dried with paper towels between each animal. Male mice were tested before female mice, and all animals underwent testing only once. Background light intensity was around 1600 lx (TES Light Meter 1337, Presisjons Teknikk AS, Oslo, Norway) and background noise was stable at 32 dB (Castle GA 112 sound level meter, Presisjons Teknikk AS, Oslo).

2.5. HPA axis responsivity test

All F0 mothers and the offspring tracked in the open field test underwent the HPA axis responsivity test. The test was done inside the housing room to eliminate transport stress. The mouse was picked up, scanned for its micro-transponder, and then restrained inside a 50 ml Falcon tube for 15 min. Blood samples were taken from the tip of the tail at 4 different time points: 0, 15, 30, and 120 min. The mouse was returned to a home-cage after the 15 min time point and retrieved when needed for sampling. All samples were taken with a 20 μL Minivette POCT capillary collecting tube coated with EDTA (Sarstedt, Ski, Norway) and transferred to an eppendorf tube on ice. The blood samples were spun at 5000 rpm at 4 °C for 10 min to obtain the plasma, which was stored at −80 °C until further analyses. Corticosterone was measured in the plasma using an MP Biomedicals ImmuChem ™ Double Antibody Corticosterone ^{125} Ria Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturers' instructions.

2.6. Statistical analyses

For mothers, we analysed differences in basal levels of corticosterone between levels of exposure using a linear regression model. Basal levels of corticosterone in pups were analysed against exposure levels, stratified by sex, using linear mixed effect models allowing for random intercepts for mother.

We also used linear regressions to model changes in the levels of corticosterone over time. Due to clear peaks in corticosterone levels at 30 min, we first analysed the change in corticosterone levels between 0 to 30 min and 30–120 min against exposure levels. These analyses were carried out in mothers and pups separately. For mothers, the level of exposure and time were the explanatory variables, while for pups we also studied the effects of sex along with exposure. To analyse longitudinal trends in corticosterone levels across exposure, we used longitudinal mixed effect models with a piecewise linear structure of the population average corticosterone levels. The piecewise linear model was preferred due to increasing corticosterone levels from 0 to 30 min and decreasing levels from 30 to 120 min. Repeated measurements of corticosterone within each mouse implies a possibility of within individual variability, which is modelled using an autoregressive correlation structure of the order one over time. In all raw profiles, we observed a variation in the starting levels of corticosterone. Further, in the case of pups, we observed a greater inter-individual variability in female mice. To account for these observations, we included a random intercept (for both mother and pup models) and a random slope for sex (in the pups model) in the mixed effect longitudinal models. As a sensitivity analysis, we repeated the longitudinal modelling stratified by sex in the pups, including exposure as the variable of interest and a random intercept for each mouse.

We also analysed the data from the open field experiments using univariate and multivariate linear regression models with sex, exposure, and the interaction between sex and exposure as the

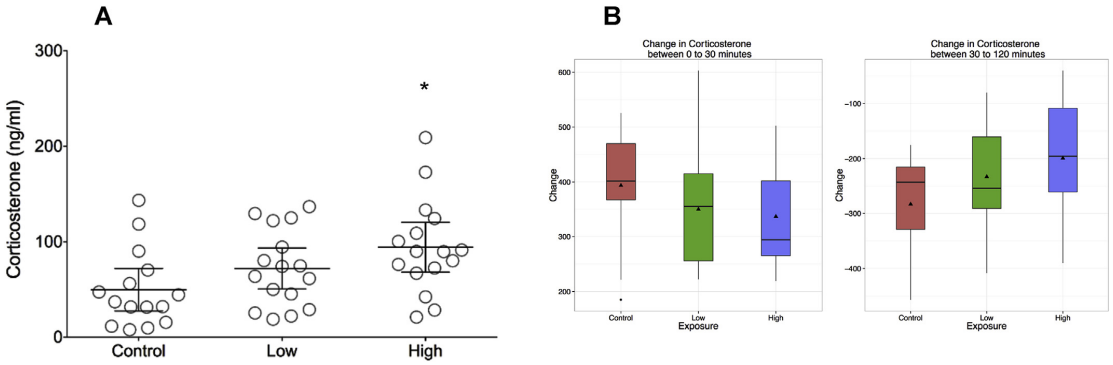

Fig. 1. Plasma corticosterone in mice mothers exposed to a mixture of persistent organic pollutants. (A) Basal corticosterone and (B) corticosterone dynamics following stress. In (A), data includes mean ± 95% CI and an asterisk represents a significant exposure effect compared to controls (Linear model, $p = .007$). In (B), each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR) and the lower whisker is the first quartile minus 1.5 × IQR. There was a significant effect of the high exposure between 30 and 120 min compared to controls ($p = <0.05$, see Table 1).

Table 3
Results of modelling maternal corticosterone changes over time (0–30 and 30–120 min) following exposure to two doses (high and low) of a mixture of persistent organic pollutants using linear regression models.

	Dependent variable:	
	Change in Corticosterone (0–30) (1)	Change in Corticosterone (30–120) (2)
Low Exposure	−43.775	50.437
	(36.884)	(34.744)
High Exposure	−57.018	84.077**
	(36.884)	(34.744)
Constant	393.547***	−283.381***
	(26.081)	(24.568)
Observations	45	45
R2	0.059	0.124
Adjusted R2	0.014	0.082
Residual Std. Error (df = 42)	101.010	95.152
F Statistic (df = 2; 42)	1.309	2.967*

Note: *p < .1; **p < .05; ***p < .01.

Table 4
Estimated parameters from the longitudinal mixed effects model for the mother and pups exposed to two doses (high and low) of a mixture of persistent organic pollutants. Sex (male vs female) and time (0–30 vs 30–120 min) were also within the model.

	Mother levels	Pup levels
Low Exposure	10.96	−31.54
	(17.57)	(19.34)
High Exposure	30.59	−14.95
	(17.57)	(17.92)
Time	12.00***	9.55***
	(0.68)	(0.29)
(Time - 30)+	−15.22***	−12.03***
	(0.81)	(0.35)
Male		−118.80***
		(14.36)
Low Exposed Male		45.69*
		(22.28)
High Exposed Male		47.64*
		(20.75)
Intercept	109.18***	129.87***
	(16.56)	(13.43)
AIC	2145.67	4863.83
BIC	2170.99	4928.48
Log Likelihood	−1064.84	−2415.92
Num. obs.	180	428
Num. groups	45	107

***p < .001, **p < .01, *p < .05.

explanatory variables. Frequency at particular locations (borders, centres, and corners) and cumulative duration at those locations were treated as bivariate outcomes in each multivariate regression. In addition, frequency of grooming and cumulative duration of grooming was also treated as a bivariate outcome. Univariate regressions were carried out with two responses, i) the ratio of time spent moving against not moving, and ii) the ratio of time spent grooming against rearing. Multivariate and univariate ANOVA was used to determine the significance of the factors and a *p*-value of <0.05 was treated as a significant finding. All statistical analyses were carried out in R statistical software (Version 3.2.1., R Development Core Team, http://www.r-project.org) and the R scripts can be found in the supplementary material.

3. Results

3.1. Corticosterone

High exposed mothers had significantly higher basal corticosterone levels than controls, whereas the low exposure showed only a trend for higher values than controls (Fig. 1A). When modelling changes in corticosterone following stress, we found a significant effect (Table 3) of the high exposure between 30 and 120 min, as the controls showed a greater level of change during this period compared to the high exposed group, with the low exposed mothers having an intermediate value (Fig. 1B). When modelling the absolute values across the whole experiment, there was no effect of any exposure (Table 4).

There was no effect of exposure on basal cortisol levels in either male or female pups (Fig. 2A). When modelling changes in corticosterone following stress, there was a significant interaction between sex and exposure. Here, high exposed males showed a significantly greater increase in corticosterone between 0 and 30 min, but also a greater decrease between 30 and 120 min, than controls (Table 5), whereas the opposite non-significant pattern was observed in females (Fig. 2B). When modelling the absolute values of corticosterone across the whole experiment, there was a clear dose response in males with both the low and high exposed groups having significantly greater values than controls, but no such trends were observed in females (Table).

3.2. Behaviour

There was no effect of exposure on any behavioural endpoint (see supplementary Figs. 1-14). There was a significant effect of sex on the bivariate outcome comprised of frequency of grooming and cumulative duration of grooming (Pillai's multivariate F-statistic, p value = .007). Both frequency of grooming and cumulative time spent in grooming were higher in female mice. In addition, there was also a significant sex effect on the ratio of the duration of grooming against rearing, with male mice spending 1.79 (95% CI = 1.06, 3.06, p = .03) times more on grooming than rearing compared to female mice. The results are summarised in supplementary Tables 1-3.

4. Discussion

The stress response in mice exposed to a human based mixture of POPs was assessed in mothers and their offspring. We found evidence that POP exposure can influence corticosterone dynamics in mothers and their offspring. However, the effects on the HPA axis were sex specific and were not evident in measures of anxiety-like behaviour. These results have important implications on neurological development in both humans and wildlife.

We found that exposed mothers had elevated basal levels and a prolonged period of corticosterone elevation following stress compared to controls. This conforms to both ecotoxicological and laboratory studies that have found associations between stress hormones and POPs across taxa (i.e. Gendron et al., 1997; Jørgensen et al., 2002; Oskam et al., 2004; Franceschini et al., 2008; Pereiro et al., 2014). However, the direction of change is not consistent between studies. For example, basal cortisol was elevated in Arctic char (Jørgensen et al., 2002) and guinea pigs (Kato et al., 1981), but decreased in polar bears (Oskam et al., 2004) and rats (Pereiro et al., 2014). Similarly, POPs have been found to either blunt (Hontela et al., 1992; Jørgensen et al., 2002; Verboven et al., 2010) or oversensitise (Zimmer et al., 2009) the stress response. These inconsistencies may be explained by the POPs studied. For example, *in vitro*, PCBs 118 and 126 were found to induce cortisol production in human H295R adrenal cells, but not PCB 153 (Kraugerud et al., 2010) or perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), or perfluorononanoic acid (PFNA) (Kraugerud et al., 2011). Similarly, a study in wild polar bears found cortisol to be

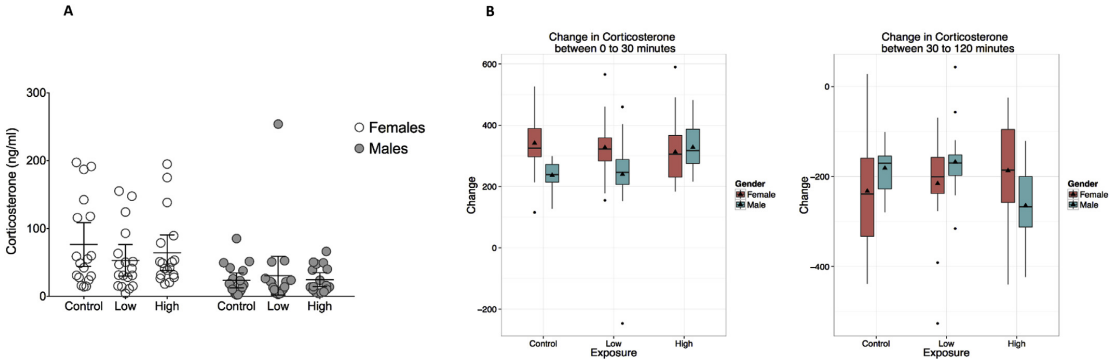

Fig. 2. Plasma corticosterone in pups exposed to a mixture of persistent organic pollutants via their mothers. (A) Basal corticosterone and (B) corticosterone dynamics following stress. In (A), data are means ± 95% CI. In (B), each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR) and the lower whisker is the first quartile minus 1.5 × IQR. There was a significant interaction between exposure and gender between 0-30 and 30–120 min ($p = <0.05$, see Table 3).

Table 5
Results of modelling corticosterone changes using linear regression models in pups exposed to two doses (high and low) of a mixture of persistent organic pollutants. Sex (male vs female) and time (0–30 and 30–120 min) were also included within the model.

	Dependent variable:	
	Change in Corticosterone (0–30) (1)	Change in Corticosterone (30–120) (2)
Low Exposure	−14.224	16.800
	(33.429)	(32.381)
High Exposure	−29.099	45.426
	(32.986)	(31.952)
Male	−104.687***	50.961
	(33.429)	(32.381)
Low Exposure and Male	17.037	−3.066
	(47.276)	(45.794)
High Exposure and Male	120.051**	−128.582***
	(47.702)	(46.206)
Intercept	341.493***	−232.442***
	(23.638)	(22.897)
Observations	107	107
R2	0.162	0.106
Adjusted R2	0.120	0.062
Residual Std. Error (df = 101)	100.287	97.143
F Statistic (df = 5; 101)	3.902***	2.399**

Note: *p < .1; **p < .05; ***p < .01.

negatively associated with PBDE 99 and 153, as well as PCB 170, 180, 190 and 201, but positively associated with PCB 66/95, α-HCH, dieldrin, PBDE 47, and para, para'-dichlorodiphenyldichloroethane (*p,p'*- DDD) (Bechshøft et al., 2012). Environmental factors may also influence the response to POPs, as Jørgensen et al. (2002) found that the PCB mixture Aroclor 1254 suppressed basal cortisol in food-deprived fish, but elevated cortisol in fed fish. Therefore, it appears the effect of POPs on the HPA axis is dependent on the mixture composition, most likely because different POPs will have different mechanistic pathways, and environmental factors.

Although they did not receive POP containing food, sex specific effects on the stress response were found in the offspring. This demonstrates the risk to offspring of POP exposure via mothers. Although POPs were found in brain tissue from both mothers and pups (Berntsen et al. in prep), we cannot conclude whether the effects in the offspring were due to direct effects of the POPs transferred via the mother or due to secondary physiological effects of exposure in the mothers. For example, POP exposure can have a wide-ranging effect on many physiological parameters, including the thyroid axis (Gilbert et al., 2012), sex steroids (Lilienthal et al., 2006), and metabolism (Swedenborg et al., 2009) that are important for neurological development in offspring. These systems were not investigated in the current study, but in the mothers, we did see alterations in corticosterone dynamics. Here, cortisol is known to regulate neurodevelopment and alterations in stress hormones during pregnancy can have long lasting effects on the levels of anxiety (Davis and Sandman, 2010) and lead to dysregulation of the HPA axis in children (Davis et al., 2011). The current sex effect on the stress response is not unexpected, as comparable results on the stress response following POP exposure have been found in other animal models (Zimmer et al., 2009; Verboven et al., 2010). Why male pups were found to have an over-sensitised stress response compared to females is currently unknown. However, previous work has demonstrated that gonadal hormones influence development of the HPA axis (reviewed in Bale and Epperson, 2015), POPs are known to interfere with testosterone production in male mice (Kaya et al., 2002), and we found effects of our POP mixture on sperm quality in a sub sample of the pups from the current experiment (Khezri et al., 2017a).

Alterations in stress levels are commonly linked with changes in behaviour, such as anxiety (Cohan et al., 2006). However, in the current study we observed no effect of POP exposure on behavioural endpoints of anxiety in mothers or offspring in the open field test. For the mothers, in which we observed alterations in basal corticosterone, this may appear contradictory to the expected. However, Cohan et al. (2006) reported that rats showing higher levels of anxiety-like behaviour did not have higher basal corticosterone compared to comparative groups. Therefore, increased basal cortisol may not necessarily be associated with increased anxiety-like behaviour. However, in the future it may be beneficial to include behavioural tests that involve the activation of the stress response to gain a more detailed insight into the link between the observed changes in the HPA axis following POP exposure and potential modification of behaviour.

High exposed mothers were found to have elevated basal corticosterone and a prolonged elevation of corticosterone following stress. This could be due to one of several possibilities. For instance, the burden of chemicals may have continuously stimulated a stress response, the feedback mechanism to reduce elevated corticosterone may have been impaired, or maybe the mothers were less capable of metabolising corticosterone. Here, we note

that in a separate experiment on CD1 mothers and their pups, exposed to the same concentrations of the POP mixture used in the current study, there were transient effects on the liver, including an early increase in relative liver size, alterations in liver morphology (centralobular hypertrophy), and the induction of detoxification enzyme activity (CYP1A1, CYP1A, CYP3A, CYP2B, CYP2E1, CYP2A) in the offspring, which disappeared over time (unpublished data). This suggests the doses used impacted on liver morphology and physiology, but were not pathological in the offspring, although the mothers remained untested. Liver endpoints were not assessed in the current study, but would be of interest with regards to the ability of the liver to metabolise corticosterone.

We find evidence of HPA dysregulation in both the mothers and their male offspring exposed to the high dose, and male pups exposed to the low dose, of our POP mixture. The implication of this finding is unclear, but such dysregulation has previously been associated with neurological disorders. For example, in laboratory studies rats with a blunted stress response were found to show more extreme responses to a stressor, similar to post traumatic stress disorder (Cohan et al., 2006). Furthermore, elevated levels of glucocorticoids have negative effects on the cognitive abilities of animals, causing neuronal cell death and reducing neurogenesis (Sapolsky et al., 2000). In humans, elevated or decreased basal cortisol levels have been associated with post-traumatic stress disorder (Yuhuda et al., 1990; Bremner et al., 1997) and dissociative disorders (Simeon et al., 2001), whereas a hypersensitive stress response has been associated with panic disorder (Abelson et al., 2006), and both an elevated and blunted cortisol response has been associated with depression (Burke et al., 2005; Van den Bergh et al., 2008). Therefore, further work should assess whether exposure to our POP mixture can lead to other neurological disorders in mice and other vertebrate models.

We used a POP mixture based on human dietary intake levels in a mouse model. Based on tissue analysis, although mice were fed 5000 × and 100 000 × the estimated daily intake of humans, the levels within the plasma, adipose tissue, and brain were in some instances comparable to the levels of POPs found in humans and wildlife (Berntsen et al. in prep). For example, compounds detected in the plasma of the low dose group of pups ranged from 0,6 (para, para'-dichlorodiphenyldichloroethylene [*p,p'*-DDE]) to 58 (perfluorodecanoic acid [PFDA]) times the levels in human blood (Berntsen et al. in prep). Similarly, concentrations of organochlorines measured in autopsy tissue from Greenland (Dewailly et al., 1999) were in between the pup and the mother brain levels of the low dose group of the present study when lipid adjusted levels were compared. Therefore, the effects on corticosterone HPA dysregulation observed in the male pups exposed to the low dose in the current study is a concern for human safety. As such, further work is required to understand which compounds are acting upon the HPA axis, and whether the effects of these compounds are mediated by the presence of other compounds similar to that seen in other species (i.e. Oskam et al., 2004; Bechshøft et al., 2012). Of note, we recently tested the effect of a second POP mixture on larval zebrafish behaviour that contained the exact same 29 compounds as used in the current study, but the concentrations were based on human blood levels rather than the estimated dietary intake (see Berntsen et al., 2017). Nevertheless, we found this alternative POP mixture could influence anxiety-like behaviour in larval zebrafish (Khezri et al., 2017b). This observed effect was found to be mimicked by the perfluorinated fraction of the mixture, and of these six compounds, only PFOS could mimic the effect. Therefore, it would be interesting to see whether PFOS itself can mimic the effects on HPA regulation we see in the present study when using the total mixture. Especially as PFOS was the dominating perfluorinated congener measured in brain tissue from the current experiment (Berntsen et al. in prep).

In conclusion, a human based POP mixture dysregulated the HPA axis in female mice and their male offspring. As HPA dysregulation is associated with neurological disease in humans, further work should be carried out to determine which compounds within the POP mixture are acting upon the HPA axis and the risk to humans.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.01.085.

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Supplementary tables S2-S4. Statistical output for open field test data.

Table S2 Univariate regressions of frequency of grooming and cumulative time spent grooming

Factors	Estimate	Std error	*p* value	
Frequency of grooming				
Intercept	7.4442	0.8088	<0.001	***
Sex (male)	0.7435	0.8275	0.371	
Exposure (low)	-0.8925	1.0272	0.387	
Exposure (high)	-0.3482	0.9954	0.727	
Cumulative grooming				
Intercept	44.208	8.822	<0.001	***
Sex (male)	23.692	9.026	0.010	*
Exposure (low)	-6.744	11.205	0.549	
Exposure (high)	-3.691	10.858	0.735	

* p < 0.05, ** p <0.01, *** p < 0.001

Table S3 Univariate regression of log grooming/rearing behaviour

Factor	Estimate	Std error	pvalue	
Intercept	-0.6118	0.2607	0.021	*
Sex (male)	0.5873	0.2679	0.0308	*
Exposure (low)	-0.2167	0.3307	0.5138	
Exposure (high)	-0.1085	0.3228	0.7374	

* p < 0.05, ** p <0.01, *** p < 0.001

Table S4 ANOVA table for log grooming and rearing behaviour

Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Sex	1	8.345	8.3447	4.7119	0.032	*
Exposure	2	0.761	0.3806	0.2149	0.807	
Residuals	95	168.244	1.771			

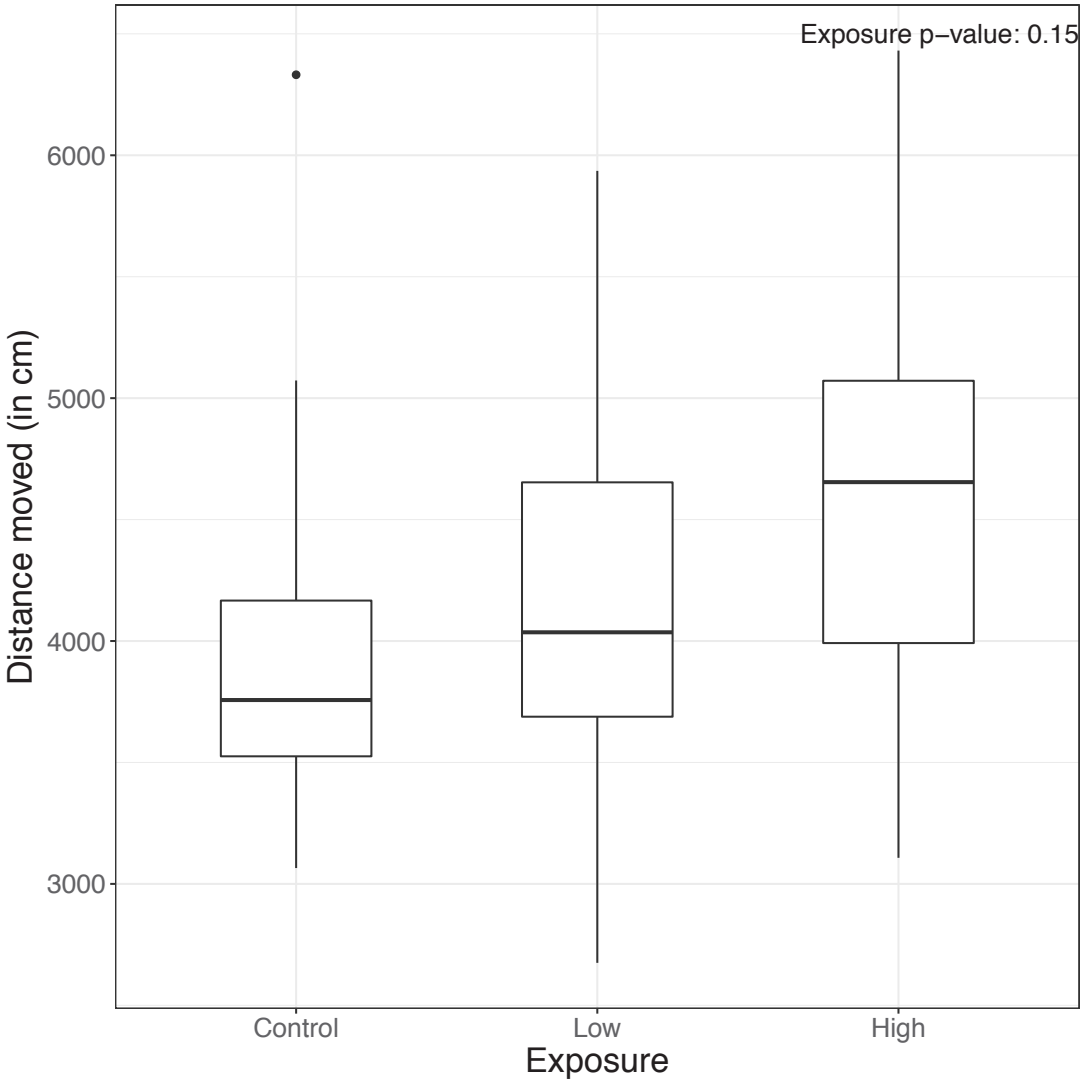


Figure S1. Box and whisker plots of the total distance moved for mothers that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

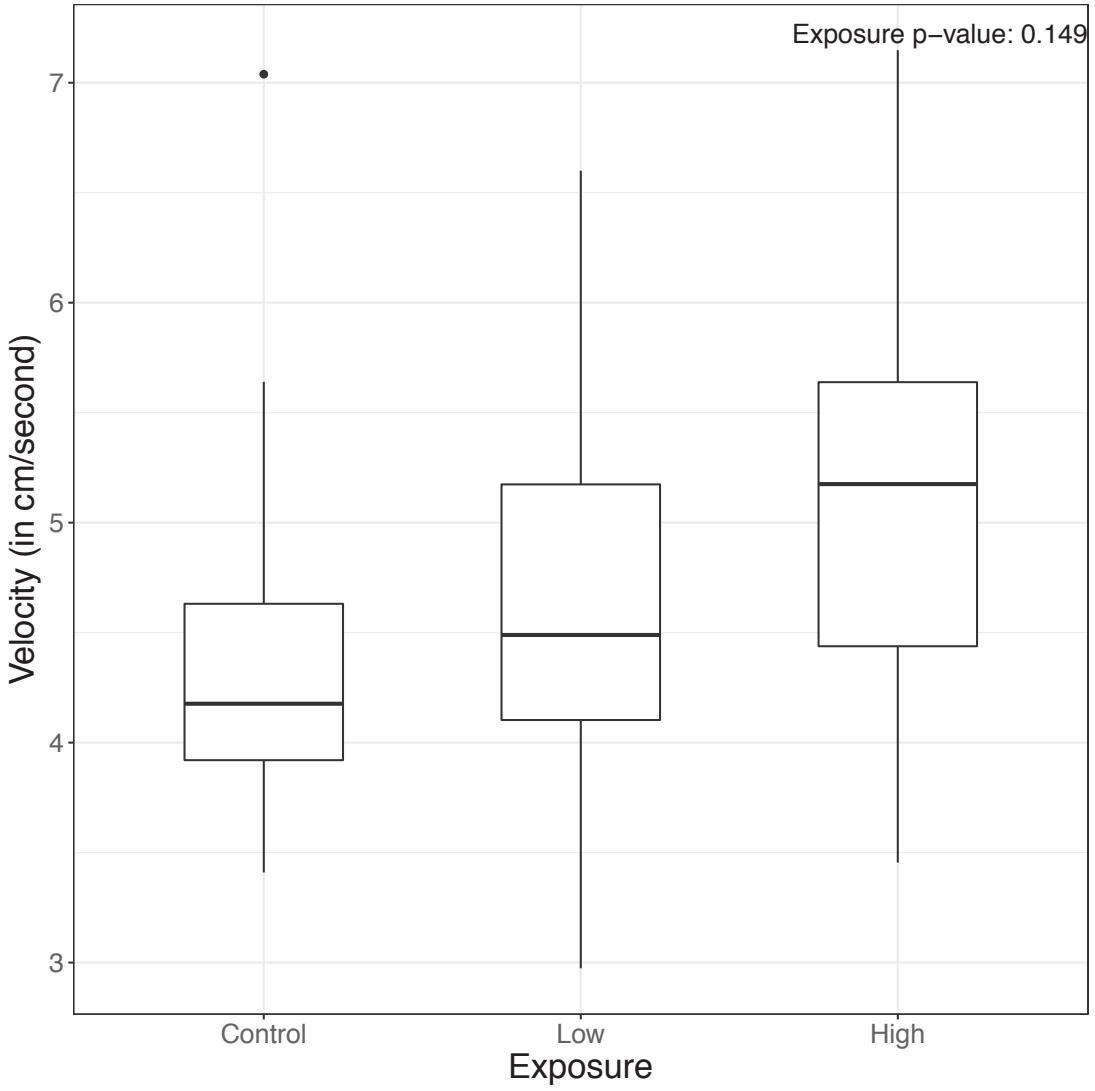


Figure S2. Box and whisker plots of the mean velocity of mothers that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

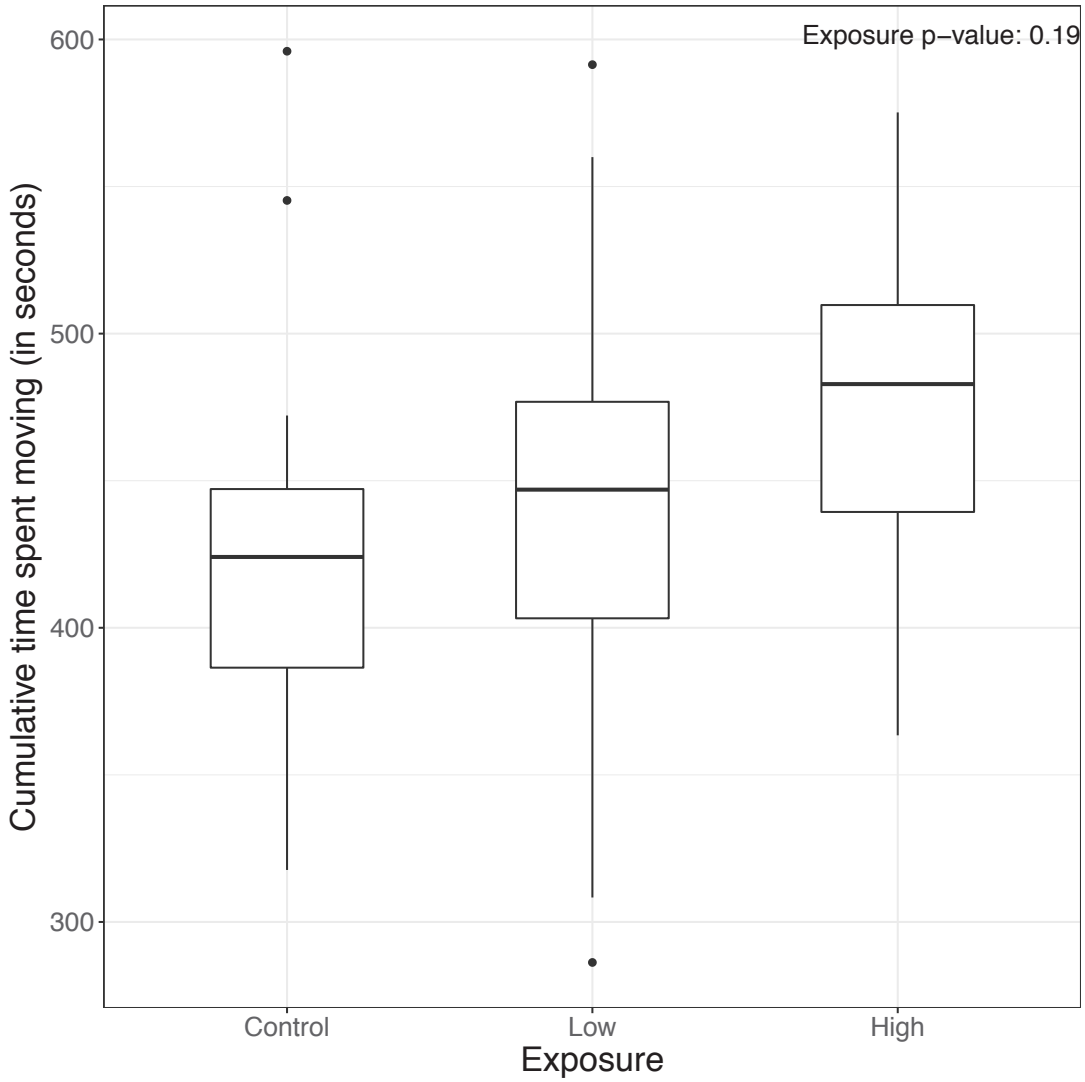


Figure S3. Box and whisker plots of the cumulative time spent moving of mothers that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

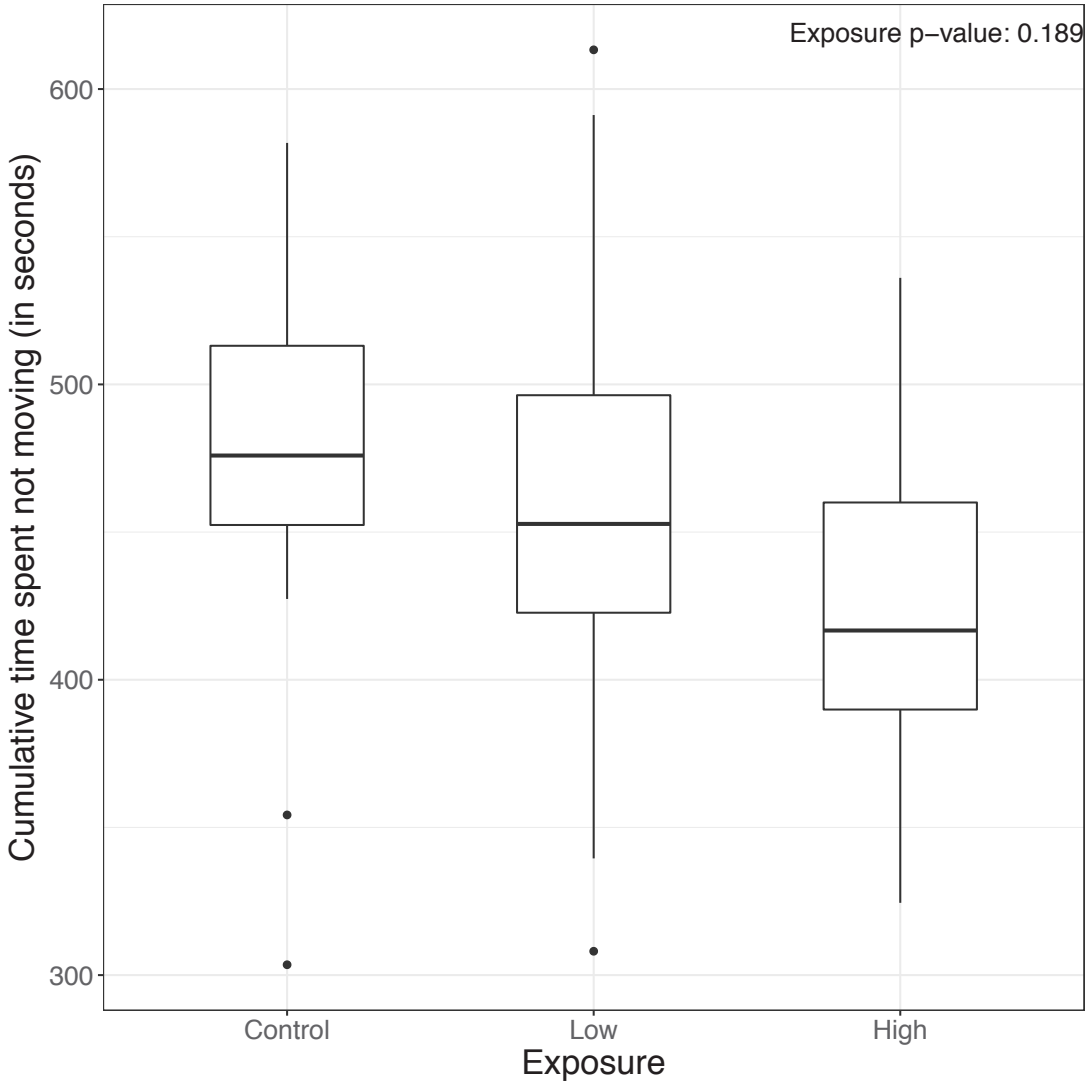


Figure S4. Box and whisker plots of the cumulative time spent not moving of mothers that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

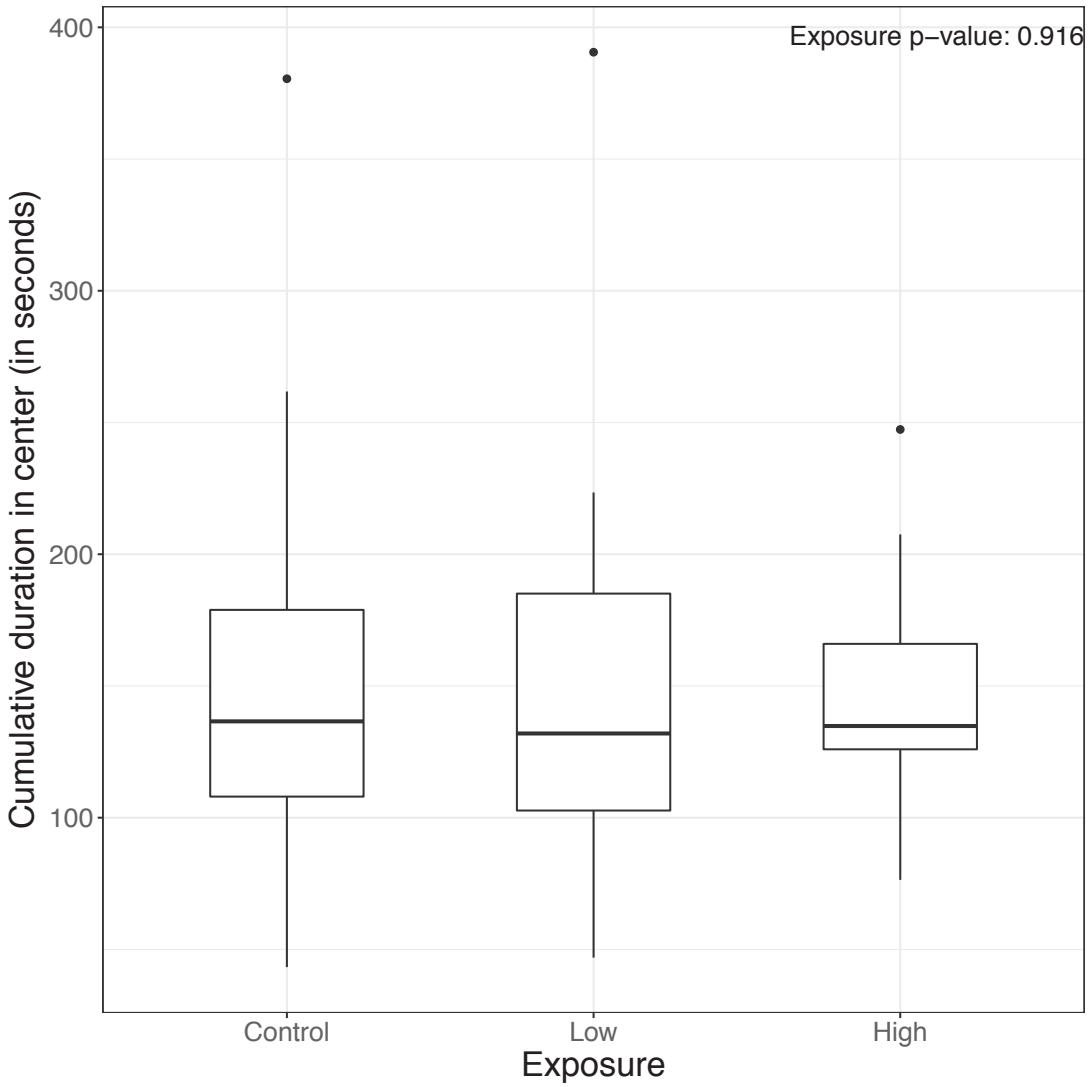


Figure S5. Box and whisker plots of the cumulative time spent in the center zone for the mothers that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 ×IQR, and the upper quartile is the third quartile plus 1.5 ×the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

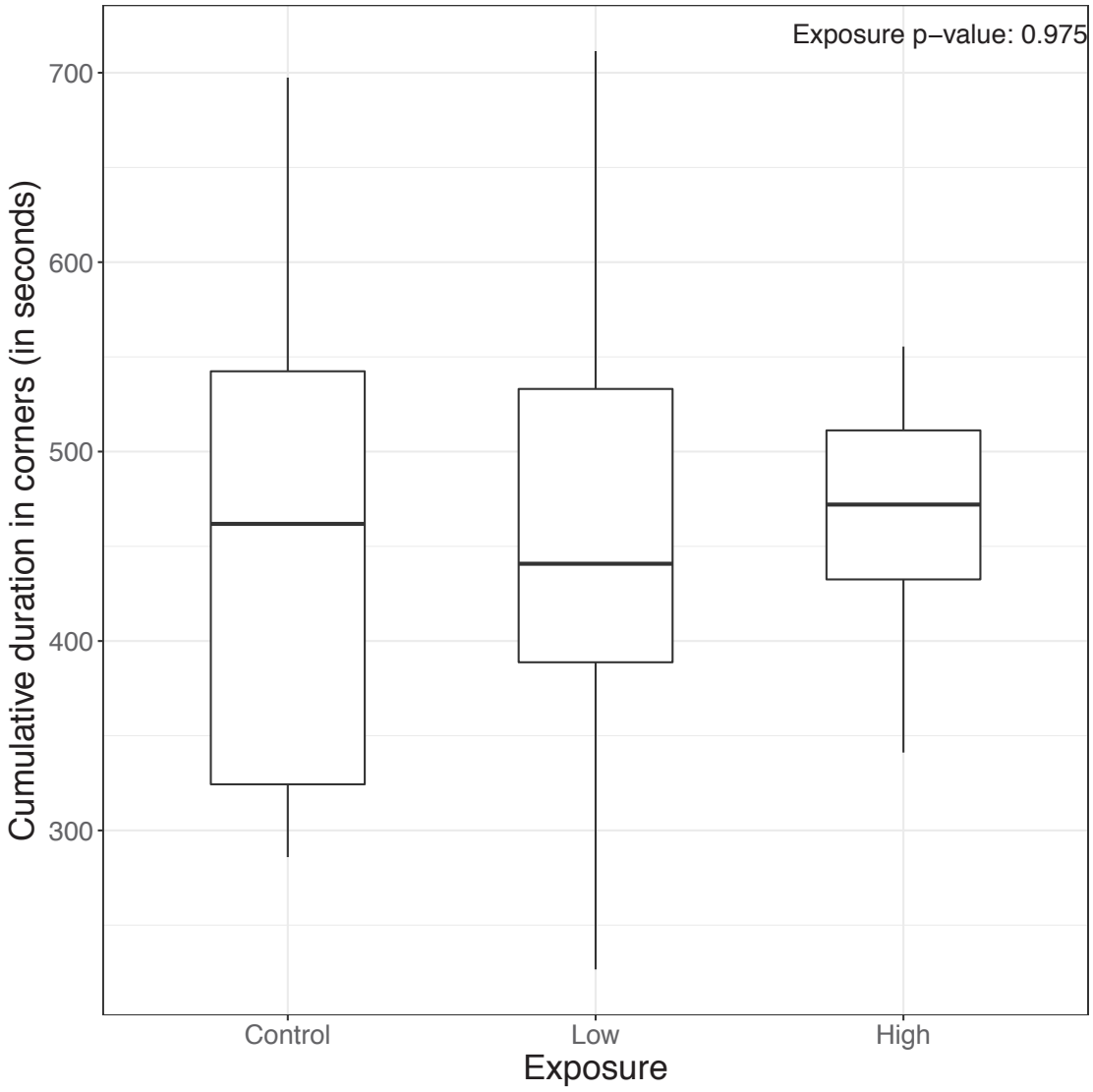


Figure S6. Box and whisker plots of the cumulative time spent in the corner zones for the mothers that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR.

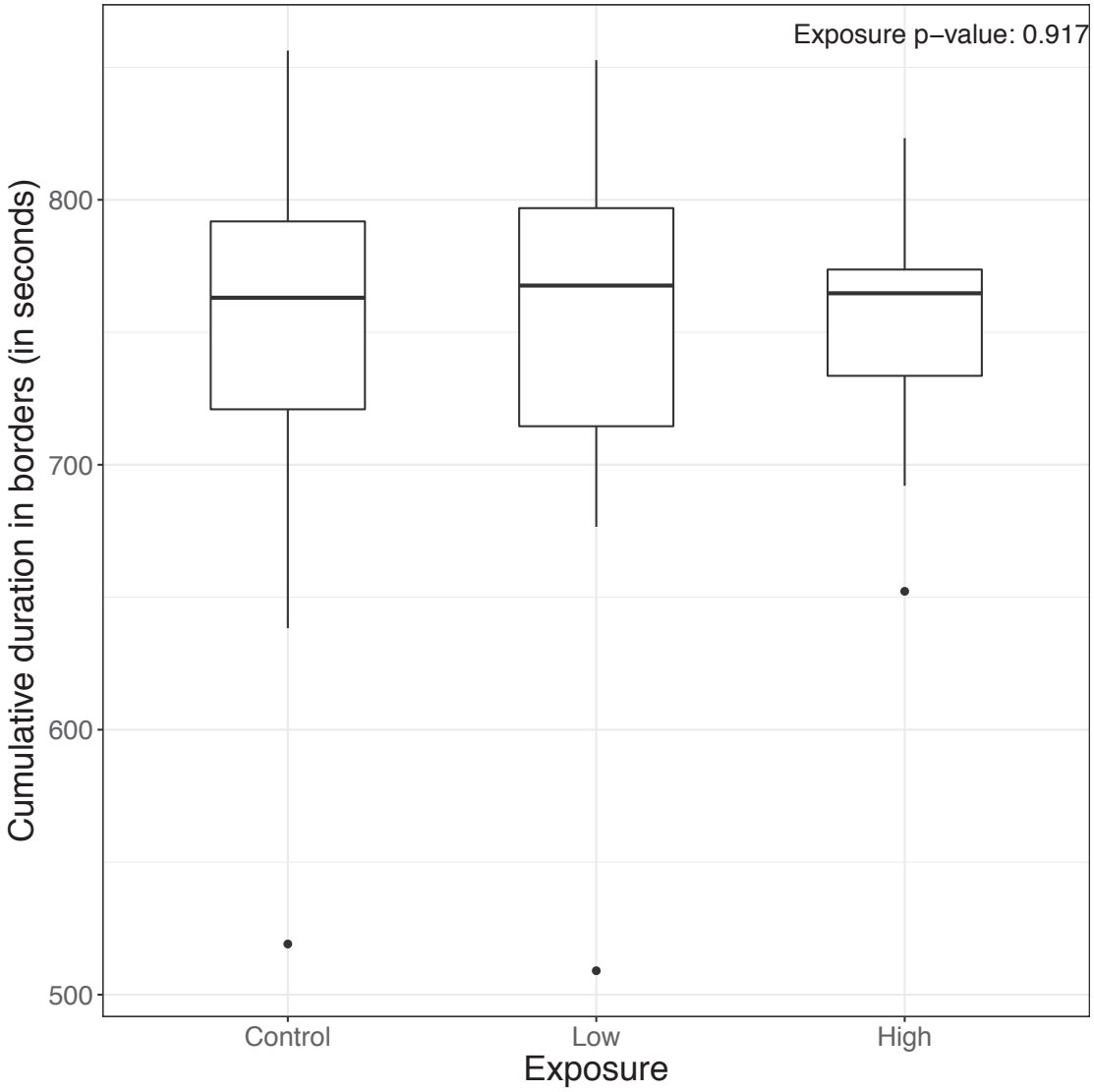


Figure S7. Box and whisker plots of the cumulative time spent in the border zones for the mothers that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

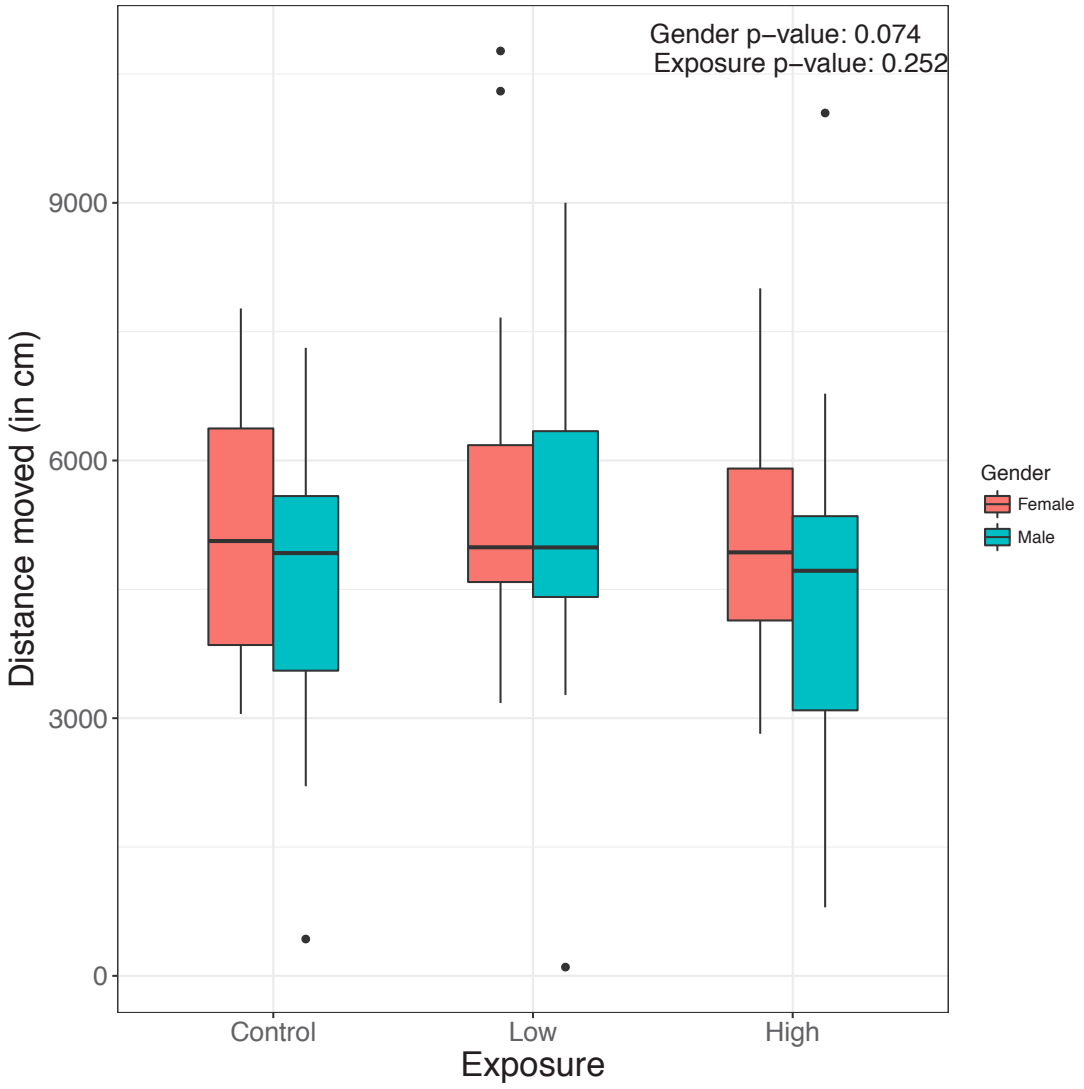


Figure S8. Box and whisker plots of the total distance moved for pups that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

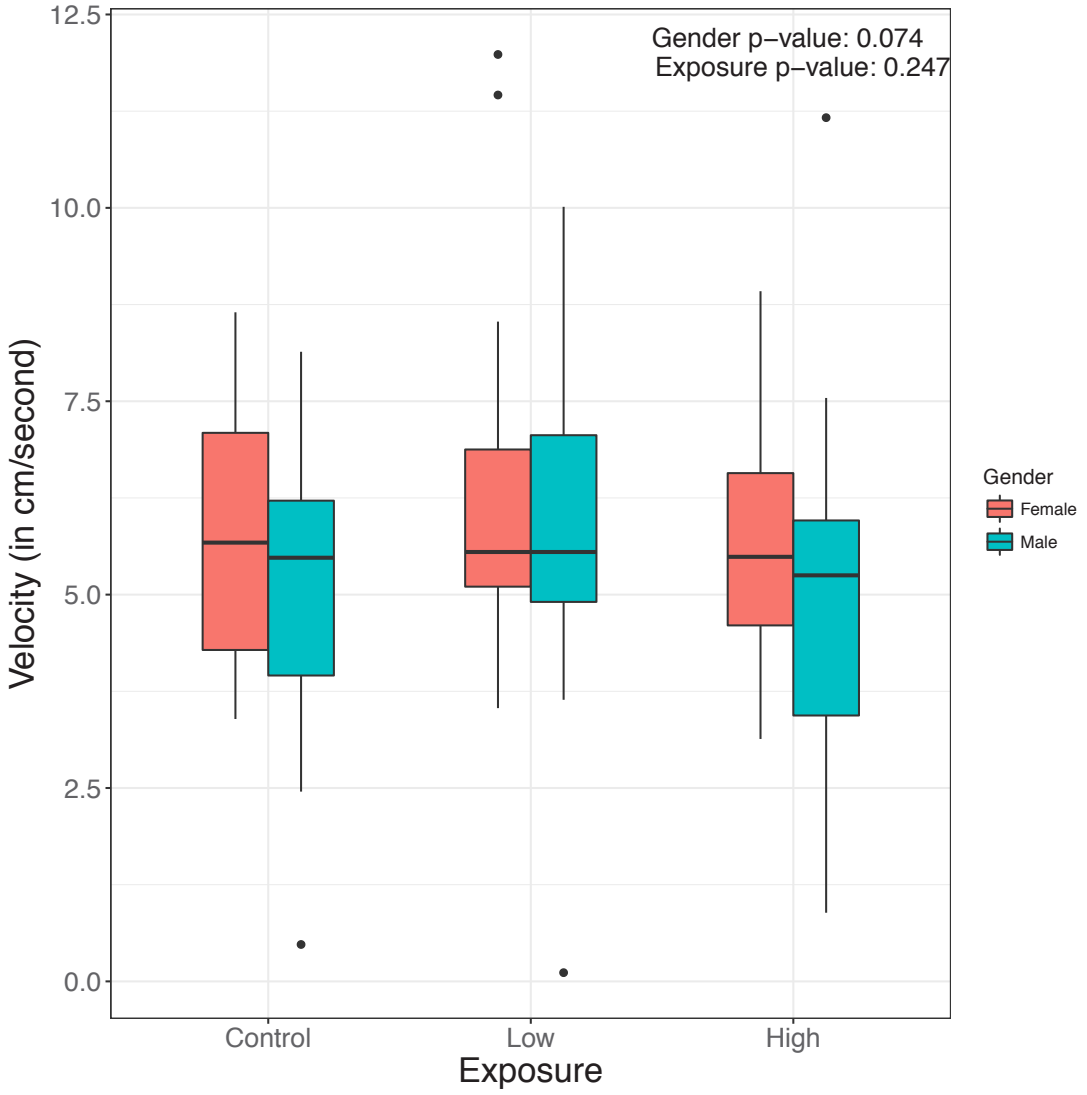


Figure S9. Box and whisker plots of the mean velocity of pups that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

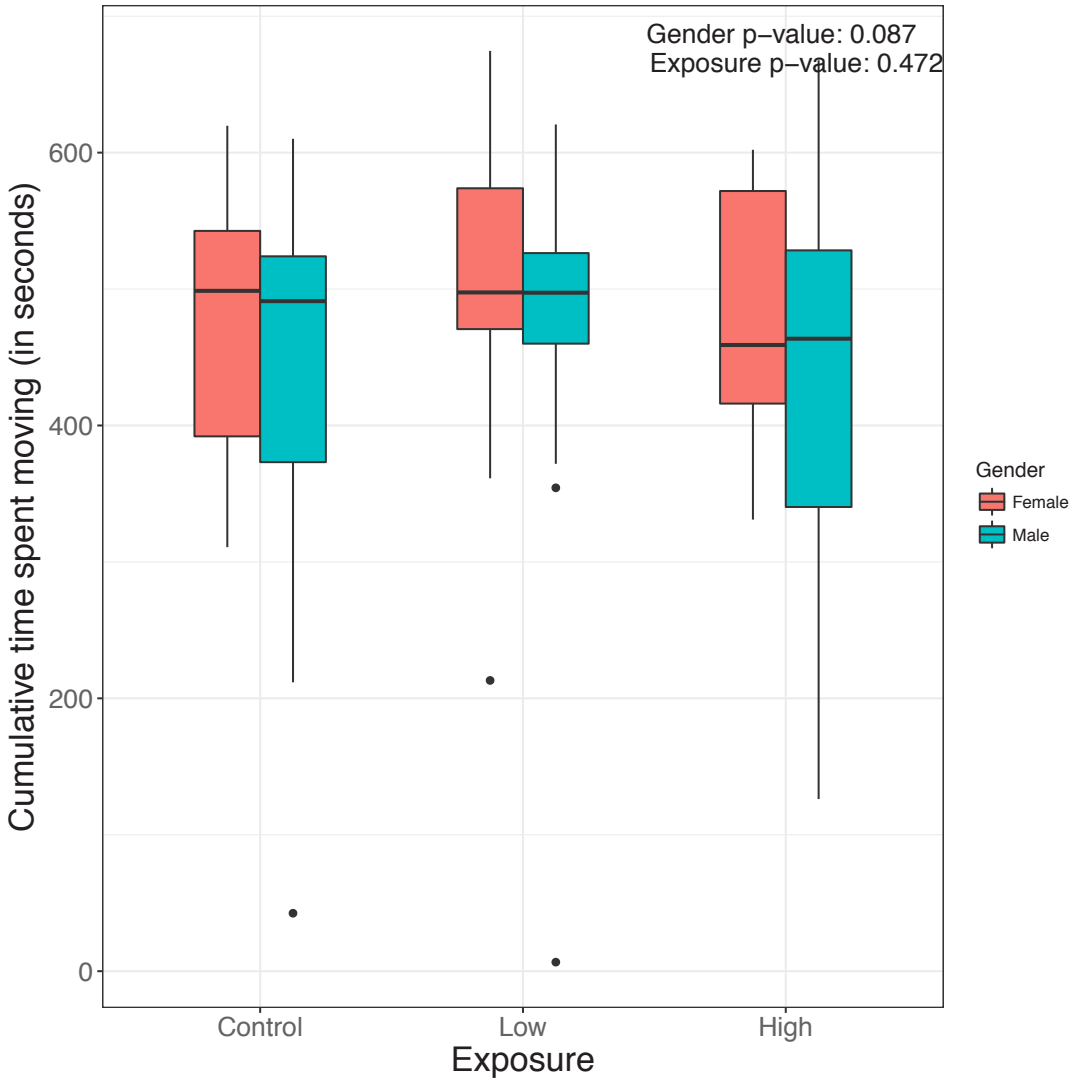


Figure S10. Box and whisker plots of the cumulative time spent moving of pups that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

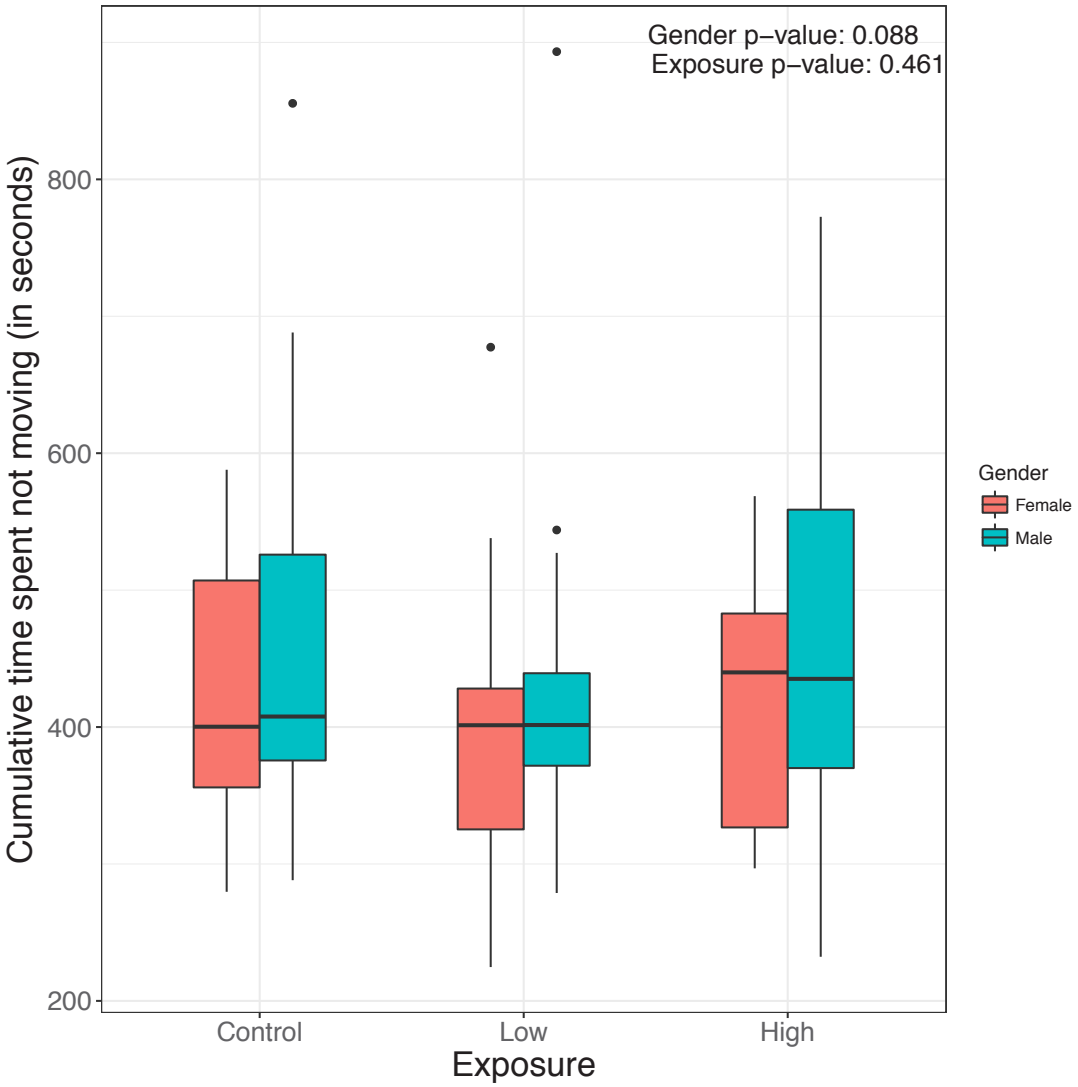


Figure S11. Box and whisker plots of the cumulative time spent not moving of pups that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 ×IQR, and the upper quartile is the third quartile plus 1.5 ×the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

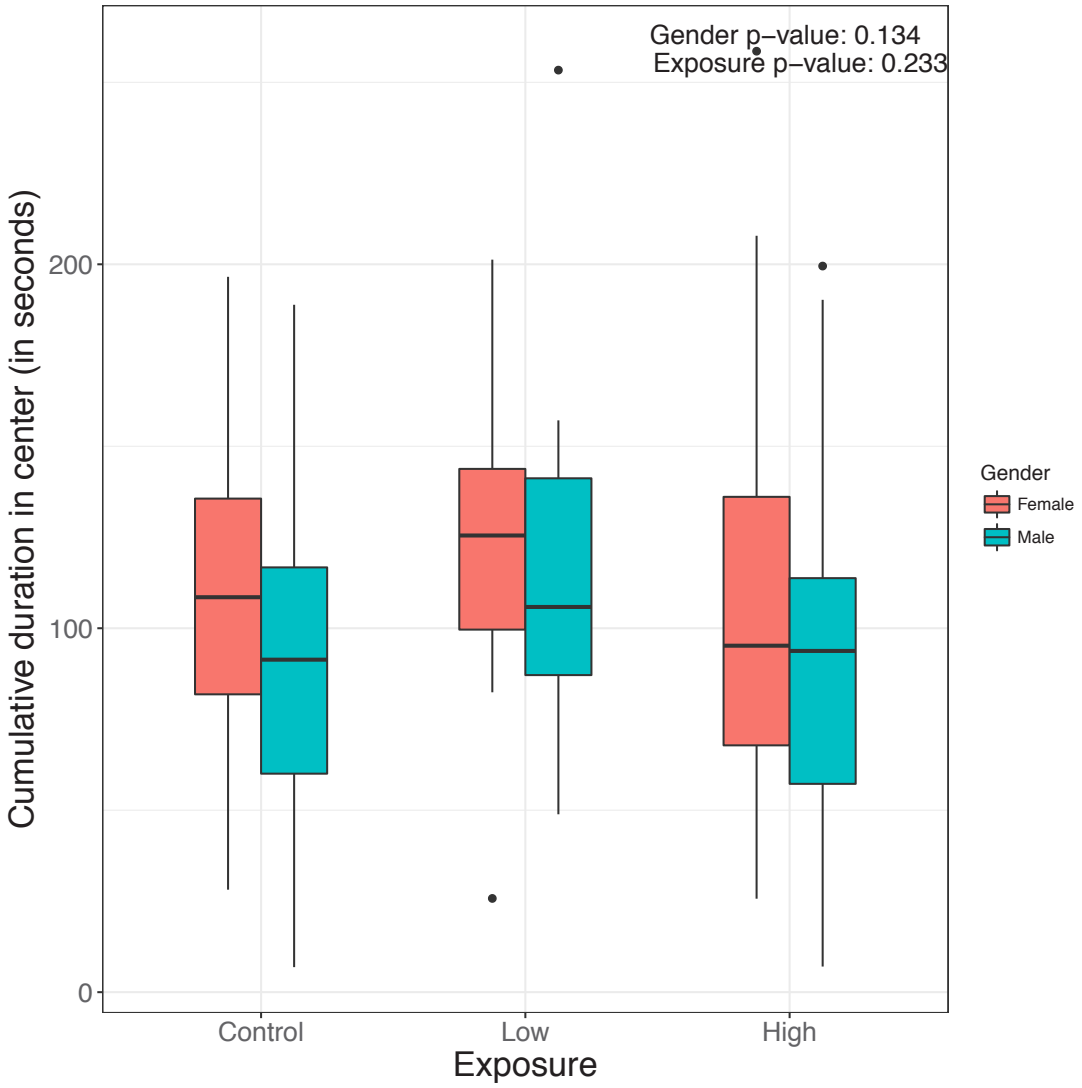


Figure S12. Box and whisker plots of the cumulative time spent in the center zone for the pups that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 ×IQR, and the upper quartile is the third quartile plus 1.5 ×the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

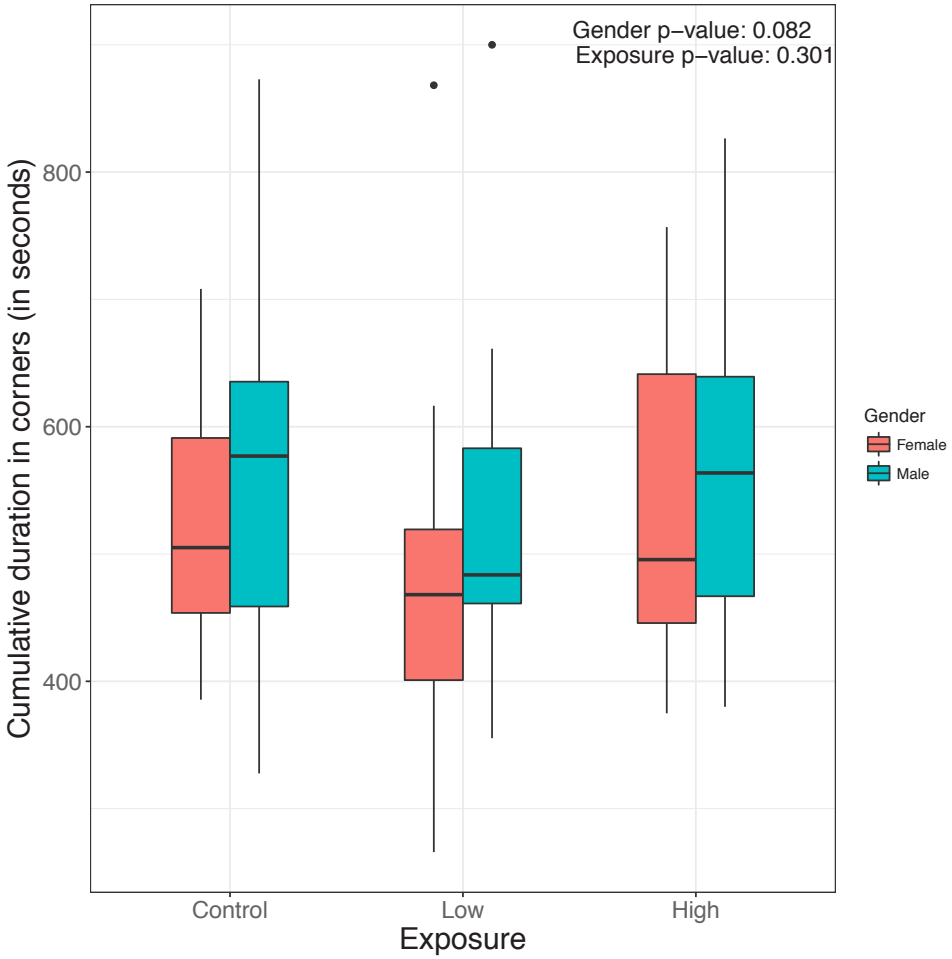


Figure S13. Box and whisker plots of the cumulative time spent in the corner zones for the pups that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

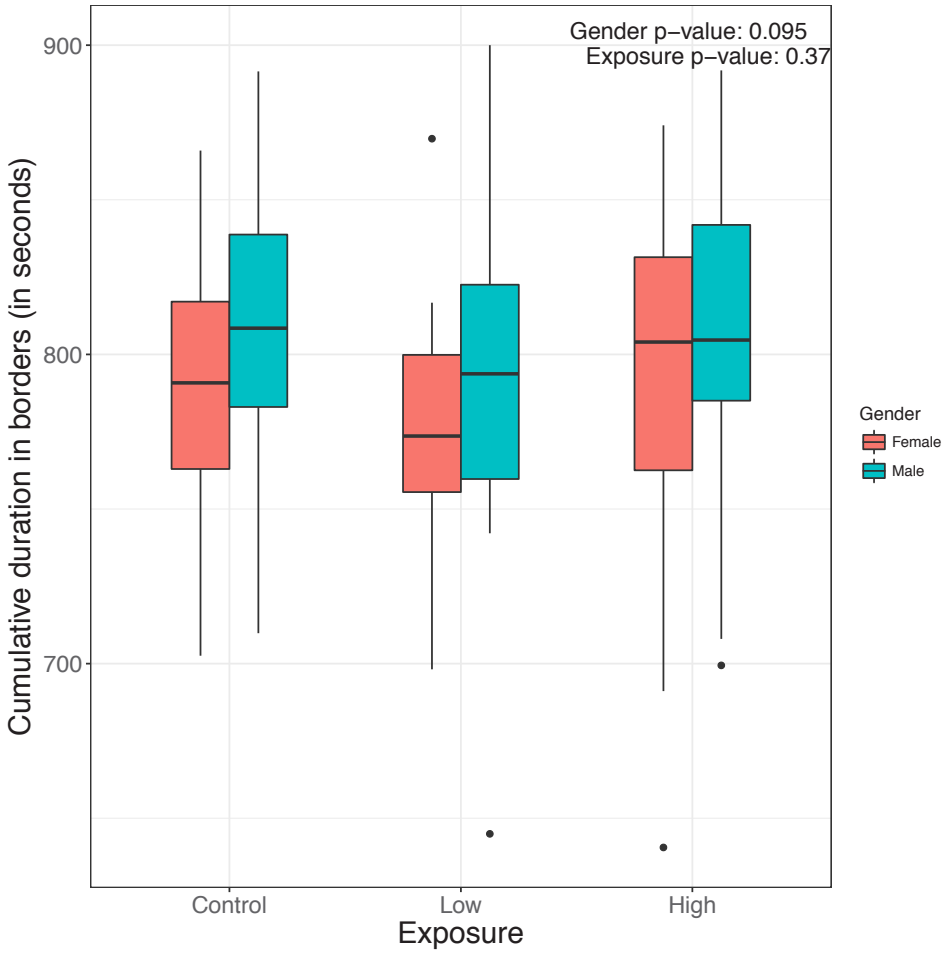
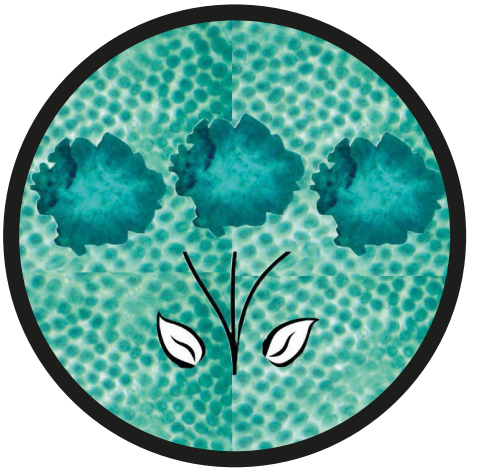


Figure S14. Box and whisker plots of the cumulative time spent in the border zones for the pups that were previously exposed to one of two doses (high and low) of persistent organic pollutants (POPs) or a control and recorded for 15 minutes in an open field test. Each box represents the first, second, and third quartile, the upper whisker is the third quartile plus 1.5 × the interquartile range (IQR), the lower whisker is the first quartile minus 1.5 × IQR, and the upper quartile is the third quartile plus 1.5 × the IQR. The outliers are those values beyond the third quartile plus 1.5 × IQR.

Paper III



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Maternal exposure to a human based mixture of persistent organic pollutants (POPs) affect gene expression related to brain function in mice offspring hippocampus

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HIGHLIGHTS

- POPs detected in the dams were found in offspring brains showing placental transfer.
- POPs exposure led to hippocampal gene expression changes related to brain function.
- External stress combined with POPs caused behavioural deficits in mice offspring.
- The human based POP mixture proved to be useful in prenatal and lactational studies.

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ABSTRACT

Male and female mice pups were exposed to a low and high dose of a human relevant mixture of persistent organic pollutants (POPs) during pregnancy and lactation. Most compounds detected in the dams were found in offspring brains. The mice offspring exhibited changed expression of hippocampal genes involved in cognitive function (*Adora2a*, *Auts2*, *Crlf1*, *Chrnb2*, *Gdnf*, *Gnal*, *Kcnh3*), neuro-inflammation (*Cd47*, *Il1a*), circadian rhythm (*Per1*, *Clock*), redox signalling (*Hmox2*) and aryl hydrocarbon receptor activation (*Cyp1b1*). A few genes were differentially expressed in males versus females. Mostly, similar patterns of gene expression changes were observed between the low and high dose groups. Effects on learning and memory function measured in the Barnes maze (not moving, escape latency) were found in the high dose group when combined with moderate stress exposure (air flow from a fan). Mediation analysis indicated adaptation to the effects of exposure since gene expression compensated for learning disabilities (escape latency, walking distance and time spent not moving in the maze). Additionally, random forest analysis indicated that *Kcnh3*, *Gnal*, and *Crlf1* were the most important genes for escape latency, while *Hip1*, *Gnal* and the low exposure level were the most important explanatory factors for passive behaviour (not moving). Altogether, this study showed transfer of POPs to the

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offspring brains after maternal exposure, modulating the expression level of genes involved in brain function.

1. Introduction

Halogenated persistent organic pollutants (POPs) are of concern to human health and wildlife because of their potential toxicity. Many are structurally closely related and share common characteristics, most notably their persistence and ubiquitous distribution in the environment, their potential for bioaccumulation in living organisms and for bio-magnification in the food chain (de Wit, 2002; Carpenter, 2006; Yogui and Sericano, 2009; Butt et al., 2010; Letcher et al., 2010; Salamova and Hites, 2011). This has led to POPs being banned from production via the Stockholm Convention or regulated through REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), however, a huge number of chemicals that we are exposed to in our daily life are not even tested for toxic properties. Due to this substantial number of compounds and their ubiquitous nature within the environment, cocktails of POPs are typically found in both humans and animals. For example, human umbilical cord blood has been reported to contain more than 200 xenobiotics. Halogenated POPs are present in the blood of children, as well as in breast milk (Aarem et al., 2016; Caspersen et al., 2016), they pass the blood-brain barrier (BBB) (Seelbach et al., 2010; Zhao et al., 2016; Wang et al., 2018) and accumulate in brain tissues (Jones and de Voogt, 1999; Olsen et al., 2007). The infant's body burden of POPs increases with length of breastfeeding, sometimes to levels higher than those of the mother. The prenatal period is a sensitive life stage for adverse effects of toxicant exposure on cognitive development, as brain development at this stage involves processes like neural tube formation and brain segmentation, proliferation and differentiation of different progenitor cell types, apoptosis, migration, phenotypic specification of neurons, and formation of receptors (Stiles and Jernigan, 2010; Kang et al., 2011; Smirnova et al., 2014; Bal-Price et al., 2018). These events create a different window of vulnerability to toxicant exposure (Rice and Barone, 2000; Smirnova et al., 2014). Thus, there is a need for understanding how environmental toxicants in neonates can affect an individual's health and development. The traditional approach has been to study single compounds at relatively high doses, but subsequent research would indicate that toxicants can have biphasic response curves, and/or additive, synergistic, or antagonistic effects on biological endpoints (Altenburger et al., 2013; Bopp et al., 2018). Therefore, effects can be difficult to predict from modelling the results of individual compounds. Consequently, there is a need to focus on the effects of human relevant mixtures.

There is a growing concern that many chemicals present in the environment are potential developmental neurotoxicants (Fritsche et al., 2018). Epidemiological literature has documented associations between neurodevelopmental disorders (Thapar et al., 2017) and prenatal and/or postnatal exposure to neurotoxicants, including lead, mercury, air pollution particulate matter ($PM_{2.5}$), pesticides and flame retardants (Jacobson and Jacobson, 1997; Bjorling-Poulsen et al., 2008; Eskenazi et al., 2008; Costa et al., 2017; Myhre et al., 2018b). However, despite decades of research, epidemiological studies have only enabled to establish associative relationships between POPs and neurodevelopmental effects. Controlled animal experiments provide a possibility to achieve mechanistic support to observational studies. There is evidence that low-level exposures during critical periods of brain development, which would have limited adverse effects in adults, can cause permanent disruptions of normal maturational processes (Rice and Barone, 2000; Grandjean and Landrigan, 2006). However, a critical data gap in human hazard information exists for several classes of POPs despite the fact that they have been commercialized for years (Lindstrom et al., 2011; Linares et al., 2015; LaKind et al., 2018; Lehmann et al., 2018). Review papers largely confirm the difficulty of appraising the body of evidence for a given neurodevelopmental or neurobehavioural outcome after exposure to mixtures of toxicants (Zhang et al., 2017; Harris et al., 2018). Collectively, when considering general effects that may be attributed to chlorinated, brominated or perfluorinated toxicants, studies suggest both pathological and sub-clinical neurodevelopmental and neurobehavioural effects. However, inconsistencies appear concerning health outcomes linked to specific toxicants (Zhang et al., 2017; Harris et al., 2018).

In humans, disorders of neurobehavioural development affect 10–15% of all births. The prevalence rates of such disorders are increasing (Bloom et al., 2010), and several reports express concerns that hazardous substances in the environment may be among the contributing causes of adverse neurodevelopment (Mendola et al., 2002; Alexander et al., 2011; Liu and Schelar, 2012; Grandjean and Landrigan, 2014; Martin et al., 2017; Tung et al., 2017). Subclinical decrements in brain function are even more common than diagnosed disorders, and can have severe consequences for quality of life, reduce academic achievement, disturb behaviour, in addition to being a huge economic burden for society (Grandjean and Landrigan, 2014; Bellanger et al., 2015).

Previously, we designed an environmentally relevant mixture of POPs for use in animal and *in vitro* experimental studies, containing 29 different chlorinated, brominated, and perfluorinated compounds (Berntsen et al., 2017). The *in vivo* POP mixture was composed based on human estimated daily intake (EDI) in Scandinavia, and aimed to provide a realistic mixture for toxicity studies based upon the relative levels of POPs to which individuals are exposed.

In mice used in the present study we recently reported that foetal and lactational exposure to the defined POP mixture affected testicular development, sperm production and sperm chromatin integrity (Khezri et al., 2017b). Additionally, in the same experiment, we found a subtle dysregulation of the hypothalamus-pituitary-adrenal axis (Hudecova et al., 2018). Recent *in vitro* studies with the POP mixture suggest adverse effects on neuronal cell function and development (Berntsen et al., 2021; Davidsen et al., 2021). In the current study, we explore whether the human based POP mixture affects the expression of hippocampal genes relevant to neuronal function and development in mouse offspring and to which extent such changes are reflected in behaviour and cognition. To support biological plausibility, we measured levels of the POPs in maternal and offspring blood and offspring brain tissue.

2. Materials and methods

2.1. Ethics statement

The study was performed at the Section for Experimental

Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The unit was licensed by the Norwegian Animal Research Authority (NARA) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC; FOTS: 5583) and NARA (2013/39783).

2.2. Chemicals and feed

A thorough description of the design and preparation of the POP mixture can be found in Berntsen et al. (2017). In brief, all polybrominated diphenyl ethers (PBDEs), PCBs and other organochlorines used for exposure were originally purchased from Chiron AS (Trondheim, Norway). All perfluorinated compounds (PFASs) and hexabromocyclododecane (HBCD) were obtained from Sigma-Aldrich (St. Louis, MO, USA), with the exception of perfluorohexane sulfonic acid (PFHxS) potassium salt which was from Santa Cruz (Dallas, US). All chemicals were dissolved in an appropriate solvent and added to corn oil (Jasmin, fully refined, Yonca Gida San A.S., Manisa, Turkey) intended for human consumption. All solvents were thoroughly evaporated under N_2-flow before the oil containing the POPs was sent to the feed company (TestDiets, St.Louis, MO) to be incorporated in the mouse feed. Four different diets were made, consisting of three exposure diets for pregnant mice (i.e. control (non-exposed), low dose (5000 $\times$ EDI) and high dose (100000 $\times$ EDI), and a reference diet for males and pups after weaning. For the control feed, solvents were added to the corn oil at identical levels to those found in the two exposure diets, whereas for the reference feed only untreated corn oil was used. In all diets, the soybean oil in the original feed recipe was exchanged with corn oil intended for human consumption. This was done to reduce background POP exposure, and to limit the amount of phytoestrogens, suspected to interfere with hormone homeostasis, and known to be present in soy-based food (Berntsen et al., 2017).

2.3. Animals, housing, exposure and necropsy

The animals were housed in open type III cages (Tecniplast, Buguggiate, Italy) on standard aspen bedding (Scanbur BK, Nittedal, Norway) and had cellulose nesting material. The animals were fed *ad libitum* and had access to tap water in standard drinking bottles (Tecniplast, Buguggiate, Italy) at all times. The animal room was on a 12:12 light–dark cycle, with a room temperature of 21 $\pm$ 2 °C with 20 air changes per hour and 45 $\pm$ 5% relative humidity.

Altogether, 47 female hybrid mice (129:C57BL/6F1) produced in our facility by 10 male C57BL/6J mice and 20 female 129S1/SvImJ (Jackson Laboratory, Maine, USA), were randomly assigned to three exposure groups, control (n = 15), low dose POP-mixture exposure (n = 16) and high-dose POP-mixture exposure (n = 16). Exposure started at weaning and lasted six months until this generation of dams was euthanized and necropsied. Four weeks after start of exposure, the female mice were mated with a non-litter mate, random hybrid male from the same generation. Two females were housed with one male for one week. One week before expected delivery, the females were single housed until weaning of their pups. After weaning at 3 weeks of age, the offspring were separated from the dams prior to testing in the BM. Power analysis based on a pilot study suggested a group size of 36 offspring (18 males and 18 females from 15 or 16 litters in each exposure group) in the BM test setup. We used randomization to decide which offspring in each litter should be used in the BM tests. These mice were then housed in non-sibling pairs with mice allocated for a different behaviour test (published in (Hudecova et al., (2018)). This randomization eliminates the effect the parents may have had on the litter and the effect of being in a specific cage may have had on the individual. The offspring were given a feed without added POP mixtures. As a result, these offspring were only exposed during foetal life and the three first weeks post-partum through milk and directly from the dam's feed when starting to nibble solid food. After completion of behavioural experiments, tissue and blood for the chemical analyses were obtained through necropsy at 9–10 weeks of age. Thus, the offspring were necropsied 6–7 weeks after end of exposure, while dams were exposed all the way through to necropsy. Body weights were not affected by exposure in either offspring or dams (Hudecova et al., 2018).

At necropsy, the mice were terminally bled from the heart under general anaesthesia (Isoflurane gas 4.5% ISO at 700 mL airflow, followed by 2.5% ISO at 100 mL airflow). Blood plasma was stored at −80 °C until pooling and analyses. The mice were decapitated and the brain gently removed from the skull and divided into two halves. The right brain half was frozen intact, while from the left half, the remaining parts after removal of hippocampus and some other regions of interest (olfactory bulb, prefrontal cortex, pituitary, cerebellum and pons), were stored for chemical analyses. Retroperitoneal adipose tissue adjacent to left kidney and dissected brain parts were frozen on dry ice and stored at −80 °C.

2.4. Behavioural testing in the Barnes maze

The Barnes maze is a commonly used test of learning and memory in rodents (Barnes, 1979). The test was set up in the animal room next to the housing room to reduce the transport time and change of environment. F1 pups exposed to POPs, in addition to control animals, were tested at 10–12 weeks of age. The horizontally placed Barnes maze table consisted of a non-reflective grey circular disk (100 cm in diameter, placed 60 cm above the floor; purchased from Noldus Information Technology, Wageningen, the Netherlands) with 20 holes (5 cm in diameter) located around the perimeter 2.5 cm from the edge. A black, stainless steel escape box (6.4 cm $\times$ 20.3 cm x 3.8 cm; equipped with an easy, accessible entrance tunnel made of steel mesh) was magnetically attached beneath one predefined hole (drawn by lottery) and its location remained constant for each mouse for the duration of the study. A 300 W halogen lamp was used as a motivator for the animals to find the escape box (all sessions), positioned above the maze to provide an aversive bright light stimulus (approximately 900 lx at the centre of the table, and 520–550 lx at the edges of the table). To further increase the motivation of the animals a fan blew air onto the maze on day three (session 5). Visual cues (black and white patterns) in plain sight of the animal were located on three of the walls surrounding the maze. An overhead video camera and Ethovision XT 11.0 tracking software (Noldus) were used to record and measure the behaviour of the animals. The animal was placed inside a transparent cylinder (Tecniplast rat play tunnel from Scanbur BK Nittedal, Norway) in the centre of the arena and left there for 30 s. When released from the cylinder, each mouse was tracked until it reached the goal box or for 4 min. Had the animal not located the goal box by 4 min, the mouse was gently guided to the box. The maze, the cylinder and the escape box were carefully cleaned using water and allowed to dry between each trial. The maze was turned 90° clockwise between each session, to avoid fragrance confounding between each session. The testing was done in the animal's light cycle, between 09.00 a.m. and 04.00 p.m. on three consecutive days, two sessions per day (morning and afternoon separated by 4 h). Endpoints studied were: time not moving (seconds), time spent in entry zone (seconds spent in the zone around the escape whole) without entering the escape box, primary latency (seconds until the nose tip touched the edge of the escape box whole), escape latency (seconds from the animal was

released at the centre of the table to entering the escape box), and distance travelled before entering the escape box (meters). All deviations were recorded continuously during testing. Male mice were tested before females. After each session, the noise intensity was recorded. The noise was measured in decibel and recorded with a Castle GA 112 Sound Level Meter (Presisjons Teknikk AS, Oslo, Norway) and background noise was stable around 32 dB.

2.5. Chemical analysis

The measurements were performed at the Norwegian University of Life Sciences (NMBU), Department of Food Safety and Infection Biology, Laboratory of Environmental Toxicology. In order to obtain enough plasma and tissue, and to reduce expenses, one pool of plasma from each exposure group (including both sexes of offspring) and tissue was made prior to chemical analyses. Plasma pools were made by pipetting 150 μl or 50 μl from each dam and offspring sample, respectively; except for from those few animals from which no or a very small sample had been obtained. The whole remaining brain part, kept for chemical analyses, were used for the pools, whereas the adipose tissue was cut with a scalpel blade while kept frozen at −10 °C in a cryostat and one half of it pooled according to exposure group.

The levels of PCBs, OCPs, brominated and PFASs were all measured in plasma and brain tissue, while only the lipophilic PCBs, OCPs and brominated compounds were also measured in adipose tissue. PFASs mainly bind to proteins and are not expected to accumulate in fat (Chen and Guo, 2009).

For the lipophilic group, extraction with cyclohexane/acetone and water was followed by gel permeation column or sulphuric acid clean-up. Separation and detection of the OCPs and PCBs were performed on a GC coupled to Electron Capture Detector (ECD) and low resolution mass spectrometry (LRMS). Detection of BDEs and HBCD was performed on a HRGC−LRMS. Plasma samples analysed for OH-metabolites were extracted as the other plasma samples, but with 1 M H_2SO_4 instead of water. The organic phases from this extraction were analysed by GC-MS like the other plasma samples. For perfluorinated compounds, the samples were extracted with methanol and clean up was accomplished using active carbon. Further, the samples were separated by high-performance liquid chromatography (HPLC), and detection achieved by tandem mass spectrometry (MS-MS). Details from the extraction, clean-up and instrument run for the samples and quality control parameters can be found in Supplementary Information.

The mixture reflected the levels of POPs found in a Scandinavian food basket. A literature review identified the most relevant POPs and the EDI levels of these compounds for a human of 70 kg. Based on the human EDI, corresponding EDIs of the different compounds for a 25 g mouse were calculated. Due to the possibility of background exposure via the mice feed and interspecies differences in compound metabolism, the feed concentration of the mixture was set to provide a mouse consuming 3g feed/day a daily dose of 5000 × and 100000 × the EDI for humans. Following exposure, the level of POPs within the feed (Berntsen et al., 2017) and various tissues (blood plasma, adipose tissue, brain) of the mothers and pups was determined. A list of the individual compounds in the mixture and their measured feed concentrations is shown in the Supplementary Table S1 for further information.

2.6. Gene transcription analysis

Quantitative real-time PCR (qPCR) was used to analyse the transcriptional responses in pooled samples of hippocampus of male and female offspring maternally exposed to the two POP mixture concentrations (low dose (n = 34), and high dose (n = 33)). We investigated a panel of 142 genes by qPCR. Samples from exposed animals were compared to the controls. From the results of initial screening, genes with expression level difference more than ± 2-fold or $p < 0.05$ between POP-mixture exposed and control mice were selected for single sample analysis. Based on these selection criteria, 20 genes were identified.

Total RNA was isolated from frozen (−80 °C) mouse hippocampi (approximately 14 mg tissue) using a ZR-Duet™ DNA/RNA MiniPrep (Zymo research) according to the manufacturer's instructions. The quantity and quality of isolated RNA was determined as previously described (Aaremet al. 2016; Dualeet al. 2014) using a NanoDrop Spectrophotometer (Thermo Scientific, Norway) and Agilent 2100 Bioanalyzer (Agilent Technologies, Norway). RNA purity was estimated by examining the OD 260/280 and the OD 260/230 ratios. RNA integrity numbers (RIN) from 1 to 10 (low to high RNA quality) were calculated using the 2100 Expert software (Agilent Technologies, Norway).

The cDNA synthesis was carried out (Aarem et al., 2016) with 100 ng total RNA from samples as template, using the miScript II RT kit including 5x miScript HiFlex Buffer (for selective conversion of mRNA into cDNA) according to the manufacturers protocol (Qiagen, Norway). A no reverse transcriptase control (NRT) was included and all cDNA samples were stored at −20 °C prior to gene expression analysis.

In the initial screening, cDNA (1:100 dilution) from each treatment group was divided into four female and four male groups and each group consists of pooled cDNA (3−4 cDNA samples) from each sex (i.e., resulting in eight pooled independent samples (4 female and 4 male samples)/treatment group), and for each pooled cDNA, two technical replicates were run. This qPCR layout allowed simultaneous measurement of all samples in one 384-well plate for each gene, reducing errors due to run-to-run variations. Gene-specific qPCR was carried out as previously described (Gutzkow et al., 2016) using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, London, UK) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Norway). Gene-specific primers were designed using the online Universal Probe Library System (Roche Applied Systems, Oslo, Norway). All PCR reactions were performed in duplicates, and data are expressed as an average of the duplicates. The raw-Cq values were analysed by the comparative Cq−method (Livak and Schmittgen, 2001; Duale et al., 2012, 2014). In brief, the stability of nine reference genes (*Actb*, *Gapdh*, *Hprt1, Pgk1*, *Rpl13a*, *Tbp*, *Tubb5, Hspb1* and *Ymhaz*) were evaluated by NormFinder algorithm (Andersen et al., 2004) and the most stably expressed reference genes were used for normalization. The results of the stability of the reference genes are presented in Supplementary Fig. S1. Prior to normalization, the raw data Cq values were pre-processed and outliers were excluded from further analysis. In addition, target genes with Cq values > 37 were considered beyond the limit of detection and excluded from further analysis. In the initial screening, target genes were normalized using the average of five stably expressed reference genes (*Actb*, *Gapdh*, *Rpl13a*, *Tbp* and *Ymhaz*) [this is given by ΔCq; where ΔCq (sample) = Cq (target gene) − Cq (average reference genes)]. The ΔΔCq values were generated by subtracting the ΔCq-value for the reference samples (calibrators; vehicle-treated control samples) from the ΔCq-value for the samples [$\Delta\Delta Cq = \Delta Cq$ (sample) − ΔCq (calibrator); fold change = $2^{-\Delta\Delta Cq}$]. The fold change values were then log2-transformed in order to make the values symmetrical around zero. From the initial screening results, 20 genes were selected and their transcriptional levels were analysed by qPCR in all samples; i.e. 33-34 mice/treatment group × 2 technical replicates. The raw Cq values were analysed by the comparative Cq-method as described above and the following reference genes (*Actb*, *Gapdh*, *Hprt1*, *Pgk1* and *Tubb5*) were used to normalize the

target genes.

2.7. Statistical analysis

Initially, the gene expression data were subjected to univariate analyses (e.g. one-way ANOVA) to investigate exposure effects (ΔCq of POP-mixture exposed samples versus ΔCq of controls). Normal distribution and equality of variances were tested for all data. For logarithm of each of the five behavioural outcomes, namely, time not moving (in seconds), time spent in entry zone (in seconds), primary latency (in seconds), escape latency (in seconds) and distance travelled (in meters), we analysed the difference between the first and fourth sessions as one outcome and the response at the fifth session as the second outcome. The association between exposure groups and behavioural outcomes were evaluated using a linear regression modelling approach, while adjusting for sex and accounting for interaction between exposure and sex. Effect estimates (along with 95% confidence intervals) reported from the analyses can be interpreted as the average change in outcome in the exposed group (High or Low) compared to the unexposed group.

In order to test whether genes are associated with exposure, we investigated the association of gene expression levels of the 20 significantly identified genes from the initial screening with exposure using linear regression models, while adjusting for sex and interaction between exposure levels and sex. Given that some genes are highly correlated with each other, we carried out a principal component analysis (PCA) and computed principal component scores that captured the variability of the gene expression. We considered the first principal component as the mediator in a causal mediation analysis to test whether the effect of exposure on behavioural outcomes were mediated through gene expression (Imai et al., 2010). Each mediation analysis was repeated for low and high exposure levels compared to the unexposed group. Average causal mediated effects, direct effects and total effects are reported for each outcome (difference between the first and fourth sessions as one outcome and the response at the fifth session as the second outcome). The implementation of Random Forests (RF) analysis on two behaviour outcomes (Log escape latency and Log time not moving) provided us with the relative importance of each gene and the exposure levels in predicting the behaviour endpoints. The RF algorithm has become a commonly used machine learning algorithm for genetic association studies, since it is a tree based model that can model interactions between multiple genes well (Goldstein et al., 2011).

Statistical analyses were carried out in R statistical software (Version 3.6.0., R Development Core Team, http://www.r-project.org) (Tingley et al., 2014). P-values < 0.05 were considered statistically significant. The R scripts can be found in the Supplementary Material.

3. Results

3.1. Concentration of chemicals

A dose-dependent increase (low vs high) was observed in both dams and offspring, for all detected compounds in all tested tissues. For exposed groups, the POP levels in dam plasma and tissues were expectedly higher than in offspring. All compounds detected in the dams were also detected in at least one, while most in all, offspring tissue compartments examined. This indicates placental and/or lactational transfer of most of the included compounds (Tables 1 and 2, Supplementary Table S1.

3.2. Concentration of POPs in mouse plasma and adipose tissue

In plasma, the perfluorinated compounds were most abundant on a wet weight basis, PFOA (ng/g) and PFOS (ng/g) dominating in both the dams and offspring generations, respectively. Additionally, PFDA was also found at high concentrations (ng/g) in all groups. For the chlorinated group of compounds, the highly chlorinated PCBs 138 and 153, as well as dieldrin dominated in all groups. Oxychlordane and PCB 180 were further among the most abundant compounds in the offspring, whereas *p,p'*-DDE was prominent in the dams (Table 2). For the brominated compounds, BDE 209, its breakdown product BDE 207 and BDE 47 dominated in the dams, whereas only a few congeners could be detected in the offspring (BDE 100 and 207, and BDE 100, 153 and 154 in the low and high groups, respectively). In adipose tissue, chemicals from the chlorinated group were observed to be the most prominent, with many of the same compounds as for blood dominating, PCB 153 and PCB 138 being the most abundant in all exposure groups (Supplementary Table S1).

3.3. Concentrations and accumulation of POPs in brain

Most compounds the dams were exposed to, were also detected in offspring brain samples. Highly chlorinated PCBs were dominating in brains from mothers and offspring followed by HCB and dieldrin (Table 1). Among the brominated compounds, BDEs 47, 99 and 100 were the most prominent congeners in the dam brains, while the higher brominated BDE 209 and 207 were the predominant in the offspring brains. PFOS, PFUnDA and PFDA were the most abundant PFAS congeners in brains from all exposure groups.

Brain/plasma ratios of measured compounds are shown in Supplementary Tables S2A and S2B. The compounds which were most efficiently transferred from blood to brain were HCB in all low exposure groups, α-HCH in dams, β-HCH in both generations, BDE 209 of the low dose group of dams and BDE 209 in offspring (Supplementary Table S2A). α-HCH shows a high brain/blood-ratio in low exposed dams, while in high exposed dams it was only detected in brain and not in blood. Further, it was not detected in brain or blood in offspring. β-HCH was found at higher levels in the brain than in blood of the low exposed offspring. BDE 209 was absent from offspring blood, but present at almost equal amounts in brain and fat when lipid adjusted, with a brain/adipose tissue ratio of 0.9 (Supplementary Table S2A). The best transferring PFAS from blood to brain was PFUnDA, with a much higher fraction of the blood levels being deposited in the brain than for any of the other PFAAs (Supplementary Table S2B).

3.4. Metabolites in plasma

Eleven chlorinated and five brominated hydroxy-metabolites were analysed in plasma from control and high exposed dams of which six chlorinated were detected in the high exposed dams (Supplementary Table S3).

3.5. Human relevance

To predict the relevance of the current exposure scenario and POP mixture for human health outcomes, we compared levels in mice plasma to the average blood levels (ng/ml) of POPs from the Scandinavian population, published in (Berntsen et al., (2017)). This comparison reveals that for some compounds, plasma levels in the low dose offspring group are human relevant. *p,p'*-DDE in humans is in the same range and even higher than the levels measured in this group. Further, most of the PCB congeners are only 10 times higher than in humans. The BDEs on the other hand, were mostly

Table 1
Levels of POPs in brains (ng/g wet weight) in pooled samples from dams and pups of the different exposure groups and generations in mice exposed to a human relevant POP mixture. The values for each group of compounds (range) are listed from the highest to lowest measured concentration. The three most prominent compounds within each exposure and compound group are highlighted in a dark grey colour.

Range	Dam						Offspring					
	Control		Low		High		Control		Low		High	
Chlorinated												
1	HCB	1.12	PCB 138	33.22	PCB 138	646.16	HCB	0.69	PCB 153	6.78	PCB 153	140.2
2	PCB 153	0.36	HCB	31.22	PCB 153	635.38	PCB 138	0.06	PCB 138	6.68	PCB 138	128.35
3	PCB 180	0.09	PCB 153	28.79	HCB	535.07	PCB 118	0.03	Dieldrin	5.51	Dieldrin	54.59
4	PCB 28	n.d.	Dieldrin	27.26	Dieldrin	344.52	PCB 28	n.d.	HCB	4.99	HCB	54.2
5	PCB 52	n.d.	*p,p'*-DDE	18.87	PCB 118	303.97	PCB 52	n.d.	β-HCH	3.22	PCB 118	38.58
6	PCB 101	n.d.	β-HCH	15.02	PCB 180	184.48	PCB 101	n.d.	PCB 180	1.81	PCB 180	35.13
7	PCB 118	n.d.	PCB 118	10.96	*p,p'*-DDE	155.21	PCB 153	n.d.	Oxychlordane	1.47	Oxychlordane	32.02
8	PCB 138	n.d.	PCB 180	8.67	Oxychlordane	125.89	PCB 180	n.d.	PCB 118	1.14	β-HCH	23.88
9	*p,p'*-DDE	n.d.	Oxychlordane	7.28	β-HCH	80.41	*p,p'*-DDE	n.d.	*trans*-Nonachlor	0.88	PCB 52	16.4
10	α-Chlordane	n.d.	*trans*-Nonachlor	5.35	*trans*-Nonachlor	65.07	α-Chlordane	n.d.	*p,p'*-DDE	0.69	*trans*-Nonachlor	13.12
11	Oxychlordane	n.d.	α-HCH	4.07	α-HCH	22.33	Oxychlordane	n.d.	PCB 28	n.d.	*p,p'*-DDE	3.02
12	*trans*-Nonachlor	n.d.	PCB 101	2.72	PCB 52	8.22	*trans*-Nonachlor	n.d.	PCB 52	n.d.	PCB 28	n.d.
13	α-HCH	n.d.	PCB 52	0.76	PCB 101	7.26	α-HCH	n.d.	PCB 101	n.d.	PCB 101	n.d.
14	β-HCH	n.d.	PCB 28	0.22	PCB 28	2.88	β-HCH	n.d.	α-Chlordane	n.d	α-Chlordane	n.d
15	γ-HCH	n.d.	α-Chlordane	n.d.	α-Chlordane	n.d.	γ-HCH	n.d.	α-HCH	n.d	α-HCH	n.d
16	Dieldrin	n.d.	γ-HCH	n.d.	γ-HCH	n.d.	Dieldrin	n.d.	γ-HCH	n.d	γ-HCH	n.d
Brominated												
1	BDE 47	n.d.	BDE 47	3.7	BDE 47	56.44	BDE 47	n.d.	BDE 209	0.96	BDE 207*	4.99
2	BDE 99	n.d.	BDE 209	2.11	BDE 99	21.51	BDE 99	n.d.	BDE 100	0.4	BDE 209	3.67
3	BDE 100	n.d.	BDE 100	1.72	BDE 100	20.27	BDE 100	n.d.	BDE 207*	0.4	BDE 153	3.28
4	BDE 153	n.d.	BDE 99	1.7	BDE 209	17.95	BDE 153	n.d.	BDE 99	0.18	BDE 100	1.44
5	BDE 154	n.d.	BDE 207*	1.35	BDE 153	17.67	BDE 154	n.d.	BDE 153	0.15	BDE 99	1.18
6	BDE 196*	n.d.	BDE 153	0.83	BDE 207*	12.88	BDE 196*	n.d.	BDE 154	0.07	BDE 154	0.39
7	BDE 202*	n.d.	BDE 154	0.57	BDE 154	6.58	BDE 202*	n.d.	BDE 47	n.d.	BDE 47	n.d.
8	BDE 206*	n.d.	BDE 196*	n.d.	BDE 208*	2.47	BDE 206*	n.d.	BDE 196*	n.d.	BDE 196*	n.d.
9	BDE 207*	n.d.	BDE 202*	n.d.	BDE 196*	n.d.	BDE 207*	n.d.	BDE 202*	n.d.	BDE 202*	n.d.
10	BDE 208*	n.d.	BDE 206*	n.d.	BDE 202*	n.d.	BDE 208*	n.d.	BDE 206*	n.d.	BDE 206*	n.d.
11	BDE 209	n.d.	BDE 208*	n.d.	BDE 206*	n.d.	BDE 209	n.d.	BDE 208*	n.d.	BDE 208*	n.d.
12	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.
Perfluorinated												
1	PFHxS	n.d.	PFOS	18.5	PFOS	266	PFHxS	n.d.	PFOS	2.12	PFOS	34.3
2	PFOS	n.d.	PFUnDA	14.4	PFUnDA	139	PFOS	n.d.	PFDA	1.21	PFDA	20.2
3	PFOA	n.d.	PFDA	13.8	PFDA	136	PFOA	n.d.	PFUnDA	1.09	PFUnDA	18.1
4	PFNA	n.d.	PFOA	4.86	PFOA	77.7	PFNA	n.d.	PFNA	0.37	PFNA	6.8
5	PFDA	n.d.	PFNA	3.44	PFNA	42	PFDA	n.d.	PFOA	0.3	PFOA	6.07
6	PFUnDA	n.d.	PFHxS	2.07	PFHxS	35	PFUnDA	n.d.	PFHxS	n.d	PFHxS	2.63
7	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d
8	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d

n.d = Not detected, *= Not added to mixture

Table 2
Plasma levels of POPs (ng/g wet weight) in pooled samples for the different exposure groups and generations of mice exposed to a human relevant POP mixture. The values for each group of compounds (range) are listed from the highest to lowest measured concentration. Human blood wet weight levels (ng/ml) gained from the corresponding *in vitro* mixture, based on the Scandinavian population (Berntsen et al., 2017), are also given for comparison. The three most prominent compounds within each exposure and compound group are highlighted in a dark grey colour.

Range	Human		Dam						Offspring					
			Control		Low		High		Control		Low		High	
Chlorinated														
1	*p,p'*-DDE	0.502	PCB 153	0.126	Dieldrin	12.884	PCB 138	90.814	PCB 153	1.160	PCB 138	3.984	PCB 138	49.756
2	PCB 153	0.362	HCB	0.088	PCB 138	12.032	PCB 153	87.31	PCB 138	1.158	PCB 153	2.891	PCB 153	48.939
3	PCB 138	0.222	PCB 138	0.079	PCB 153	7.435	*p,p'*-DDE	51.444	PCB 180	0.330	Dieldrin	1.355	Dieldrin	16.829
4	PCB 180	0.194	PCB 118	0.070	*p,p'*-DDE	5.337	Dieldrin	50.394	PCB 118	0.282	Oxychlordane	1.285	PCB 180	14
5	HCB	0.117	PCB 180	0.033	HCB	2.874	PCB 118	42.126	HCB	0.274	PCB 180	0.774	Oxychlordane	13.659
6	PCB 118	0.064	PCB 28	n.d.	PCB 180	2.723	HCB	40.551	PCB 28	n.d.	HCB	0.747	PCB 118	11.829
7	β-HCH	0.053	PCB 52	n.d.	PCB 118	2.474	PCB 180	24.4	PCB 52	n.d.	PCB 118	0.369	β-HCH	10
8	*trans*-Nonachlor	0.041	PCB 101	n.d.	Oxychlordane	1.579	Oxychlordane	21.522	PCB 101	n.d.	*trans*-Nonachlor	0.359	HCB	9.512
9	Dieldrin	0.024	*p,p'*-DDE	n.d.	β-HCH	1.547	β-HCH	14.436	*p,p'*-DDE	n.d.	*p,p'*-DDE	0.299	*trans*-Nonachlor	6.22
10	Oxychlordane	0.022	α-Chlordane	n.d.	PCB 101	1.179	trans-Nonachlor	9.843	α-Chlordane	n.d.	β-HCH	0.149	PCB 52	2.805
11	PCB 28	0.013	Oxychlordane	n.d.	PCB 52	1.084	PCB 52	4.724	Oxychlordane	n.d.	PCB 52	0.12	*p,p'*-DDE	1.707
12	α-Chlordane	0.011	*trans*-Nonachlor	n.d.	*trans*-Nonachlor	1.084	PCB 101	2.362	*trans*-Nonachlor	n.d.	PCB 28	n.d.	PCB 28	n.d.
13	PCB 52	0.010	α-HCH	n.d.	α-HCH	0.211	PCB 28	n.d.	α-HCH	n.d.	PCB 101	n.d.	PCB 101	n.d.
14	PCB 101	0.008	β-HCH	n.d.	PCB 28	n.d.	α-Chlordane	n.d.	β-HCH	n.d.	α-Chlordane	n.d.	α-Chlordane	n.d.
15	α-HCH	0.006	γ-HCH	n.d.	α-Chlordane	n.d.	α-HCH	n.d.	γ-HCH	n.d.	α-HCH	n.d.	α-HCH	n.d.
16	γ-HCH	0.006	Dieldrin	n.d.	γ-HCH	n.d.	γ-HCH	n.d.	Dieldrin	n.d.	γ-HCH	n.d.	γ-HCH	n.d.
Brominated														
1	HBCD	0.025	BDE 47	n.d.	BDE 209	4.884	BDE 209	26.115	BDE 47	n.d.	BDE 100	0.09	BDE 154	1.951
2	BDE 209	0.011	BDE 99	n.d.	BDE 47	1.232	BDE 47	10.761	BDE 99	n.d.	BDE 207*	0.159	BDE 153	1.098
3	BDE 47	0.009	BDE 100	n.d.	BDE 207*	0.642	BDE 207*	4.724	BDE 100	n.d.	BDE 47	n.d.	BDE 100	0.61
4	BDE 153	0.010	BDE 153	n.d.	BDE 100	0.526	BDE 99	3.806	BDE 153	n.d.	BDE 99	n.d.	BDE 47	n.d.
5	BDE 99	0.004	BDE 154	n.d.	BDE 99	0.495	BDE 100	3.806	BDE 154	n.d.	BDE 153	n.d.	BDE 99	n.d.
6	BDE 100	0.002	BDE 196*	n.d.	BDE 154	0.263	BDE 153	2.49	BDE 196*	n.d.	BDE 154	n.d.	BDE 196*	n.d.
7	BDE 154	0.002	BDE 202*	n.d.	BDE 153	0.179	BDE 154	0.919	BDE 202*	n.d.	BDE 196*	n.d.	BDE 202*	n.d.
8	BDE 206*	N/A	BDE 206*	n.d.	BDE 196*	n.d.	BDE 196*	n.d.	BDE 206*	n.d.	BDE 202*	n.d.	BDE 206*	n.d.
9	BDE 207*	N/A	BDE 207*	n.d.	BDE 202*	n.d.	BDE 202*	n.d.	BDE 207*	n.d.	BDE 206*	n.d.	BDE 207*	n.d.
10	BDE 208*	N/A	BDE 208*	n.d.	BDE 206*	n.d.	BDE 206*	n.d.	BDE 208*	n.d.	BDE 208*	n.d.	BDE 208*	n.d.
11	BDE 209	N/A	BDE 209	n.d.	BDE 208*	n.d.	BDE 208*	n.d.	BDE 209	n.d.	BDE 209	n.d.	BDE 209	n.d.
12	HBCD	N/A	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.
Perfluorinated														
1	PFOS	29.43	PFOS	0.89	PFOA	345	PFOA	6980	PFOS	15.9	PFOS	28.8	PFOS	635
2	PFOA	4.52	PFOA	0.53	PFDA	246	PFOS	3403	PFDA	14.2	PFDA	27.5	PFOA	598
3	PFHxS	3.45	PFNA	0.32	PFOS	223	PFHxS	3381	PFNA	13	PFOA	26.1	PFDA	503
4	PFNA	0.80	PFDA	0.25	PFNA	176	PFDA	2580	PFOA	11.5	PFNA	23.5	PFNA	470
5	PFDA	0.50	PFUnDA	0.16	PFHxS	161	PFNA	2531	PFHxS	6.39	PFHxS	15.5	PFHxS	317
6	PFUnDA	0.56	PFHxS	n.d	PFUnDA	63	PFUnDA	724	PFUnDA	3.15	PFUnDA	6.22	PFUnDA	114
7	PFDoDA*	N/A	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	0.81	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d
8	PFTrDA*	N/A	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d

n.d = Not detected, N/A = Not available, *=Not added to mixture

not detected in the offspring, whereas they were up to 450 times higher than human blood levels in the low dose exposed dams. For the perfluorinated compounds, PFOS levels were similar to human blood levels in the low dose offspring. PFHxS and PFOA were 5–6 times higher and the remaining three PFAAs were 10–60 times higher (Supplementary Table S4).

3.6. Gene analysis

The genes selected from the initial screening are presented in Supplementary Fig. S2. Initial screening identified 20 genes (*Adora2a, Alk, Auts2, Bnip1, Cd47, Chrnb2, Clock, Crlf1, Cyp1b1, Gdnf, Gnal, Hip1, Hmox2, Il1a, Kcnh3, Ier3, Mecp2, Per1, Pnpt1* and *Tnf*) that were analysed in all samples (N = 101 samples; i.e., 33 or 34 mice/treatment group). The relative transcript levels are shown in Fig. 1. Thirteen genes (*Adora2a, Alk, Auts2, Cd47, Chrnb2, Clock, Crlf1, Cyp1b1, Gnal, Hmox2, Il1a, Kcnh3 and Per1*) were identified as significant differentially expressed in POP-mixture exposed groups compared to the control group (Fig. 1A). Initial univariate analysis indicated that the expression levels of eight genes (downregulated genes*: Auts2, Adora2a, Gnal, Per1*, and upregulated genes: *Kcnh3, Cyp1b1, Hmox2, Cd47*) were changed more than 2-fold relative to control samples (Supplementary Table S5). Interestingly, three genes (Auts2, *Kcnh3 and Cyp1b1)* were differentially expressed

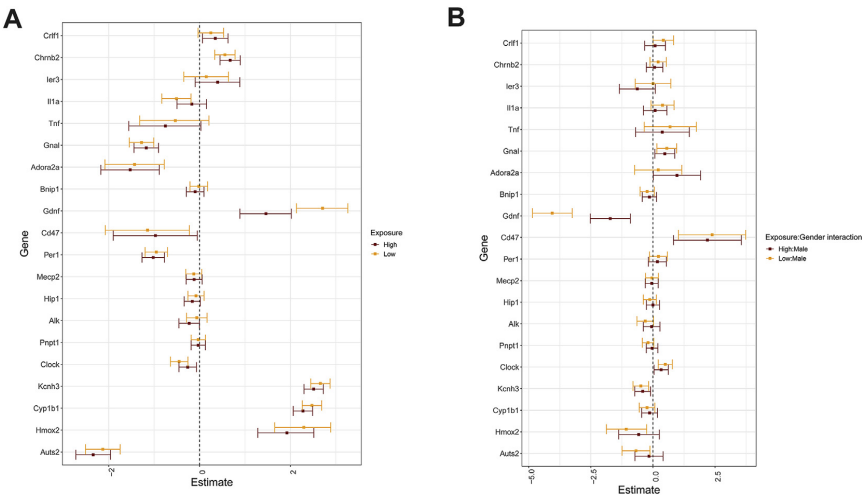

Fig. 1. Average change in gene expression levels in mouse offspring maternally exposed to a human relevant mixture of POPs at two dose levels (high and low) at 10–12 weeks of age, adjusted for sex and interaction between exposure and sex. Fig. 1A illustrates the average change in gene expression between the levels of exposure against the control group. Fig. 1B describes the average change in gene expression between highly exposed males (and low exposed males) compared to females in the control group, representing the interaction effect.

more than 9-fold relative to control samples in both low and high groups. In general, the transcript levels of most of the 20 genes showed similar expression pattern in high and low exposed offspring when compared with controls (Fig. 1). Further, there were no statistical significant differences between genders (males and females) and exposure groups, except for the *Gdnf* gene (Fig. 1). Estimates (fold change) and 95% confidence intervals of association between each gene with main effects of gender and exposure and interaction between gender and exposure under a generalized linear model framework is shown in Supplementary Table S6.

Correlation analysis was conducted with the transcript level of the 20 identified genes. Visual inspection of the correlation heatmap (Supplementary Fig. S3) reveals that some of these genes were highly correlated with each other. Therefore, a PCA was carried out to capture the variability of the gene expression levels.

The first principal component (PC1) of clustered genes explained 36.4% of the variation and was used as a mediator variable in a mediation model. PC1 is dominated by increased and decreased levels of gene expression as shown in the biplot of the components (Supplementary Fig. S4).

3.7. Behavioural testing in offspring

After repeated behaviour testing of the offspring in four consecutive sessions, there was no exposure effect on learning ability, expressed as the difference in behavioural endpoints between session 1 and 4 (Fig. 2A). However, in session 5, when a fan was introduced, offspring of high exposed mothers showed significantly longer time not moving and longer escape latency (Fig. 2B). A similar trend was found for the low dose exposure group, although not statistically significant. Sex did not affect behaviour outcomes.

Mediation analysis indicated that the direct effect of gene transcription levels as expressed by PC1 from PCA contributed with an adaptive response in several behaviour endpoints, including escape latency, distance travelled and time not moving, thereby bringing the total effect towards the normal (e.g. control; Fig. 3). Fig. 4 shows rank ordering of gene expression after random forest analysis, where *Hip1*, *Gnal* and exposure (low dose) were the most important three predictors for not moving, while *Kcnh3*, *Gnal* and *Crlf1* for escape latency.

4. Discussion

4.1. Internal tissue dose levels of the POPs

The toxicants in the POP mixture used in our study were selected for their presence in Scandinavian food products, breast milk or blood, and the mixture was designed based on defined human estimated daily intake levels (Berntsen et al., 2017). The measured concentrations in mouse blood and tissue showed that our exposure regime resulted in concentrations in the low dose group of maternally exposed offspring, comparable to levels in the Scandinavian population. Generally, compounds with high content in the feed were also detected at high concentrations in the tissues, though a few compounds were not detected at all (HBCD, α-chlordane and γ-HCH). The concentration of several POPs in offspring plasma were higher than in the dams (Table 2). All offspring were placed in a separate room after weaning and given the reference feed, which was not added POPs. The reason for the higher levels in control offspring is unknown.

Some compounds showed greater affinity for the brain than other chlorinated (β-HCH, α-HCH, HCB), brominated (BDE 209) and perfluorinated (PFUnDA) compounds.

4.2. Concentration and accumulation of the POPs in brain

The abundance of potential hazardous chemicals in the brains of exposed offspring is of special relevance for brain development and may induce behavioural effects. In this context it is important to emphasize that the mice were terminally bled, and little blood was likely to be left in brain capillaries. It is therefore assumed that the measured concentrations reflect the levels in brain tissues. Although difficult to retrieve for humans, measurements of

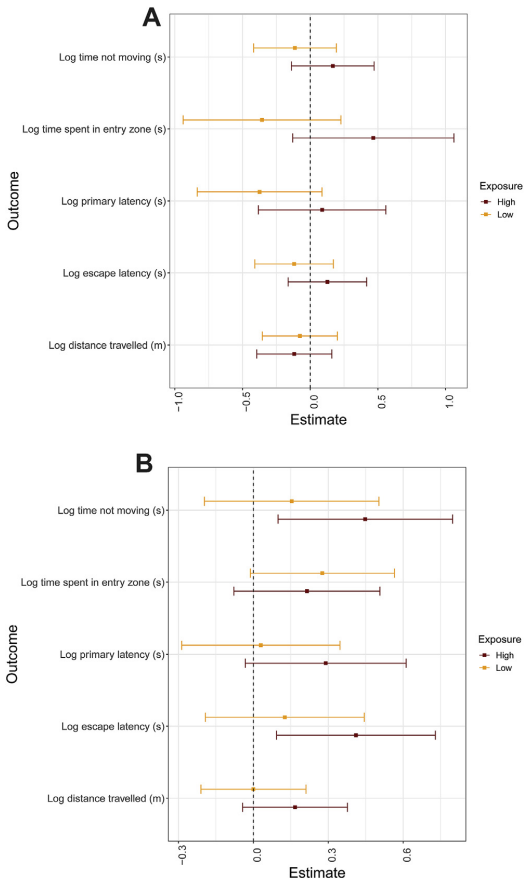

Fig. 2. Average change in behavioural performance in mouse offspring maternally exposed to a human relevant mixture of POPs at two dose levels (high and low) and subjected to the Barnes maze test repeated five times (5 sessions) at 10–12 weeks of age. Fig. 2A illustrates the difference in behavioural outcomes between session 1 and 4 (e.g. the learning effect) and 2B the same outcomes when a noisy fan was introduced to increase the motivation for hiding (session 5).

organochlorine (OC) or PBDE concentrations in human brain tissues (Dewailly et al., 1999) associated to neurological disorders (Corrigan et al., 1996, 1998; Hatcher-Martin et al., 2012; Mitchell et al., 2012) have been performed. OCs included in our experiment occurred in human brains at levels comparable with concentrations in low exposed offspring and low exposed dams in the current experiment, confirming human relevance of the exposure in the present study.

Most compounds detected in dams were also found in offspring brain samples, indicating transfer of these compounds across the placenta as well as the BBB. Small lipid-soluble compounds will diffuse across the BBB, while other compounds would need carrier- or receptor-mediated transport (Goasdoue et al., 2017). Thus, the lipid-soluble POPs such as the chlorinated and brominated compounds will easily cross this barrier, whereas one would expect the less lipophilic, protein binding PFAS to need another transport mechanism.

We observed that the brain/adipose tissue ratio was much higher for BDE 209 than for the other BDEs (Supplementary Table 2A). This may seem contradictory to published data, suggesting highly brominated congeners to penetrate the BBB less efficiently than congeners with lower bromine levels (Zhao et al., 2016). Foetal exposure may lead to higher brain levels of this compound than after postnatal exposure, as shown in rats (Zhang et al., 2011). This seems likely based on the current data where lipid adjusted levels of BDE 209 in brain were almost similar in the dams and offspring of the low exposure group despite a longer and continuous exposure period in the dams. Still, such an effect is difficult to explain as the foetal BBB is functional at an early stage in many mammals, including rodents (Goasdoue et al., 2017).

The fact that PFUnDA transferred best from blood to brain, compared with the other PFAAs (Supplementary Table 2B), could according to Greaves and colleagues be due to higher lipophilicity and brain accumulation of longer chained PFAAs (Greaves et al., 2013). This group also hypothesized that PFASs with different chain length had specific affinity to different proteins and tissues, with certain proteins binding preferentially to PFCAs with a certain chain length (Greaves et al., 2012). Furthermore, the same authors indicated that longer chained PFCAs (C_{10}–C_{15}) may be transported across the BBB via mechanisms resembling the transport of saturated fatty acids (Greaves et al., 2013) which may perhaps explain the increased accumulation of the longer chained PFCAs in the brain. Literature on PFUnDA in the brain is sparse, but it was found to be one of the most abundant PFAS measured in the brains of polar bears from Greenland (Eggers Pedersen et al., 2015). Here, PFUnDA was detected at same average concentrations as PFOS across different brain regions, but at lower levels than PFTrDA (Eggers Pedersen et al., 2015). Also, in brain samples from harbour seals and red-throated divers the longer chained PFAAs such as PFUnDA have been found to accumulate to a higher extent in brain relative to blood, than shorter chained compounds (Ahrens et al., 2009; Rubarth et al., 2011).

4.3. Metabolites of POPs in relation to developmental neurotoxicity

It has been suggested that highly brominated BDEs like BDE 209 may debrominate to more toxic, lower brominated compounds (Martin et al., 2017). Of the five congeners likely to be debromination products and which are marked with an asterisk in Tables 1 and 2 and Supplementary Table S1, only BDE 207 was detected in all measured compartments, whereas BDE 208 was detected less frequently and at lower concentrations. Since brominated congeners detected in the mouse tissues were not present in the feed, debromination has most likely taken place in the animals. BDE 207 is presumably a frequent *in vivo* debromination product of BDE 209, as found in both rats (Wang et al., 2010), cows (Kierkegaard et al., 2007), human (Qu et al., 2007), and in the mouse tissues in the present study.

Hydroxylated (OH) metabolites of the chlorinated and brominated POPs were identified in the current study (Supplemental Table 3), where three out of six OH-metabolites detected in the high exposed dams (4-OH-PCB 107, 3′OH-PCB 138, 4-OH-PCB 146) are among the five most frequently found in human blood (Grimm et al., 2015). The presence of OH-metabolites like 4-OH-CB107 may have toxicological implications since they have shown greater toxicity and increased abundancy compared to the parent compounds (Antunes-Fernandes et al., 2011; Grimm et al., 2015). However, it is an uncommon finding in humans that levels of these metabolites exceed the most persistent and accumulating PCB parent compounds (Grimm et al., 2015). PFASs do not undergo metabolism in the liver or other tissues (Pizzurro et al., 2019), precluding any concern about species extrapolation after

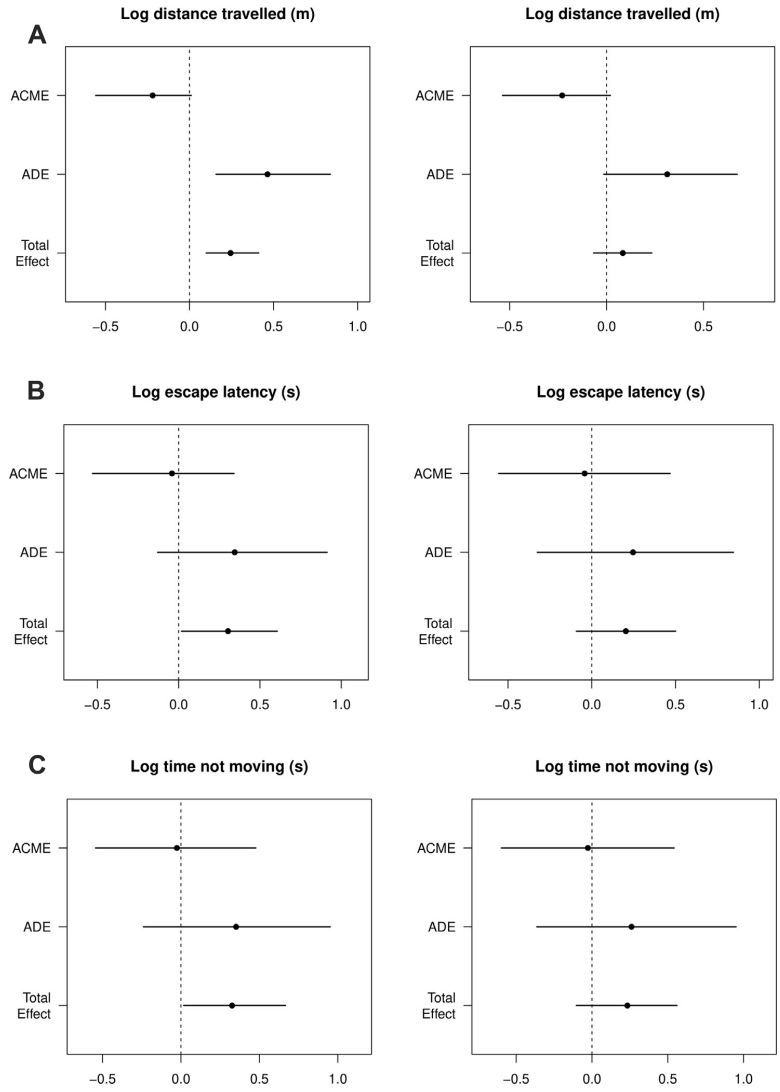

Fig. 3. Estimated average causal mediated effect (ACME), average direct effect (ADE) and total effect of exposure, with mediation by gene transcription levels, on behavioural endpoints in session 5 of the Barnes maze test for high (left panel) and low dose mice (right panel) maternally exposed to a human relevant mixture of POPs. The plots show mean estimates with confidence limits. The zero stippled line is the reference (e.g. control group). Three behavioural endpoints are shown: A) Log transformed escape latency, B) Log transformed distance travelled, C) Log transformed time not moved.

metabolism. Two perfluorinated compounds, perfluorododecanoic acid (PFDoDA) and perfluorotridecanoic acid (PFTrDA), were not added to the feed, but were already in the test panel and therefore measured. Interestingly, they were detected at low levels in the high dose feed (unpublished data) and PFDoDA also in high dose dam plasma (Table 2). As these compounds are longer than the PFAAs added to the mixtures, they cannot be break down products. Rather, they must be contaminants, and as they were only detected in the high dose feed, contamination of the PFAAs used with these longer-chained congeners is the most likely explanation for their presence. The design of our mouse study did not allow determination of the relative importance of metabolites compared to the parent compounds for the observed gene expression changes and behavioural outcomes.

4.4. Hippocampal gene expression levels

An approach to study how cells respond to a stress condition is to investigate how the gene transcription is altered, for instance in relation to disturbed neurodevelopmental and cognitive processes. In exposed mice we observed differential expression of hippocampal genes related to cognitive function with increased

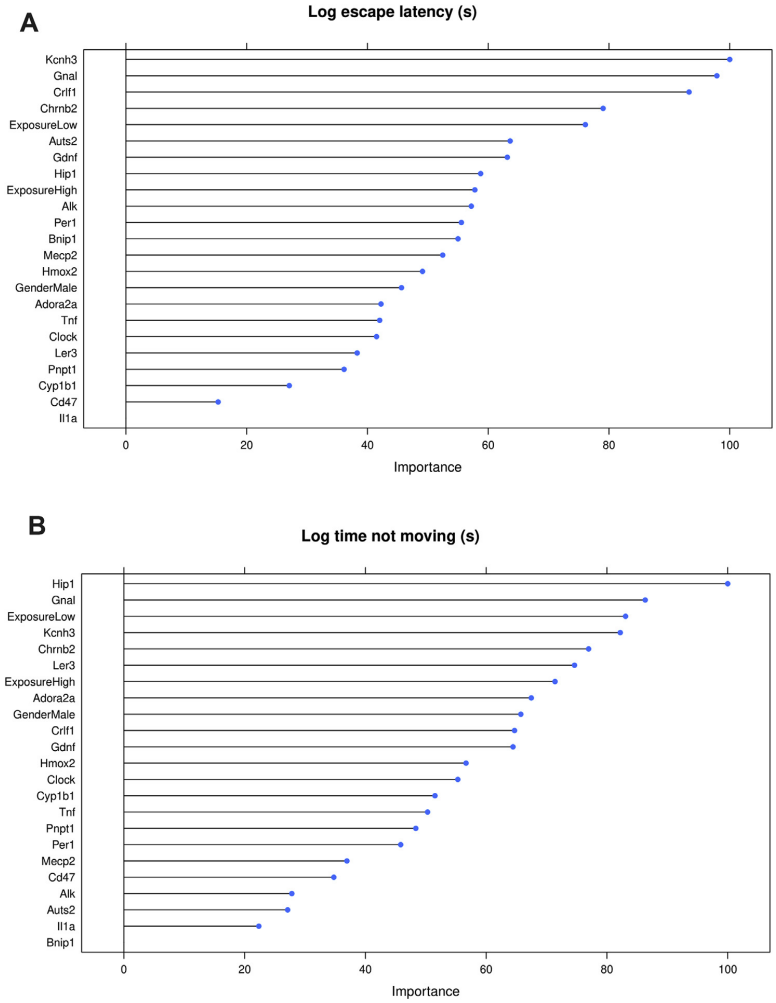

Fig. 4. Random Forest Plots showing the relative importance of gene expression and exposure for behavioural endpoints (log transformed escape latency, Fig. 4A), and the log transformed time spent not moving, Fig. 4B) in session 5 of the Barnes maze test for high and low dose mice maternally exposed to a human relevant mixture of POPs.

expression of *Crlf1*, *Chrnb2*, *Gdnf*, *Kcnh3*, and decreased expression of *Auts2*. Additionally, we found that *Hip1*, *Gnal* and exposure (low dose) were the most important three predictors for not moving, while *Kcnh3*, *Gnal* and *Crlf1* for escape latency. Considering reported neurological function of these genes, the associations between behavioural changes and gene expression adds biological plausibility and mechanistic support to our findings. *Crlf1* encodes a protein that supports differentiation and survival of a wide range of neural cell types during embryonic development and in adult neural tissues (Rousseau et al., 2006). Likewise, in the rodent hippocampal dentate gyrus, the majority of mature and immature granule cells express *Chrnb2* cholinergic receptors (Kaneko et al., 2006), and increased expression of this gene may reflect dysfunctional cholinergic signalling via *Chrnb2* on hippocampal neurogenesis and learning performance. The *Gdnf* gene encodes a neurotrophic factor that contributes to normal hippocampal development (Irala et al., 2016), and hippocampal GDNF protein levels are maintained throughout the lifespan (Werry et al., 2010) suggesting that this protein has a continuous regulatory role of hippocampal function including learning and memory (Cunha et al., 2010).

The *Kcnh3* gene expression level increased significantly in both exposure groups, which is interesting in the context of cognitive function since it is reported that cognitive function is changed when this gene is knocked out (Miyake et al., 2009). It has also been suggested that *Kcnh3* is a potent regulator of excitability in hippocampal pyramidal neurons (Zhang et al., 2010). We further observed a highly significant change in expression level of the *Auts2* gene in exposed animals versus controls. Interestingly, dysregulation of this gene is associated with e.g. autism, mental retardation and developmental delay (Bedogni et al., 2010; Fan et al., 2016). Knockout of both coding and noncoding sequences of the *Auts2*

gene in zebrafish caused microcephaly and a decreased number of neuronal cells (Oksenberg et al., 2013), also seen in ASD patients (Liu et al., 2015).

Gnal encodes a stimulatory G protein alpha subunit which couples dopamine type 1 receptors (D1R) and adenosine A2A receptors (Adora2a). The Adora2a protein has important roles in the regulation of glutamate and dopamine release, and inhibition of this protein has been shown to enhance spatial memory and hippocampal plasticity (Laurent et al., 2016). It has further been reported that *Gnal* ± mice displayed a clear reduction in acute locomotor response to psychostimulant drugs (Corvol et al., 2007). Also, expression levels of e.g. *Gnal* was altered in PCB-exposed rats (DasBanerjee et al., 2008). The expression level of *Gnal* was decreased in both exposure groups compared to control group (Fig. 1).

Hip1 knockout mice show neurological deficits (Metzler et al., 2007), and overexpression of *Hip1* is reported to induce neuronal cell death through apoptosis (Choi et al., 2006). Changes in *Hip1* expression in our study may therefore be a biological attempt to counteract apoptosis.

Altogether, this support that the differentially expressed genes in the exposed animals compared to the controls are likely to affect learning and memory processes in the mouse brain, either directly or as a compensatory mechanism for learning deficits. However, complex processes like cognitive functions are likely to act through networks of genes rather than through single genes.

Our results further show that prenatal exposure to the POP mixture led to dysregulation of genes associated with inflammation, disturbances of circadian rhythm, AhR activation and redox signalling. Interleukins modulate inflammatory responses, and interestingly, several interleukins are linked to cognitive deficits (Misiak et al., 2018). In this respect, negative relationship between *IL-1α* levels in the hippocampus may indicate inflammatory responses and possibly cognitive impairment. CD47 together with SIRPα mediate the interplay between microglia and other brain cells, are important in neuroinflammatory processes and in several CNS disorders (Zhang et al., 2015). Organic compounds can bind the aryl hydrocarbon receptor (AhR), and this may in turn lead to an increased expression of genes linked to inflammation and xenobiotic metabolism (Esser and Rannug, 2015). Our results show that the AhR regulated gene *Cyp1b1* had higher expression in both exposure groups than in the control. Heme oxygenase-1 (*Hmox1*) can be induced in response to toxicants like PCBs (Lee et al., 2006), BFR (Zou et al., 2013), and perfluorinated compounds (Shi and Zhou, 2010), in addition to other stimuli that cause oxidative stress (Keyse and Tyrrell, 1989; Stocker, 1990; Dwyer et al., 1992; Hoshida et al., 1996). Astrocytes and microglia are potent inducers of *Hmox1*. To counter neuroinflammation and oxidative stress, these cells respond by inducing *Hmox1* expression, as shown in our study.

We observed that the clock genes *Per1* (period circadian clock 1) and *Clock* (clock circadian regulator) were downregulated as a response to POP exposure. The proteins encoded by these genes play central roles in the regulation of circadian rhythms. Genes in the Per1 family encode components of the circadian rhythms of locomotor activity, metabolism, and behaviour. *Per1* is upregulated by Clock/Arntl heterodimers. Disruption of several individual clock genes throughout the brain can impair hippocampal long-term memory in young animals, possibly gating memory formation dependent on the time of day (Kwapis et al., 2018).

Estimates of gene expression patterns comparing sex effects revealed higher expression of *Gnal*, *Adora2a*, *Cd47*, *Clock*, and lower expression of *Kcnh3*, *Hmox2*, and *Auts2* in males versus females (Fig. 1B). The biological importance of these differences is challenging to explain based on our mouse study, since we did not reveal any statistically significant differences in cognitive performance between the sexes. However, it could be that these genes affect other neurodevelopmental cognitive outcomes than those measured in the Barnes maze. For example, males are more susceptible to ADHD and autism while females suffer more from mood disorders such as depression and anxiety (Pinares-Garcia et al., 2018; May et al., 2019). The association between sex- and age-dependent vulnerability to neuropsychiatric disorders has been suggested to relate to immaturity at birth in addition to immune activation in the brain, including complex interactions between sex hormones, brain transcriptome, activation of glial cells, and cytokine production (for review see (Ardalan et al., 2019). Thus, our study showing sex differences in expression of genes involved in cognitive processes and inflammatory/redox signalling support the notion that there may be sex-dependent differences in neurocognitive deficits after exposure to environmental toxicants during pregnancy and lactation.

4.5. Neurobehavioral effects in maternally POP-exposed offspring

We have previously reported that moderate X-ray exposure restored learning and memory deficits (as measured in the Barnes maze) in C57BL/6NTac 8-oxoguanine DNA glycosylase 1 (Ogg1)+/− (heterozygote) mice (Hofer et al., 2018), while exposure to algae toxins in adult mice did not affect performances in the Barnes maze or the open field test (Myhre et al., 2018a). Despite clear effects and similarity in gene expression between exposure groups in the present study, this was not manifested in equally clear effects on behaviour outcomes in the Barnes maze. Only two significant learning and memory outcomes (not moving, escape latency) were affected in high exposed offspring subjected to a stressful environment. Interestingly, mediation analysis indicated that changes in gene expression to some extent brought behaviour outcomes back to the normal situation. This may have impaired expression of exposure related behaviour effects in the Barnes maze.

The results on behavioural outcomes are to some extent contradictory to results obtained in human observational studies. Adverse neurodevelopmental effects are reported after exposure to OC compounds (Urabe et al., 1979; Kilburn and Thornton, 1995; Schantz, 1996; Winneke et al., 1998; Ribas-Fito et al., 2003, Ribas-Fito et al., 2007; Eskenazi et al., 2006; Korrick and Sagiv, 2008; Park et al., 2010; Torres-Sanchez et al., 2013), PCBs (Fein et al., 1984; Rogan et al., 1986; Gladen et al., 1988; Tilson et al., 1990; Jacobson and Jacobson, 1996; Seegal, 1996; Longnecker et al., 1997), and BDEs (Roze et al., 2009; Herbstman et al., 2010; Gascon et al., 2011; Cowell et al., 2015). The epidemiological evidence for adverse neurodevelopmental effects of PFASs seems less convincing compared to chlorinated and brominated POPs, and some results are contradictory. For example, PFOA exposure in children were associated with increased reading ability and reduced hyperactivity (Quaak et al., 2016; Zhang et al., 2018), cognitive dysfunction, language processing and social developmental abilities, as well as perturbing the fine and gross motor abilities (Goudarzi et al., 2016). Prenatal exposure to PFNA has been shown to decrease scores in tests related to verbal reasoning (Wang et al., 2015), and disturbances related to impulsivity (Gump et al., 2011). In a recent cohort study, no consistent evidence was found to conclude that prenatal exposure to PFASs (PFHpS, PFOS, PFHxS, PFOA, PFDA, PFUnDA and PFNA) are associated with ADHD symptoms or cognitive dysfunctions in preschool children aged three and a half years (Skogheim et al., 2020). The results showed negative relationships with nonverbal working memory, however, on the other hand positive relationships with verbal working memory. The relationships were weak which suggests no clear association and according to the authors a need for replication (Skogheim et al., 2020).

Animal studies support epidemiological associations in humans reported between pre- and postnatal POP exposure and neurobehavioral effects. Studies of PCB exposure during both gestation and lactation indicate behavioural effects in rodents (for review, see (Eriksson and Fredriksson, 1996b, a; Holene et al., 1998; Berger et al., 2001; Branchi et al., 2005; Mariussen and Fonnum, 2006) and monkeys (Rice, 1999). Mice or rats pre- or postnatally exposed to brominated POPs exhibited learning and memory deficits (Viberg et al., 2003; Eriksson et al., 2006; Koenig et al., 2012; Sun et al., 2017). To our knowledge, developmental neurobehavioral effects are not previously reported for the highly brominated BDE-100 or BDE-207 which are found at high levels in the offspring brain in the present study. Other hippocampus-associated effects have been reported for brominated POPs, like disturbed long-term potentiation (LTP) (Dingemans et al., 2007) and disturbed sensorimotor behaviours in neonates (Miller-Rhodes et al., 2014).

Recent gene-expression studies in rat cerebellar granular neurons using a sub-toxic and marginally toxic concentration of a POP mixture with similar composition as the one used in the present study, revealed differential expression of genes involved in apoptosis, oxidative stress, neurotransmission and cerebellar development, with more genes affected at the marginally toxic concentration (Berntsen et al., 2021). Additionally, we observed increased proliferation and decreased synaptogenesis at human relevant concentrations in human neuronal stem cells using the same POP mixture (Davidsen et al., 2021).

The POP mixture also increased swimming speed of larval zebrafish (Khezri et al., 2017a). This behavioural effect was similar to that observed with perfluorooctanesulfonic acid (PFOS), although the gene expression profile differed between exposures. Some studies report lack of effects on learning and memory after PFOS exposure (Lau et al., 2003), while others reported increased motor activity and reduced habituation in gestationally and lactationally exposed neonatal rats (Butenhoff et al., 2009). Johansson and collaborators exposed mice to single doses of PFOS, PFOA and PFDA, and effects on spontaneous behaviour and habituation were observed with dose-response trends (Johansson et al., 2008). In another study, adult mice exhibited a dose dependent disturbed locomotor activity following a single neonatal exposure to PFHxS (Viberg et al., 2013).

To our knowledge, very few studies have investigated how POP exposure changes behaviour to an acute stressor, however, similarly exposed siblings of the mice in the current study were subjected to a restraint stress test in which male mice from the high exposed group showed an elevated corticosterone response compared to controls (Hudecova et al., 2018). This shows that exposure can elicit adverse reactions to stressors. Other studies have reported effects of combined POP and stress exposure on behavioural outcomes. In these studies, exposure to both PFOS and stress occurred in pregnant mice, thus before behaviour was tested (Fuentes et al., 2006, 2007a, 2007b; Ribes et al., 2010). In detail, Fuentes and colleagues reported that prenatal mortality in mice concurrently exposed to PFOS and restraint was higher than in unrestrained mice exposed to PFOS (Fuentes et al., 2006). The same group further found that mice prenatally exposed to PFOS and restraint exhibited a reduced mobility in the open-field test (Fuentes et al., 2007a). In the Morris water maze test an interaction between sex and restraint was observed with a worse learning rate in female mice born from dams concurrently exposed to PFOS and restraint (Fuentes et al., 2007a). In another study, PFOS and maternal stress interaction did not cause adverse effects on physical maturation or in neuromotor development, however, it caused a reduced distance travelled in the open-field test in adult offspring (mainly observed in females) indicating long lasting functional alterations (Fuentes et al., 2007b). In Ribes et al., mice prenatally exposed to PFOS spent more time in the centre of an open field when compared to the prenatally stressed mice, while no sex differences or interactions between PFOS exposure and restrain were reported (Ribes et al., 2010). The findings reported by (Fuentes et al., 2006, 2007a, 2007b; Ribes et al., 2010) support the present study since the maternally exposed mice exhibited spatial learning and memory deficits in the Barnes maze when subjected to air flow stress from a fan.

4.6. Statistical approaches used to estimate predictors for neurobehavioral outcomes

The present results also illustrate how the variable importance in RF differs from traditional variable selection procedures. When several variables are highly collinear (Fig. 4, Supplementary Fig. S4) but good predictors of the response, as are the expression of hippocampal genes, stepwise and criterion-based variable selection procedures will typically retain one or two of the collinear variables but discard the rest. In contrast, RF "spreads" the variable importance across all explanatory variables. This approach guards against the elimination of variables which are good predictors of the response, and may be biologically important (Cutler et al., 2007).

5. Conclusions

We conclude that, despite species differences, the POP mixture used in the present study is useful for realistic human exposure-scenario studies that aim to increase the understanding of how defined, complex mixtures affect biological functions in females and their developmentally exposed offspring. Human relevant plasma and brain concentrations were obtained in low dose offspring and was associated with changes in hippocampal gene expression relevant to brain function. Behavioural endpoints obtained in the Barnes maze were less affected by POP exposure.

Credit author 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.chemosphere.2021.130123.

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Supplementary Information

Chemical analyses

Two methods were used for extraction of the chemicals, one for the lipophilic compounds, and one for the perfluorinated group only. For the lipophilic groups of chemicals, extraction of POPs has been described by Polder and collaborators (Polder et al. 2014). Briefly the samples were weighed, and added internal standards (PCB 29, 112 and 207 (Ultra Scientific, RI, USA); BDE 77, 119 and 181 and $^{13}C_{12}$-BDE 209 (Cambridge Isotope Laboratories, Inc., MA, USA)) and solvents (cyclohexane/acetone/water), followed by homogenization using a T25 Ika Ultra-Turrax®. Lipid % was determined gravimetrically using 1 ml aliquots of the fat extracts, except for the plasma samples, where all of the extracts were used. The removal of lipids for the determination of dieldrin was performed using a gel permeation column, filled with Bio-Beads S-X3, 200–400 mesh (Bio-Rad Laboratories, Inc., CA, USA) installed on a Gilson Model 233 combined injector and fractionating system (Gilson, Inc., WI, USA). The removal of lipids for the determination of the rest of the OCPs, PCBs, BDEs and HBCD was performed using ≥ 97.5% H_2SO_4 (Fluka Analytical®).

Separation and detection of the OCPs and PCBs were performed on a GC coupled to Electron Capture Detector (ECD) and low resolution mass spectrometry (LRMS) (Agilent6890 Series; agilent Technologies), as described by Polderet al. (2014). PCB 28, 52 and 101, and dieldrin were quantified using a 63Ni micro μ-ECD (Agilent 6890 μ-ECD). The rest of the PCBs and pesticides were quantified, using a MS detector (Agilent 5975C; Agilent Technologies), which was operated by negative chemical ionization (NCI) in selected ion monitoring (SIM) mode. The target ions used were at m/z 71 (HCHs), 284 (HCB), 359 (oxychlordane), 410 (α-chlordane), 444 (*trans*-nonachlor), 318 (*p,p*'- DDE), 326 (PCB 118), 360 (PCB 138 and 153), 396 (PCB 180). Detection of BDEs and HBCD was performed on a HRGC–LRMS (Agilent 6890 Series; Agilent Technologies), equipped with an autosampler (Agilent 7683 Series;

Agilent Technologies) and coupled to a MS detector (Agilent 5973 Network; Agilent Technologies) (Polderet al. 2014). The BDEs and HBCD were monitored using negative chemical ionization (NCI) in selected ion monitoring (SIM) mode at m/z 79/81. BDE 209 was monitored at m/z 484/486 and 13C12-BDE-209 at m/z 495/497. Samples were analyzed for perfluorinated compounds according to Bytingsviket al. (2012) and references therein. In brief, the samples were extracted with methanol and clean up was accomplished using active carbon. Further, the samples were separated by high-performance liquid chromatography (HPLC), and detection achieved by tandem mass spectrometry (MS-MS).

OH-metabolites were measured in control and high exposed dam pooled samples according to Berget al. (2010). Plasma samples analyzed for OH-metabolites were extracted as the other plasma samples, but initially the internal standards: 4′-OH-[$^{13}C_{12}$]CB159, PCB-29, -112 and -207 were added. Also the water phase was replaced with 10 ml 1 M H_2SO_4. The organic supernatants were transferred to 10 mL glass tubes, before extraction with 2 × 5 ml 1 M KOH in 50% ethanol. The organic phases from this extraction were analyzed by GC–ECD/GC-MS like the other plasma samples. The alkaline phases were acidified with 96% H_2SO_4 to pH between 1 and 2, and re-extracted with 3 × 5 ml cyclohexane. These organic phases, containing the OH-metabolites, were then evaporated to ∼1 ml, before derivatization with acetic anhydride:pyridine (1:1). The derivatised samples were subsequently analyzed by an Agilent 6890 Series GC system with Agilent autosampler, Agilent 7683 Series split/splitless injector and Agilent 5973 quadrupole mass spectrometer. The instrument conditions (column, gas and temperature program) has been described in Berget al. (2010). The laboratory is accredited by the Norwegian Accreditation for testing the analyzed chemicals in biological material according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137). The details of the analytical quality system have been described in Polderet al. (2014). Briefly, every analytical series included three procedural blanks (solvents), one blind (non-spiked clean feed), two

spiked samples of clean feed for recoveries and the laboratory's own reference materials (LRMs) of blubber of harp seal (*Pagophilus groenlandicus*). The lowest levels of detection (LODs) for individual compounds were defined as three times the noise level. The LODs (ng/g wet weight (ww)) and relative recoveries (%) were for HCB 0.03 (97 %), HCHs 0.02 (87-103%), *p,p'*- DDE 0.05 (106%), dieldrin 0.5 (99%), PCBs 0.03–0.1 (89-109%), chlordanes 0.03 (87-93%), BDEs 0.03-0.2 (90-122%), HBCD 0.03 (108%) and perfluorinated compounds 0.06-0.28 (83-90%). Detection limit in feed with contaminants and exposed tissues were 10 times higher due to dilution of samples. Positive consistent blanks were found for dieldrin (0.46 ng/g), PCBs 138, 153 and 180 (0.03, 0.19 and 0.16 ng/g respectively), Dieldrin (0.46 ng/g) and BDEs 47 and 209 (0,08 and 0.11 respectively), and results were corrected for these blanks. The quality control parameters were within the accepted ranges for the methods applied. In addition to the RLM, analytical quality was successfully approved by routinely analysing relevant Certified Reference Materials (CRM) such as mackerel oil (CRM 350) and by participation in relevant intercalibration tests such as the 2011 MOE Interlaboratory study for the Northern Contaminants Program (NCP) III — phase 6 on lake trout (*Salvelinus namaycush*) and brown trout organized by the Ontario Ministry of the Environment, Laboratory Services Branch.

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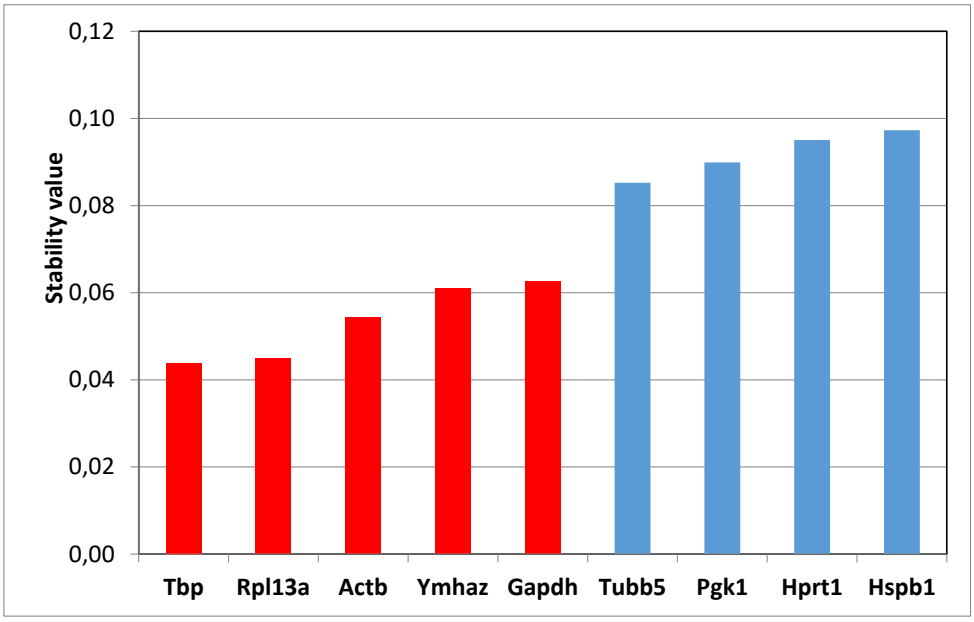
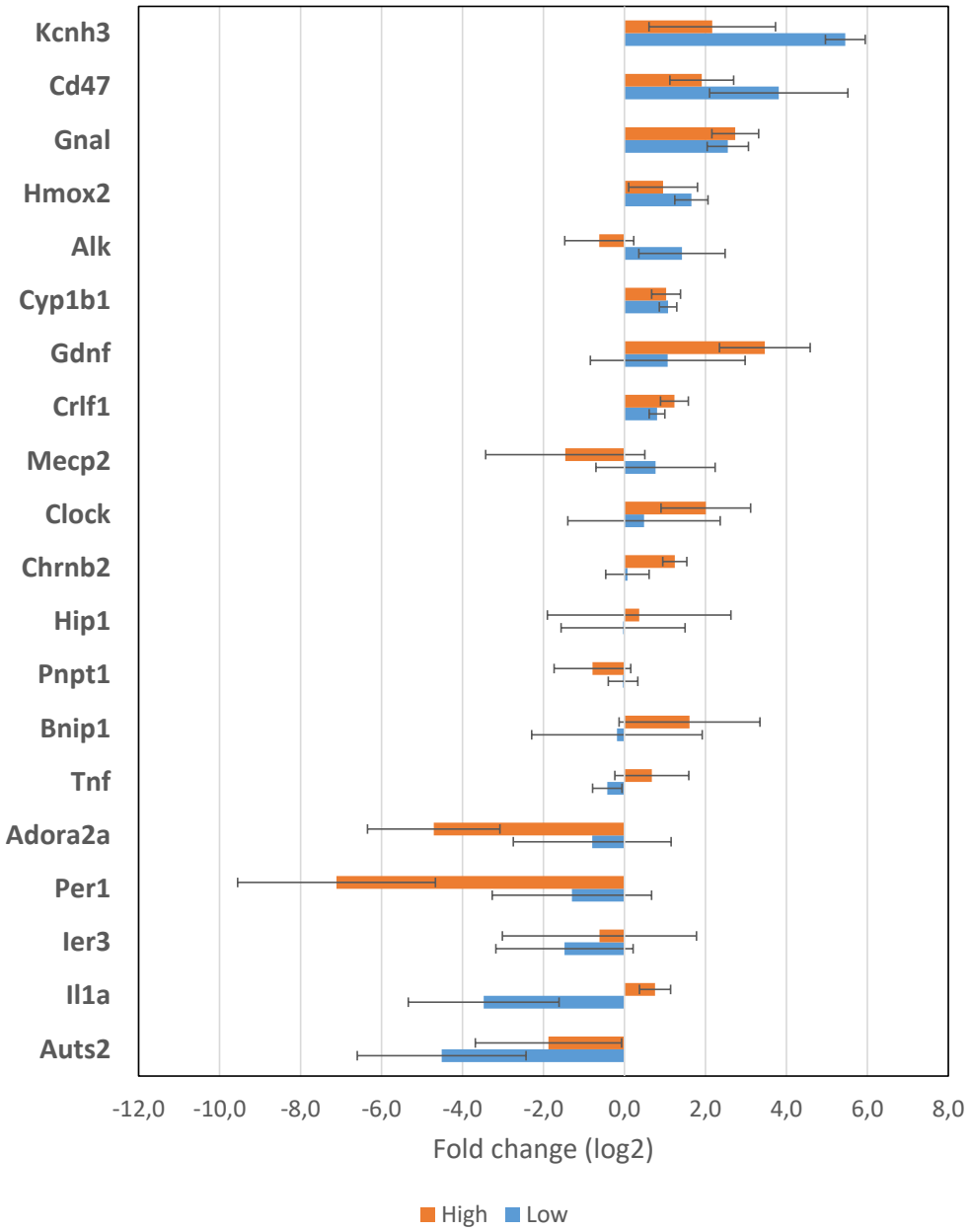
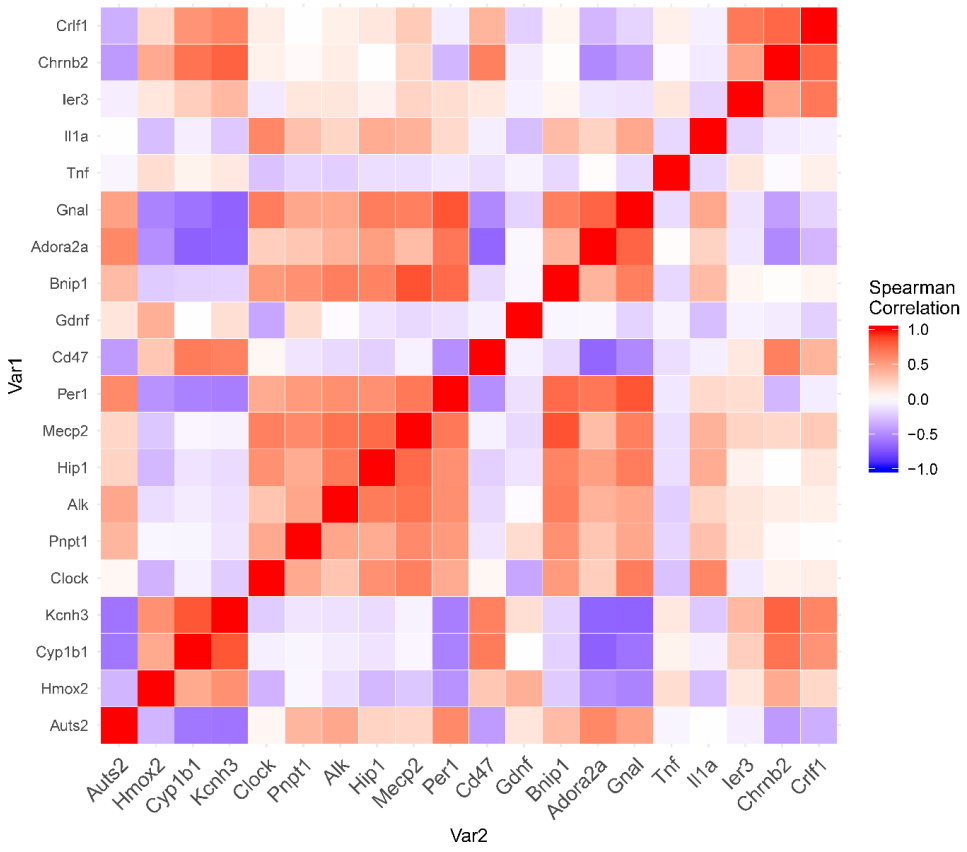


Supplementary Figure S1: Stability of nine reference genes, evaluated by NormFinder algorithm, in mice maternally exposed to a human relevant mixture of POPs. Five reference genes with the lowest stability values are marked with red, and the geometric mean of these five reference genes was used for normalization.



Supplementary Figure S2: The transcription level of 20 genes selected from 142 screening genes. Initial screening of gene expression profile of POP-mixture exposure in mouse hippocampus. qPCR was used to analyze the transcriptional response of hippocampus samples from mice following the exposure of a low dose, a high dose of POP-mixture and vehicle treated controls. Selected differentially expressed genes from screening genes (N=20 genes); selection is based on $P \leq 0.05$ or fold change $> \pm 2.0$ compared to control samples in one or more treated

groups. Each bar represents the average log2-transformed fold change values and the error bar indicates ±SE.



Supplementary Figure S3: Correlation heatmap showing Spearman correlation coefficients between transcript levels of 20 genes from mouse offspring maternally exposed a human relevant mixture of POPs at two dose levels (high and low).

Supplementary Table S1: Adipose tissue levels of POPs (ng/g wet weight) in pooled samples from dams and pups of the different exposure groups and generations of mice exposed to a human relevant POP mixture. The values for each group of compounds (range) are listed from the highest to lowest measured concentration. PFAAs were not measured in adipose tissue. The three most prominent compounds within each exposure and compound group are highlighted in a dark grey color.

Range	Dam						Offspring					
	Control		Low		High		Control		Low		High	
Chlorinated												
1	HCB	18.7	PCB 138	2450	PCB 153	56200	HCB	31	PCB 153	904	PCB 153	16200
2	PCB 153	3.37	PCB 153	2330	PCB 138	52800	PCB 153	5.04	PCB 138	841	PCB 138	13100
3	PCB 138	1.8	*p,p'*-DDE	1200	PCB 118	22600	PCB 138	2.75	Dieldrin	336	PCB 180	4280
4	PCB 180	1.11	Dieldrin	860	*p,p'*-DDE	22200	PCB 180	1.28	PCB 180	243	PCB 118	3260
5	PCB 118	0.692	HCB	828	Dieldrin	19500	PCB 118	1.12	HCB	199	Dieldrin	2630
6	*p,p'*-DDE	0.49	PCB 118	771	HCB	17200	*p,p'*-DDE	0.79	Oxychlordane	168	Oxychlordane	2040
7	PCB 28	n.d.	PCB 180	762	PCB 180	15900	PCB 28	n.d.	*trans*-Nonachlor	163	β-HCH	1800
8	PCB 52	n.d.	Oxychlordane	440	Oxychlordane	10500	PCB 52	n.d.	PCB 118	115	*trans*-Nonachlor	1440
9	PCB 101	n.d.	*trans*-Nonachlor	431	*trans*-Nonachlor	8380	PCB 101	n.d.	β-HCH	113	HCB	1390
10	α-Chlordane	n.d.	β-HCH	350	β-HCH	8190	α-Chlordane	n.d.	*p,p'*-DDE	70.3	*p,p'*-DDE	205
11	Oxychlordane	n.d.	PCB 101	106	PCB 101	2100	Oxychlordane	n.d.	PCB 101	4.7	PCB 52	26.8
12	*trans*-Nonachlor	n.d.	PCB 52	71	PCB 52	239	*trans*-Nonachlor	n.d.	PCB 52	3.75	PCB 101	18.2
13	α-HCH	n.d.	α-HCH	30.1	α-HCH	132	α-HCH	n.d.	PCB 28	n.d	PCB 28	n.d
14	β-HCH	n.d.	PCB 28	9.96	PCB 28	49.2	β-HCH	n.d.	α-Chlordane	n.d	α-Chlordane	n.d
15	γ-HCH	n.d.	α-Chlordane	n.d.	α-Chlordane	n.d.	γ-HCH	n.d.	α-HCH	n.d	α-HCH	n.d
16	Dieldrin	n.d.	γ-HCH	n.d.	γ-HCH	n.d.	Dieldrin	n.d.	γ-HCH	n.d	γ-HCH	n.d
Brominated												

1	BDE 209	0.23	BDE 47	345	BDE 47	4700	BDE 100	0.33	BDE 100	24.5	BDE 153	289
2	BDE 207*	0.15	BDE 99	151	BDE 209	2240	BDE 99	0.21	BDE 99	23.4	BDE 99	159
3	BDE 100	0.09	BDE 100	149	BDE 99	1980	BDE 47	0.15	BDE 153	19.8	BDE 209	158
4	BDE 47	n.d.	BDE 209	109	BDE 100	1850	BDE 153	0.11	BDE 209	15.2	BDE 100	139
5	BDE 99	n.d.	BDE 153	62.1	BDE 153	1190	BDE 154	0.07	BDE 154	11.1	BDE 207*	98.2
6	BDE 153	n.d.	BDE 154	55.1	BDE 207*	1190	BDE 196*	n.d.	BDE 207*	7.81	BDE 154	82.6
7	BDE 154	n.d.	BDE 207*	41.5	BDE 154	636	BDE 202*	n.d.	BDE 47	5.94	BDE 47	19.4
8	BDE 196*	n.d.	BDE 208*	5.3	BDE 208*	82.29	BDE 206*	n.d.	BDE 196*	n.d.	BDE 208*	14.96
9	BDE 202*	n.d.	BDE 196*	n.d.	BDE 196*	n.d.	BDE 207*	n.d.	BDE 202*	n.d.	BDE 196*	n.d.
10	BDE 206*	n.d.	BDE 202*	n.d.	BDE 202*	n.d.	BDE 208*	n.d.	BDE 206*	n.d.	BDE 202*	n.d.
11	BDE 208*	n.d.	BDE 206*	n.d.	BDE 206*	n.d.	BDE 209	n.d.	BDE 208*	n.d.	BDE 206*	n.d.
12	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.

n.d = Not detected, *= Not added to mixture

Supplementary Table S2A: Lipid adjusted values (ng/g lipid weight) from brain, plasma and adipose tissue as well as brain/plasma and brain/adipose tissue ratios from low dose dams and offspring in mice exposed to a human relevant POP mixture. The tissue ratios for each group of compounds are listed from the highest to the lowest value (range).

Dam							Offspring						
Compound	**Brain**	**Plasma**	**Adipose**	**B/P ratio range**		**B/A ratio range**	**Compound**	**Brain**	**Plasma**	**Adipose**	**B/P ratio range**		**B/A ratio range**	
Chlorinated														
PCB 28	2.7	n.d.	13.87	α-HCH	0.76	α-HCH	1.21	PCB 28	n.d.	n.d.	n.d.	β-HCH	1.17	β-HCH	0.41
PCB 52	9.5	343.1	98.56	HCB	0.43	β-HCH	0.38	PCB 52	n.d.	40	4.8	HCB	0.36	HCB	0.36
PCB 101	34	373.1	148.13	β-HCH	0.38	HCB	0.34	PCB 101	n.d.	n.d.	6.02	Dieldrin	0.22	Dieldrin	0.23
PCB 118	137	782.8	1074.08	*trans*-Nonachlor	0.19	Dieldrin	0.28	PCB 118	20.7	123.3	147.25	PCB 118	0.17	PCB 118	0.14
PCB 153	359.9	3807.5	3245.13	PCB 138	0.18	PCB 101	0.23	PCB 153	123.3	1332.5	1157.63	PCB 138	0.13	*p,p'*-DDE	0.14
PCB 138	414.9	2352.8	3412.26	Oxychlordane	0.18	PCB 28	0.2	PCB 138	121.5	967	1077.34	PCB 180	0.13	Oxychlordane	0.12
PCB 180	108.3	861.8	1061.3	PCB 118	0.17	Oxychlordane	0.15	PCB 180	32.9	258.8	311.24	*p,p'*-DDE	0.13	PCB 153	0.11
p,p'-DDE	235.9	1688.9	1671.31	*p,p'*-DDE	0.14	*p,p'*-DDE	0.14	*p,p'*-DDE	12.6	99.9	90.03	*trans*-Nonachlor	0.13	PCB 138	0.11
HCB	390.2	909.4	1152.86	PCB 180	0.13	PCB 118	0.13	HCB	90.7	249.8	254.88	PCB 153	0.09	PCB 180	0.11
α-Chlordane	n.d.	n.d.	n.d.	PCB 101	0.09	PCB 138	0.12	α-Chlordane	n.d.	n.d.	n.d.	Oxychlordane	0.06	*trans*-Nonachlor	0.08
Oxychlordane	91	499.7	613.17	PCB 153	0.09	PCB 153	0.11	Oxychlordane	26.7	429.7	214.47	PCB 28	N/A	PCB 28	N/A
trans-Nonachlor	66.8	343.1	600.48	Dieldrin	0.08	*trans*-Nonachlor	0.11	*trans*-Nonachlor	16	119.9	209.07	PCB-52	N/A	PCB 52	N/A
α-HCH	50.9	66.6	41.9	PCB 52	0.03	PCB 52	0.1	α-HCH	n.d.	n.d.	n.d.	PCB 101	N/A	PCB 101	N/A
β-HCH	187.8	489.7	488.06	PCB 28	N/A	PCB 180	0.1	β-HCH	58.5	50	144.25	α-Chlordane	N/A	α-Chlordane	N/A
γ-HCH	n.d.	n.d.	n.d.	α-Chlordane	N/A	α-chlordane	N/A	γ-HCH	n.d.	n.d.	n.d.	α-HCH	N/A	α-HCH	N/A
Dieldrin	340.8	4077.3	1197.12	γ-HCH	N/A	γ-HCH	N/A	Dieldrin	100.1	453	429.94	γ-HCH	N/A	γ-HCH	N/A
Brominated														
BDE 47	46.2	389.7	480.68	BDE 153	0.18	BDE 209	0.17	BDE 47	n.d.	n.d.	7.6	BDE 100	0.24	BDE 209	0.9

BDE 99	21.2	156.6	210.09	BDE 99	0.14	BDE 153	0.12	BDE 99	3.3	n.d.	30.01	BDE 47	N/A	BDE 100	0.23
BDE 100	21.5	166.6	207.44	BDE 100	0.13	BDE 47	0.1	BDE 100	7.3	30.1	31.41	BDE 99	N/A	BDE 99	0.11
BDE 153	10.3	56.6	86.46	BDE 47	0.12	BDE 99	0.1	BDE 153	2.7	n.d.	25.41	BDE 153	N/A	BDE 153	0.11
BDE 154	7.1	83.3	76.72	BDE 154	0.08	BDE 100	0.1	BDE 154	1.3	n.d.	14.2	BDE 154	N/A	BDE 154	0.09
BDE 209	26.4	n.d.	152.26	BDE 209	N/A	BDE 154	0.09	BDE 209	17.5	n.d.	19.41	BDE 209	N/A	BDE 47	N/A
HBCD	n.d.	n.d.	n.d.	HBCD	N/A	HBCD	N/A	HBCD	n.d.	n.d.	n.d.	HBCD	N/A	HBCD	N/A

The lipid % used for conversion of concentrations from wet weight values to lipid adjusted values were 0.3, 8 and 72 % in dams, and 0.3, 5.5 and 78 % in offspring, for brain, plasma and adipose tissue, respectively

B/P ratio – Brain to plasma ratio – ranged from highest to lowest ratio within each chemical group

B/A ratio – Brain to adipose tissue ratio – ranged from highest to lowest ratio within each chemical group

N/A = Not applicable

Supplementary Table S2B: Brain/plasma ratios of the perfluorinated compounds in high and low dose dams and offspring, based on wet weight levels, in mice maternally exposed to a human relevant POP mixture. The ratios are listed from the highest to the lowest value (range).

Range	**Dam**				**Offspring**			
	Low		**High**		**Low**		**High**	
1	PFUnDA	0.23	PFUnDA	0.19	PFUnDA	0.18	PFUnDA	0.16
2	PFOS	0.08	PFOS	0.08	PFOS	0.07	PFOS	0.05
3	PFDA	0.06	PFDA	0.05	PFDA	0.04	PFDA	0.04
4	PFNA	0.02	PFNA	0.02	PFNA	0.02	PFNA	0.01
5	PFHxS	0.01	PFHxS	0.01	PFOA	0.01	PFHxS	0.01
6	PFOA	0.01	PFOA	0.01	PFHxS	N/A	PFOA	0.01

Supplementary Table S3: Hydroxy-metabolite concentrations (ng/g wet weight) in control and high exposed dams in mice exposed to a human relevant POP mixture. The three most prominent chlorinated metabolites are highlighted in a dark grey color.

Range		Dam	
	Compound	Control	High
Chlorinated			
1	4-OH-CB107	n.d.	104
2	3-OH-CB118	n.d.	16.6
3	3'-OH-CB138	n.d.	11.2
4	4-OH-CB146	n.d.	5.43
5	4'-OH-CB172	n.d.	3.96
6	3'-OH-CB180	n.d.	2.37
7	4'-OH-CB106	n.d.	n.d.
8	4'-OH-CB108	n.d.	n.d.
9	4'-OH-CB130	n.d.	n.d.
10	4'-OH-CB159	n.d.	n.d.
11	4-OH-CB187	n.d.	n.d.
Brominated			
12	4-OH-BDE42	n.d.	n.d.
13	3-OH-BDE47	n.d.	n.d.
14	6-OH-BDE47	n.d.	n.d.
15	4'-OH-BDE49	n.d.	n.d.
16	2'-OH-BDE68	n.d.	n.d.
n.d = Not detected			

Supplementary Table S4: Measured plasma levels of POPs in dams and offspring of the control, low and high exposed groups expressed relative to average human blood levels of POPs from the Scandinavian population as presented in Berntsen et al. (2017).

Compound	Control		Low		High	
	Dam	Pup	Dam	Pup	Dam	Pup
	x Human levels					
Chlorinated						
PCB 28	N/A	N/A	N/A	N/A	N/A	N/A
PCB 52	N/A	N/A	108	12	472	281
PCB 101	N/A	N/A	147	N/A	295	N/A
PCB 118	1.1	4.4	39	6	658	185
PCB 138	0.4	5.2	54	18	409	224
PCB 153	0.3	3.2	21	8	241	135
PCB 180	0.2	1.7	14	4	126	72
***p,p'*-DDE**	N/A	N/A	11	0.6	102	3
HCB	0.8	2.3	25	6	347	81
α-Chlordane	N/A	N/A	N/A	N/A	N/A	N/A
Oxychlordane	N/A	N/A	72	58	978	621
***trans*-Nonachlor**	N/A	N/A	26	9	240	152
α-HCH	N/A	N/A	35	N/A	N/A	N/A
β-HCH	N/A	N/A	29	3	272	189
γ-HCH (Lindane)	N/A	N/A	N/A	N/A	N/A	N/A
Dieldrin	N/A	N/A	537	56	2100	701
Brominated						
BDE 47	N/A	N/A	137	N/A	1196	N/A
BDE 99	N/A	N/A	124	N/A	952	N/A
BDE 100	N/A	N/A	263	45	1903	305
BDE 153	N/A	N/A	18	N/A	249	110
BDE 154	N/A	N/A	132	N/A	460	976
BDE 209	N/A	N/A	444	N/A	2374	N/A
HBCD	N/A	N/A	N/A	N/A	N/A	N/A
Perfluorinated						
PFHxS	N/A	1.9	47	5	994	93
PFOS	0.03	0.5	8	1	116	22
PFOA	0.12	2.5	76	6	1543	132
PFNA	0.4	16	220	29	3164	588
PFDA	0.5	29	497	56	5212	1016
PFUnDA	0.3	5.6	113	11	1293	204

N/A = Not applicable – As a result of the compound not being detected
x = times

Supplementary Table S5: Expression level of 20 selected genes in hippocampus of mice maternally exposed to a human relevant POP mixture.

Gene Name	Description	Low			High		
		FC	SE	P-value	FC	SE	P-value
Auts2	AUTS2, activator of transcription and developmental regulator	-10.27	0.01	5.31E-09	-10.29	0.01	5.26E-09
Adora2a	adenosine A2a receptor	-3.70	0.02	2.71E-04	-2.67	0.13	2.04E-05
Gnal	G protein subunit alpha L	-2.74	0.02	1.25E-08	-2.59	0.02	7.08E-09
Per1	period circadian regulator 1	-2.27	0.02	5.12E-09	-2.47	0.02	5.23E-09
Il1a	interleukin 1 alpha	-1.33	0.07	5.90E-01	-1.12	0.06	5.08E-02
Alk	ALK receptor tyrosine kinase	-1.23	0.04	6.60E-03	-1.30	0.05	3.11E-02
Clock	clock circadian regulator	-1.22	0.05	3.14E-01	-1.11	0.03	2.12E-02
Hip1	huntingtin interacting protein 1	-1.15	0.04	4.50E-02	-1.17	0.03	9.10E-02
Tnf	tumor necrosis factor	-1.14	0.12	4.10E-01	-1.66	0.07	9.07E-01
Mecp2	methyl-CpG binding protein 2	-1.14	0.04	1.24E-01	-1.13	0.04	1.06E-01
Pnpt1	polyribonucleotide nucleotidyltransferase 1	-1.13	0.04	6.66E-01	-1.05	0.04	1.03E-01
Bnip1	BCL2 interacting protein 1	-1.12	0.04	9.95E-02	-1.16	0.03	2.50E-01
Ier3	immediate early response 3	1.17	0.17	7.43E-01	1.16	0.16	6.93E-01
Crlf1	cytokine receptor like factor 1	1.61	0.12	3.09E-03	1.52	0.12	3.35E-04
Chrnb2	cholinergic receptor nicotinic beta 2 subunit	1.95	0.10	5.11E-09	2.05	0.10	5.39E-09
Gdnf	glial cell derived neurotrophic factor	2.09	0.49	9.26E-01	1.18	0.14	6.87E-02
Cd47	CD47 molecule	2.10	0.11	5.11E-09	2.25	0.08	6.20E-09
Hmox2	heme oxygenase 2	6.12	1.01	1.91E-03	5.04	0.65	6.07E-05
Cyp1b1	cytochrome P450 family 1 subfamily B member 1	10.82	0.51	5.10E-09	9.30	0.49	5.10E-09
Kcnh3	potassium voltage-gated channel subfamily H member 3	11.69	0.59	5.10E-09	10.42	0.58	5.10E-09

Note. *Fold change values given for the different POP-mixture groups compared to controls. Data analyzed by one-way ANOVA with Tukey HSD post hoc test. P<0.05 were marked with red and color gradient show the degree of fold change (red color (upregulated) and green color (downregulated) more than 2-fold. N= 101 mice; i.e., 33/34 hippocampus samples/treatment group.*

Supplementary Table S6: Associations (estimates, 95% confidence intervals and associated *p* -values) of gene expression levels with gender and exposure group according to separate generalized linear models for each of the 20 genes.

Gene	Category	Estimate	Low CI	Up CI	pvalue
Auts2	High	-2.342	-2.724	-1.961	0.000
Auts2	Low	-2.130	-2.512	-1.749	0.000
Auts2	Male	0.125	-0.280	0.535	0.549
Auts2	High:Male	-0.157	-0.724	0.409	0.588
Auts2	Low:Male	-0.681	-1.243	-0.120	0.019
Hmox2	High	1.923	1.282	2.516	0.000
Hmox2	Low	2.296	1.655	2.889	0.000
Hmox2	Male	0.307	-0.356	0.932	0.349
Hmox2	High:Male	-0.574	-1.377	0.256	0.170
Hmox2	Low:Male	-1.077	-1.875	-0.253	0.011
Cyp1b1	High	2.277	2.063	2.490	0.000
Cyp1b1	Low	2.475	2.261	2.688	0.000
Cyp1b1	Male	0.140	-0.088	0.368	0.233
Cyp1b1	High:Male	-0.136	-0.453	0.181	0.403
Cyp1b1	Low:Male	-0.238	-0.553	0.076	0.141
Kcnh3	High	2.513	2.300	2.726	0.000
Kcnh3	Low	2.661	2.448	2.874	0.000
Kcnh3	Male	0.220	-0.006	0.448	0.061
Kcnh3	High:Male	-0.414	-0.731	-0.098	0.012
Kcnh3	Low:Male	-0.488	-0.802	-0.175	0.003
Clock	High	-0.261	-0.454	-0.068	0.009

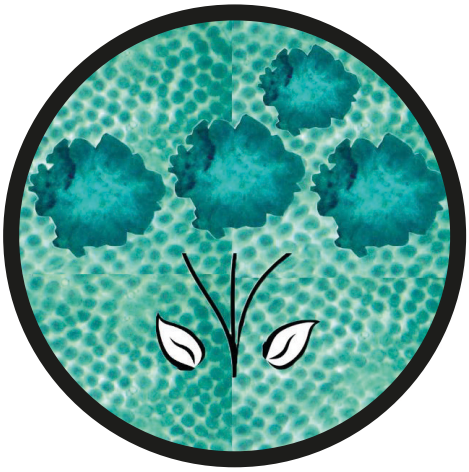
Clock	Low	-0.449	-0.641	-0.256	0.000
Clock	Male	-0.184	-0.389	0.022	0.083
Clock	High:Male	0.330	0.044	0.616	0.026
Clock	Low:Male	0.496	0.212	0.779	0.001
Pnpt1	High	-0.029	-0.187	0.128	0.717
Pnpt1	Low	-0.028	-0.186	0.130	0.730
Pnpt1	Male	0.053	-0.115	0.221	0.542
Pnpt1	High:Male	-0.036	-0.270	0.198	0.763
Pnpt1	Low:Male	-0.198	-0.431	0.034	0.097
Alk	High	-0.229	-0.455	-0.003	0.050
Alk	Low	-0.061	-0.287	0.165	0.600
Alk	Male	0.111	-0.129	0.353	0.369
Alk	High:Male	-0.054	-0.389	0.282	0.754
Alk	Low:Male	-0.310	-0.643	0.022	0.071
Hip1	High	-0.160	-0.338	0.018	0.082
Hip1	Low	-0.078	-0.256	0.099	0.390
Hip1	Male	0.093	-0.096	0.284	0.337
Hip1	High:Male	0.000	-0.265	0.264	0.998
Hip1	Low:Male	-0.127	-0.389	0.135	0.344
Mecp2	High	-0.120	-0.295	0.056	0.184
Mecp2	Low	-0.127	-0.302	0.048	0.159
Mecp2	Male	0.026	-0.161	0.214	0.786
Mecp2	High:Male	-0.049	-0.310	0.211	0.711
Mecp2	Low:Male	-0.042	-0.300	0.216	0.751

Per1	High	-1.020	-1.267	-0.773	0.000
Per1	Low	-0.953	-1.200	-0.706	0.000
Per1	Male	-0.248	-0.510	0.016	0.067
Per1	High:Male	0.178	-0.185	0.542	0.338
Per1	Low:Male	0.221	-0.140	0.582	0.232
Cd47	High	-0.971	-1.901	-0.050	0.039
Cd47	Low	-1.146	-2.075	-0.224	0.015
Cd47	Male	-2.058	-3.033	-1.052	0.000
Cd47	High:Male	2.192	0.826	3.556	0.002
Cd47	Low:Male	2.383	1.026	3.733	0.001
Gdnf	High	1.462	0.888	2.020	0.000
Gdnf	Low	2.708	2.134	3.266	0.000
Gdnf	Male	2.027	1.425	2.624	0.000
Gdnf	High:Male	-1.719	-2.522	-0.910	0.000
Gdnf	Low:Male	-4.054	-4.851	-3.251	0.000
Bnip1	High	-0.097	-0.291	0.097	0.331
Bnip1	Low	-0.016	-0.211	0.178	0.869
Bnip1	Male	0.189	-0.018	0.397	0.077
Bnip1	High:Male	-0.143	-0.432	0.145	0.334
Bnip1	Low:Male	-0.234	-0.520	0.052	0.112
Adora2a	High	-1.528	-2.173	-0.887	0.000
Adora2a	Low	-1.431	-2.083	-0.778	0.000
Adora2a	Male	-0.149	-0.829	0.545	0.669
Adora2a	High:Male	0.966	0.013	1.917	0.049

Adora2a	Low:Male	0.213	-0.740	1.160	0.660
Gnal	High	-1.174	-1.444	-0.904	0.000
Gnal	Low	-1.277	-1.547	-1.007	0.000
Gnal	Male	-0.273	-0.560	0.017	0.067
Gnal	High:Male	0.478	0.076	0.879	0.022
Gnal	Low:Male	0.556	0.158	0.954	0.007
Tnf	High	-0.752	-1.558	0.030	0.064
Tnf	Low	-0.534	-1.317	0.207	0.168
Tnf	Male	-0.524	-1.331	0.257	0.194
Tnf	High:Male	0.375	-0.700	1.464	0.496
Tnf	Low:Male	0.688	-0.353	1.747	0.200
Il1a	High	-0.171	-0.498	0.154	0.305
Il1a	Low	-0.509	-0.832	-0.189	0.003
Il1a	Male	0.070	-0.272	0.412	0.690
Il1a	High:Male	0.087	-0.384	0.560	0.717
Il1a	Low:Male	0.385	-0.080	0.851	0.108
Ier3	High	0.398	-0.096	0.887	0.114
Ier3	Low	0.148	-0.346	0.637	0.555
Ier3	Male	0.138	-0.383	0.665	0.605
Ier3	High:Male	-0.630	-1.352	0.093	0.090
Ier3	Low:Male	0.004	-0.713	0.720	0.990
Chrnb2	High	0.674	0.450	0.897	0.000
Chrnb2	Low	0.558	0.334	0.782	0.000
Chrnb2	Male	-0.144	-0.382	0.096	0.241

Chrnb2	High:Male	0.066	-0.267	0.398	0.698
Chrnb2	Low:Male	0.210	-0.120	0.539	0.215
Crlf1	High	0.346	0.065	0.627	0.018
Crlf1	Low	0.248	-0.033	0.529	0.086
Crlf1	Male	-0.204	-0.503	0.097	0.184
Crlf1	High:Male	0.085	-0.333	0.502	0.692
Crlf1	Low:Male	0.418	0.004	0.832	0,05

Paper IV



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A mixture of Persistent Organic Pollutants (POPs) and Azoxymethane (AOM) show potential synergistic effects on intestinal tumorigenesis in the A/J Min/+ mouse model

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HIGHLIGHTS

- Can a mixture of POPs affect intestinal tumorigenesis in the A/J Min/+ mouse?
- Mice were exposed to POPs through the diet and received an injection of Azoxymethane.
- Results show an increased intestinal tumorigenesis in the A/J Min/+ mouse model.

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ABSTRACT

A multitude of cancer types, including breast, testicular, liver and colorectal cancer, have associations with exposure to Persistent Organic Pollutants (POPs). The present study aimed to investigate whether a mixture of POPs could affect intestinal tumorigenesis in the A/J Min/+ mouse, a model for human colorectal cancer (CRC). Pollutants were selected for their presence in Scandinavian food products and the mixture was designed based on defined human estimated daily intake levels. Mice were exposed through the diet, at control, low and high mixture concentrations, for 10 weeks. In a separate experiment, mice also received one subcutaneous injection of Azoxymethane (AOM) to explore whether this carcinogenic compound influenced the effect of the POPs. Intestinal tumorigenesis was examined by surface microscopy and histopathology. Moderate and dose-dependent increases in tumorigenesis were observed after dietary POP exposure. The AOM treatment alone stimulated the growth of colonic lesions, but did not increase the formation of new lesions. Combined AOM treatment and POP exposure demonstrated a synergistic effect on lesion formation in the colon, and to a lesser extent in the small intestine. This synergy was also evident by an increased number of malignant colonic tumors (carcinomas). In conclusion, the study shows that a mixture of POPs interacted synergistically with a known carcinogen (AOM), causing increased intestinal tumorigenesis in the A/J Min/+ mouse model.

1. Introduction

Persistent Organic Pollutants (POPs) are man-made chemicals that are toxic to humans and wildlife, resistant to degradation and have the potential to bioaccumulate and biomagnify in living organisms (UNEP, 2015). The compounds have adverse health effects and have been associated with an increased risk of breast cancer (Hoyer et al., 2000; Cameron and Foster, 2009), testicular cancer (McGlynn et al., 2008; Giannandrea et al., 2011), liver cancer (Filgo et al., 2015), and colorectal cancer (Howsam et al., 2004; Song et al., 2014). The main route of non-occupational exposure to POPs in

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humans is through ingestion (Darnerud et al., 2006; Vestergren et al., 2012), which makes the GI tract the first organ of exposure. Traditional animal experiments only assess the impact of POPs using single compounds (Sethi et al., 2017) or compounds belonging to the same chemical group (Colter et al., 2018). However, carcinogenesis is a multistep process, so focus on individual compounds may prevent the discovery of potential synergism between multiple chemicals.

Colorectal cancer (CRC) is the third most common cancer in humans worldwide and exposure to carcinogens through the diet is an essential risk factor (IARC, 2016). CRC develops as a result of several genetic and epigenetic changes that cause a transformation of intestinal epithelium from normal tissue, via benign neoplasms, into carcinomas (Kinzler and Vogelstein, 1996; Sancho et al., 2004). Up to 85% of CRC cases are considered sporadic and 1% are attributed to the hereditary CRC syndrome known as familial adenomatous polyposis (FAP) (Burt, 2000). Mutations in the tumor-suppressor gene adenomatous polyposis coli (*APC*) are responsible for FAP, and patients develop a vast number of adenomatous polyps in the intestine, which are likely to progress into malignant tumors (Kinzler and Vogelstein, 1996). In addition, dysfunctional *APC* alleles have been found in the majority of sporadic colorectal lesions (Fodde, 2002). Research on CRC caused by *APC* mutations is therefore highly relevant to human health.

The most widely used animal model for human CRC is the multiple intestinal neoplasia (Min/+) mouse. This mouse has a heterozygous mutation in the *Apc* gene, resulting in a truncated gene product at amino acid 850 (Su et al., 1992). Inactivation of the remaining functional allele in the intestinal epithelium appears to be the rate-limiting step in tumorigenesis (Luongo et al., 1994). Loss of *Apc* inhibits the formation of the β-catenin destruction complex, leading to accumulation of β-catenin in the cytoplasm and subsequent translocation to the nucleus. Here, it interacts with the transcription factor Tcf-4, creating an active complex that transcribes specific target genes (Fodde, 2002; Kretzschmar and Clevers, 2017). The conventional Min/+ mouse model, bred on a C57BL/6 genetic background (Moser et al., 1990), develops lesions primarily in the small intestine (Mollersen et al., 2004). The A/J Min/+ mouse, on the other hand, also develops a large number of lesions in the colon, many of which progress to carcinomas over time (Sødring et al., 2016b). Therefore, the A/J Min/+ mouse model more closely resembles CRC development in humans and was therefore chosen for the present study.

The A/J strain has been shown to be more susceptible to the induction of colorectal cancer by Azoxymethane (AOM) than its C57BL/6 counterpart (Nambiar et al., 2003; Meunier et al., 2011). AOM is a genotoxic chemical used to mimic sporadic CRC and to study the underlying mechanisms of sporadic colorectal carcinogenesis (Venning FA, 2013). Following metabolic activation by cytochrome P450 enzymes (mostly CYP2E1), AOM reacts with DNA and causes adduct formation, leading to DNA mutations initiating colorectal carcinogenesis (Takahashi and Wakabayashi, 2004).

The aim of this study was to investigate whether dietary POP exposure, alone or following AOM treatment, could affect intestinal tumorigenesis in the A/J Min/+ mouse model. The mixture was designed to simulate a real-life exposure scenario relevant to humans (Berntsen et al., 2017).

2. Animals, materials and methods

2.1. Ethics statement

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The animal facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (https://www.aaalac.org/). The animal experiment was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) and the Food Safety Authority (application ID: FOTS 8127) and executed in compliance with the local and national regulations associated with laboratory animal experiments. The rodent and rabbit section of the facility is a Specific Pathogen Free (SPF) unit and follows a health monitoring program recommended by Federation of European Laboratory Animal Science Associations/FELASA (http://www.felasa.eu/). The care of the animals was carried out by two veterinary nurses with FELASA B certification and the study was performed by a veterinarian with FELASA C certification.

2.2. Chemicals and experimental diet

A thorough description of the design and preparation of the POP mixture can be found in Berntsen et al. (2017). A list of the individual compounds can be found in Table 1. In brief, compounds occurring in Scandinavian food products reported in studies prior to 2012 were selected for the POP mixture. Human estimated daily intake (hEDI) levels were defined and adjusted to a 25 g mouse consuming 3 g feed/day. However, due to the possibility of background exposure and interspecies differences in compound metabolism, concentrations were adjusted up to 5000× (low dose) and 100 000× (high dose) hEDI. All polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and other organochlorines were purchased from Chiron AS (Trondheim, Norway). All perfluorinated compounds (PFCs) and hexabromocyclododecane (HBCD) were obtained from Sigma-Aldrich (St. Louis, MO, USA), with the exception of perfluorohexane sulfonic acid (PFHxS) potassium salt which was purchased from Santa Cruz (Dallas, US). All chemicals were dissolved in an appropriate solvent and added to corn oil (Jasmin, fully refined, Yonca Gida San A.S., Manisa, Turkey) intended for human consumption. Solvents were thoroughly evaporated under N_2-flow and the remaining oil was incorporated in AIN-93G mouse feed (TestDiets, St.Louis, MO) at the low and high mixture concentrations. The control diet contained only corn oil from which the solvent had been evaporated.

2.3. Study design

In Experiment 1, 66 mice were used and each litter was randomly divided into 3 exposure groups (control, low and high POP diet) at weaning and exposed for 10 weeks (Fig. 1). In Experiment 2, 21 mice were exposed to the mixture of POPs in the same way, but in addition, these mice were also given one subcutaneous injection of 8.5 mg/kg AOM (Sigma-Aldrich, St. Louis, MO, USA) during their second week after birth. After 10 weeks of POP exposure, all mice were sacrificed and sampled. Because of high offspring mortality after the AOM injection, the breeding of mice for Experiment 2 was terminated for animal welfare reasons prior to completion of breeding the individuals for the study. This resulted in a lower number of animals compared to Experiment 1.

2.4. Animal model

The A/J Min/+ mouse model was established by backcrossing the Min/+ trait onto the genetic background of the A/J strain for >12 generations (Sødring et al., 2016b). In the present study, a total of 87 A/J Min/+ mice were used. The animals were bred in-house. Female A/J +/+ mice were mated with male A/J Min/+ mice and their A/J Min/+ offspring were used in the present study. The pups were marked with ear punches and genotyped at weaning, as

Table 1
A mixture of persistent organic pollutants (POPs) based on a literature review on estimated daily intake (EDI) values in the Scandinavian population (Berntsen et al., 2017). Average EDI values for a 70 kg human and corresponding values for a 25 g mouse are shown. EDI values for a 25 g mouse consuming 3 g of feed designed to provide daily doses of POPs corresponding to the low (5000× human EDI) and high (100,000× human EDI) doses are shown in grey, and are based on measured feed concentrations. The table is adapted from Berntsen et al. (2017).

Compound	Average EDI [a]	Daily intake human	EDI [b] 25 g	EDI [c] 25 g mouse	EDI [d] 25 g mouse	Feed measured [e]	Feed measured [f]	EDI [g] 25 g mouse	EDI [h] 25 g mouse
	70 kg person ng/day	ng/kg/day	mouse pg/day	5000× ng/day	100,000× ng/day	5000× ng/g feed	100,000× ng/g feed	5000× ng/day	100,000× ng/day
Chlorinated									
PCB 28	10	0.14	3.5	18	350	3.1	46	9	138
PCB 52	23	0.33	8.3	41	825	15.0	182	45	546
PCB 101	39	0.56	14.0	70	1400	25.4	377	76	1131
PCB 118	68	0.97	24.3	121	2425	37.2	612	112	1836
PCB 138	97	1.38	34.5	173	3450	53.8	957	161	2871
PCB 153	97	1.38	34.5	173	3450	61.4	981	184	2943
PCB 180	26	0.37	9.3	46	925	17.4	263	52	789
∑PCBs	360	5.13	128.4	642	12,825	213.3	3418	640	10,254
p,p'-DDE	201	2.87	71.8	359	7175	136.0	2390	408	7170
HCB	84	1.20	30.0	150	3000	37.4	588	112	1764
a-Chlordane	63	0.90	22.5	113	2250	45.0	723	135	2169
Oxychlordane	21	0.30	7.5	38	750	9.8	297	29	891
trans-Nonachlor	21	0.30	7.5	38	750	14.9	264	45	792
a-HCH	36	0.52	13.0	65	1300	21.2	421	64	1263
b-HCH	29	0.42	10.5	53	1050	22.3	398	67	1194
g-HCH (Lindane)	40	0.57	14.3	71	1425	31.4	435	94	1305
Dieldrin	126	1.80	45.0	225	4500	70.4	1470	211	4410
∑OCPs	621	8.88	222.1	1112	22,200	388.4	6986	1165	20,958
∑PCBs + OCPs	981	14.01	350.5	1754	35,025	601.7	10,404	1805	31,212
Brominated									
PBDE 47	68	0.97	24.3	121	2425	39.7	642	119	1926
PBDE 99	13	0.19	4.8	24	475	8.6	126	26	378
PBDE 100	11	0.15	3.8	19	375	5.6	91	17	272
PBDE 153	2	0.03	0.8	4	75	1.5	22	5	67
PBDE 154	4	0.06	1.5	8	150	2.8	38	8	114
PBDE 209	105	1.50	37.5	188	3750	64.8	1141	194	3423
HBCD	21	0.30	7.5	38	750	9.9	203	30	609
∑BFRs	224	3.2	80.2	402	8000	132.9	2263	399	6789
Perfluorinated									
PFHxS	1.2	0.017	0.4	2	43	1.7	42	5	125
PFOS	18	0.26	6.5	33	650	3.2	74	10	222
PFOA	31	0.44	11.0	55	1100	6.0	121	18	363
PFNA	9.5	0.14	3.5	18	350	2.1	42	6	127
PFDA	13	0.19	4.8	24	475	3.1	57	9	172
PFUnDA	6.7	0.096	2.4	12	240	1.6	28	5	84
∑PFAAs	79.4	1.14	28.6	144	2858	17.7	364	53	1094

Abbreviations: PCBs (polychlorinated biphenyls); OCPs (organochlorine pesticides); BFRs (brominated flame retardants); PFAAs (perfluoroalkyl acids).
[a] Average EDI (Estimated daily intake) values of POPs for a 70 kg human e based on a literature review of Scandinavian EDI values (Berntsen et al., 2017).
[b] EDI values for a 25 g mouse corresponding to human EDI values.
[c] EDI values for a 25 g mouse corresponding to human EDI values * 5000
[d] EDI values for a 25 g mouse corresponding to human EDI values * 100,000.
[e] Measured concentrations of the various compounds in the 5000× feed.
[f] Measured concentrations of the various compounds in the 100,000× feed.
[g] EDI values for a 25 g mouse consuming 3 g of the 5000× feed/day e based on concentrations measured in the feed of the current project.
[h] EDI values for a 25 g mouse consuming 3 g of the 100,000× feed/day e based on concentrations measured in the feed of the current project.

previously described in Sødring et al. (2015).

2.5. Housing and husbandry

During mating, animals were housed in groups in open type III cages (Tecniplast, Buguggiate, Italy). During exposure and AOM injection animals were housed in closed type III IVC-cages (Allentown Inc, USA) for health and safety reasons. All cages contained standard aspen bedding, cellulose nesting material and red polycarbonate houses (Tecniplast, Buguggiate, Italy). The animals were given their assigned feed, and tap water in standard drinking bottles (Tecniplast, Buguggiate, Italy), *ad libitum*. The animal room was on a 12:12 light–dark cycle, with a room temperature of 21 ± 2 °C with 20 air changes per hour and 45 ± 5% relative humidity. The cages, bedding, nesting material and water bottles were changed once a week.

2.6. Sample collection and identification of intestinal lesions

The A/J Min/+ offspring were sacrificed at 13 weeks of age. They were anesthetized with isoflurane gas (Isoflurane Baxter, San Juan, Puerto Rico), bled by cardiac puncture and euthanized by cervical dislocation. The small intestine and colon were collected, fixed and dyed as previously described in Sødring et al. (2016a). Briefly, the intestines were rinsed with PBS, fixed flat, and stored in 10% neutral buffered formalin for at least 24 h, before being stained with 0.2% methylene blue dissolved in formalin. The liver was collected and weighed. All tumors that were found (one in the liver, one from the

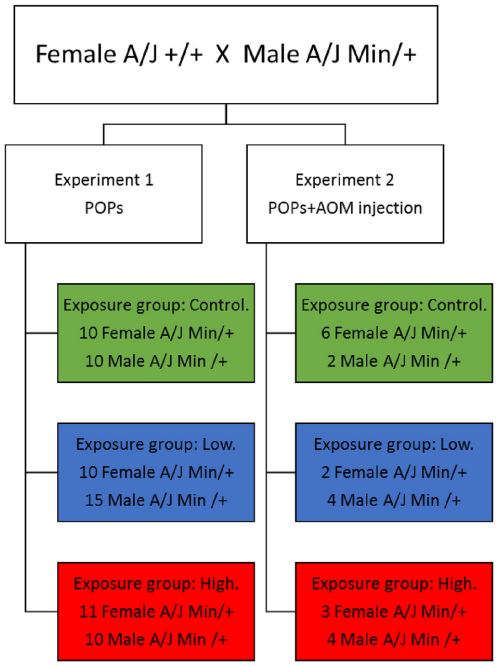

Fig. 1. Study design of the two experiments, including exposure groups (control, low and high), breeding of A/J Min/+ mice and the number of animals (females and males) in each group. In both experiments, A/J Min/+ mice were exposed to a mixture of POPs through feed for 10 weeks. In addition, mice in Experiment 2 received one subcutaneous injection of AOM (8.5 mg/kg) during the second week after birth.

forelimb, one sub-mandibular and one from the abdomen) were also collected and fixed in 10% neutral buffered formalin. The blood, cecum, spleen and retroperitoneal adipose tissue were collected and stored for analysis in another project. For surface microscopy and transillumination of the intestines, an inverted light microscope (CKX41, Olympus Inc., Hamburg, Germany) with a digital color camera (DP25, Olympus) was used. In the colon, lesions were identified as either flat aberrant crypt foci (flat ACF; <30 crypts) or tumors (>30 crypts covering more than approximately 0.4 mm^2) as explained by Sødring et al. (2015).

2.7. Histology

After scoring, the intestines were prepared using the Swiss roll technique as described earlier by Sødring et al. (2016b). The Swiss rolls were embedded in paraffin and 3 μm thick histological sections were cut and stained with haematoxylin eosin (HE) and periodic acid Schiff (PAS). All Swiss rolls were sectioned at three different random levels in the paraffin block. Examination was conducted in a microscope and lesions were identified, counted and classified as preneoplastic lesions (hyperplastic and dysplastic cells), adenomas or carcinomas. Tumors with distinct infiltrative growth through the muscularis mucosa and into the submucosa were classified as carcinomas, whereas tumors confined to the mucosa without infiltrative growth were classified as adenomas. Tumors that were found outside the intestine were also embedded in paraffin, sectioned and stained with HE and PAS, and examined in the microscope.

2.8. Statistical analyses

Statistical analyses were performed using JMP Pro 13® (SAS, Cary, NC, USA). Least squares analyses were used to analyze data on body measures. Experiment 1 and 2 were analyzed separately by the following model:

$$Y_{ijpmn} = \mu + G_i + E_j + e_{ij}$$

where:

Y_{ij} = observation of either body weight, relative liver weight, relative colon length or relative small intestine length.
μ = overall mean of body weight, relative liver weight, relative colon length and relative small intestine length.
G_j = effect of sex, $i = 1$ (Male) or 2 (Female).
E_j = effect of exposure group, $j = 1$ (control), $j = 2$ (low), $j = 3$ (high).
e_{ij} = error term.

Measures of histological changes and visually scored lesions did not meet the assumption of normality. Log transformation provided an improved, but not satisfactory, fit to the normal distribution. Initially least squares analyses were performed on log-transformed data with sex and exposure group as explanatory variables. Some sex differences were noticed, but few interactions were found between the exposure group and the sex of the animal. Thus, exposure effects were not dependent on the sex. In the final analyses, univariate non-parametric tests were used. Differences between exposure groups and the control were assessed using Steel's test, which controls for the overall experiment wise error rate (Type I). Differences between sexes were investigated using the Wilcoxon two-sample test. The level of significance was set to 5%. Size and location distribution figures were produced using Excel 2013®.

3. Results

3.1. Effects on body weight, liver weight and intestinal length

The high mixture concentration of POPs significantly decreased the terminal body weight of both the mice who only were exposed to POPs (Experiment 1) and also the mice that were injected with AOM (Experiment 2), compared to the control group (Table 2). In addition, there was a significant increase in liver weight, relative to body weight, in the high group of both experiments. Colon length, relative to body weight, was not affected by AOM or POPs. However, the length of the small intestine was significantly increased by the high concentration of POPs after the AOM injection (Experiment 2). Notably, AOM alone did not change any of the parameters measured.

3.2. Scoring of intestinal lesions

The effects of dietary exposure to the mixture of POPs on intestinal tumorigenesis was examined in mice by scoring of intestinal lesions (Table 3). High levels of POPs (Experiment 1) significantly increased the number of flat ACF in colon, when compared to the control group. Although not significant, a trend was observed towards increased flat ACF load in the high and the low groups ($p = 0.051$ and $p = 0.058$, respectively). The low mixture concentration increased the number of colonic tumors, compared to the control group. However, this was not evident after exposure to the high mixture concentration ($p = 0.096$). No other parameters measured in the small intestine and colon were affected by dietary POPs alone.

Table 2
Least square mean (±SE) of body weight (BW), relative liver weight (LW), relative colon length and relative small intestine (SI) length at necropsy in Experiment 1 (POP exposure) and Experiment 2 (POP exposure + AOM injection). The table included effects of exposure group control, low and high in both experiments. Bold letters indicate significant difference from the control group (Dunnet's test; $p \leq 0.05$).

Exposure		BW at necropsy (g)	Relative LW	Relative colon length (cm/g)	Relative SI length (cm/g)
Experiment 1	Control	23.54 ± 0.71	$0.05 \pm 0.08 \times 10^{-2}$	0.31 ± 0.01	1.41 ± 0.03
POPs	Low	23.26 ± 0.79	$0.05 \pm 0.07 \times 10^{-2}$	0.31 ± 0.01	1.46 ± 0.03
	High	**21.47 ± 0.49**	$\mathbf{0.07 \pm 0.08 \times 10^{-2}}$	0.33 ± 0.01	1.49 ± 0.03
Experiment 2	Control	24.68 ± 1.09	$0.05 \pm 0.12 \times 10^{-2}$	0.31 ± 0.01	1.36 ± 0.05
POPs + AOM	Low	25.43 ± 1.44	$0.05 \pm 0.14 \times 10^{-2}$	0.31 ± 0.03	1.49 ± 0.05
	High	**20.99 ± 1.47**	$\mathbf{0.07 \pm 0.13 \times 10^{-2}}$	0.33 ± 0.02	**1.62 ± 0.05**

Table 3
Summary of results from scoring of lesions in colon and small intestine (SI) of A/J Min/+ mice from Experiment 1 (POP exposure) and Experiment 2 (POP exposure + AOM injection). Colonic lesions are categorized as either flat ACF (<30 aberrant crypts) or tumors (>30 aberrant crypts). Load equal the total area of intestine covered by lesions. Results are presented as means (±SE). Differences between exposed groups (low and high) and control were assessed with Steel's test and indicated in bold when significant ($p \leq 0.05$). Trends with $p \leq 0.07$ are denoted *.

Exposure		Colon						SI		
		Number of flat ACF	Average size of flat ACF (mm^2)	Load of flat ACF (mm^2)	Number of tumors	Average size of tumors (mm^2)	Tumor load (mm^2)	Number of tumors	Average size of tumors (mm^2)	Tumor load (mm^2)
Experiment 1	Control	4.75 ± 0.98	$0.01 \pm 0.03 \times 10^{-1}$	0.06 ± 0.02	0.70 ± 0.24	0.75 ± 0.23	1.07 ± 0.37	27.95 ± 5.12	0.62 ± 0.04	20.39 ± 4.77
POPs	Low	8.60 ± 1.97	$0.02 \pm 0.04 \times 10^{-1}$	0.18 * ± 0.04	**2.04 ± 0.47**	0.65 ± 0.15	2.52 ± 0.93	25.56 ± 4.50	0.58 ± 0.04	17.92 ± 4.76
	High	**8.38 ± 1.42**	$0.02 \pm 0.02 \times 10^{-1}$	0.13 * ± 0.03	1.24 ± 0.23	0.47 ± 0.11	0.81 ± 0.23	26.57 ± 3.76	0.55 ± 0.03	15.85 ± 3.51
Experiment 2	Control	2.25 ± 0.84	0.05 ± 0.02	0.15 ± 0.06	3.63 ± 0.78	0.77 ± 0.20	3.47 ± 1.37	18.75 ± 5.44	0.70 ± 0.03	13.70 ± 4.39
POPs + AOM	Low	6.17 ± 1.30	0.06 ± 0.01	0.35 ± 0.09	12.33 ± 3.33	1.72 ± 0.50	25.80 ± 9.67	18.33 ± 2.33	0.63 ± 0.05	11.52 ± 1.57
	High	**11.57 ± 2.03**	0.06 ± 0.01	**0.69 ± 0.15**	**29.86 ± 4.83**	**1.99 ± 0.17**	**56.62 ± 6.70**	**48.00 ± 9.85**	0.74 ± 0.10	40.85 ± 13.44

In combination with the AOM injection (Experiment 2), POPs significantly increased the number of flat ACF, flat ACF load, number of tumors, average tumor size and tumor load in the colon of mice in the high group (Table 3). In addition, the number of tumors in the small intestine was significantly higher in the high group compared to the controls. No significant changes were observed after exposure to the low mixture concentration of POPs in Experiment 2. However, there were clear trends towards increases in several parameters, including a 7-fold increase in the colonic tumor load.

3.3. Size distribution and location of intestinal lesions

To demonstrate the distribution of size, lesions were divided into five different size categories. Fig. 2a presents the number of lesions per size category for each exposure group in both experiments and clearly illustrates the shift towards larger lesions observed in AOM treated animals. The AOM treatment alone did not appear to give any new lesions, but instead stimulated the growth of the colonic lesions. Notably, the increase in the number of lesions provoked by POPs seemed to be more pronounced in

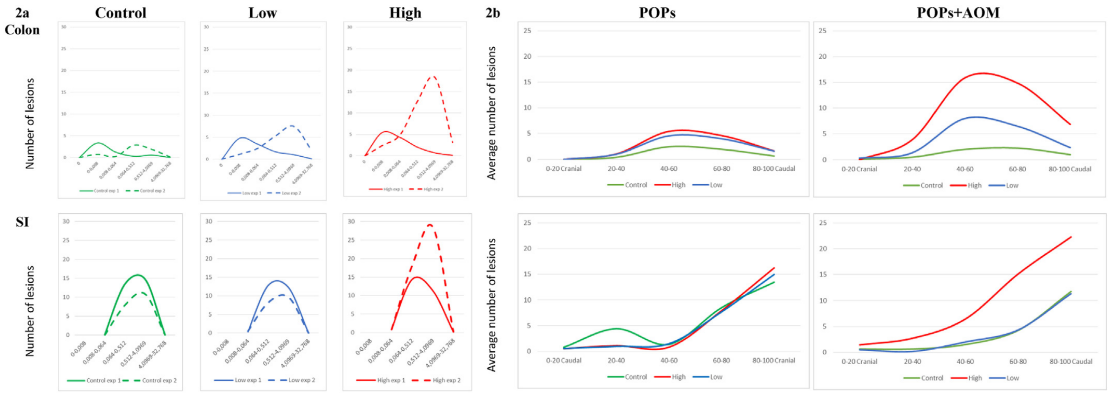

Fig. 2. a). Size distribution of lesions in colon and small intestine (SI) of A/J Min/+ mice exposed to POPs in control, low and high mixture concentrations, without AOM (Exp 1) and with AOM injection (Exp 2). Size categories (mm^2) are described by Søring et al 2015 and represented on the X axis. The Y axis shows the number of lesions. b). Location of lesions in colon and small intestine (SI) of A/J Min/+ mice exposed to POPs in control, low and high mixture concentrations, without AOM (Exp 1) and with AOM injection (Exp 2). Location categories (20% sections) are represented on the X axis. The Y axis shows the average number of lesions.

AOM treated animals than in untreated animals, particularly in the colon. This implies a synergistic effect of AOM and POPs. In the small intestine, AOM alone did not induce any apparent changes, but a moderate synergistic effect on tumor formation seemed to occur between AOM and the high level of POPs.

Location of lesions along the intestine (Fig. 2b) shows an increased number of lesions in the middle and caudal parts of the colon and caudally in the small intestine, in both experiments. In addition, the figure illustrates how dietary exposure to POPs enhances the number of lesions in both experiments, represented by more lesions in the high and low groups compared to the control group.

3.4. Histopathology

Histology from tumors collected from non-intestinal tissue showed no metastases originating from the intestinal lesions. Instead, they were either hyperplastic lesions or metastases from the local tissue.

In Experiment 1, lesions were found in the intestines of animals from all exposed groups. The total number of lesions in the small intestine was higher than that of the colon (Table 4). No significant differences were observed between the control group and the exposed groups. Preneoplastic changes and adenomas were the most frequent lesions, and only a few animals had carcinomas. Fig. 3 illustrates the types of lesions in the colon in Experiments 1 and 2.

In Experiment 2, the mice fed the high concentration of POPs had significantly more colonic lesions of all types compared to the control group (Table 4). A trend was also evident towards increases in the number of small intestinal preneoplastic lesions ($p = 0.067$). Interestingly, this increase of lesions appeared to be due to the synergistic effect between AOM and the high level of POPs, as suggested above.

4. Discussion

In the present study, we investigated whether a mixture of POPs could affect intestinal tumorigenesis in the A/J Min/+ mouse model. In a separate experiment, we also investigated whether a sub-carcinogenic exposure of AOM could influence the effect of POPs. We found that POPs alone increased the intestinal tumorigenesis moderately and in a dose-dependent manner. Comparing the two experiments, AOM alone did not seem to increase the formation of new lesions, or have a deleterious effect on the mice. However, the growth of colonic lesions was stimulated by AOM treatment. A strong synergistic effect was apparent between POPs and AOM on the formation of colonic lesions, and to a lesser extent

Table 4
Histopathological examination of lesions (preneoplastic, adenoma or carcinoma) in colon and small intestine (SI) of A/J Min/+ mice from Experiment 1 (POP exposure) and Experiment 2 (POP exposure + AOM injection). Results are presented as mean (±SE). Differences between exposed groups (low and high) and control were assessed with Steel's test and indicated in bold when significant ($p \leq 0.05$). Trends with $p \leq 0.07$ are denoted *.

Exposure		Colon			SI		
		Preneoplastic	Adenoma	Carcinoma	Preneoplastic	Adenoma	Carcinoma
Experiment 1	Control	0.70 ± 0.25	0.35 ± 0.25	0.05 ± 0.05	9.05 ± 1.32	6.80 ± 1.33	0.90 ± 0.42
POPs	Low	0.84 ± 0.29	0.32 ± 0.19	0.12 ± 0.09	7.00 ± 1.21	3.96 ± 1.04	0.44 ± 0.22
	High	1.05 ± 0.30	0.05 ± 0.05	0.00 ± 0.00	6.86 ± 1.58	3.86 ± 1.09	0.29 ± 0.16
Experiment 2	Control	1.38 ± 0.42	0.75 ± 0.31	0.00 ± 0.00	4.50 ± 1.59	5.13 ± 1.75	0.50 ± 0.27
POPs + AOM	Low	2.00 ± 0.58	2.17 ± 0.70	0.67 ± 0.49	6.00 ± 1.21	2.67 ± 0.67	0.00 ± 0.00
	High	**5.57 ± 1.11**	**6.57 ± 1.51**	**2.57 ± 0.92**	10.29 * ± 2.35	9.43 ± 2.06	0.71 ± 0.36

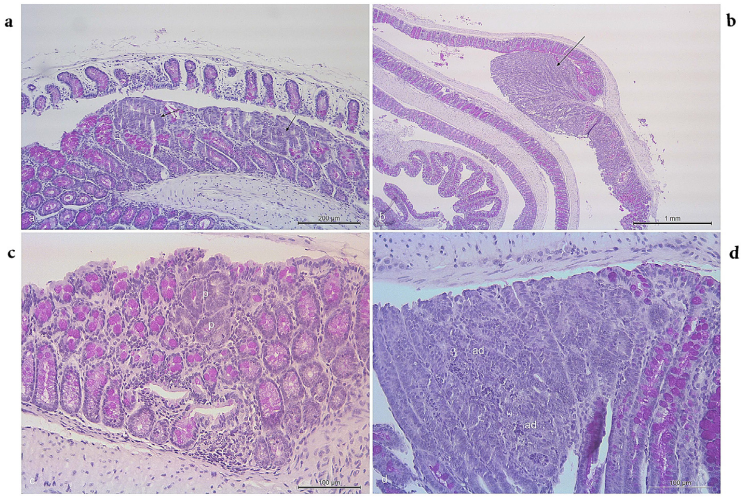

Fig. 3. Histological lesions observed in colon. a. Preneoplastic lesions (dysplasia and hyperplasia) are present in the luminal part of crypts (arrows) of a mouse of the low exposure group of Experiment 1. **b**. A carcinoma (arrow) in the mucosa infiltrates Muscularis Mucosae and Submucosa. Mouse of the low exposure group of Experiment 1. **c**. A small focus with preneoplastic crypt lesions in a mouse of the low exposure group of Experiment 2. **d**. Mucosal adenoma (ad) in a mouse from the high exposure group of Experiment 2.

on lesions in the small intestine. Interestingly, this synergy was also associated with a significant increase of malignant tumors (carcinomas) in the colon.

4.1. Effects of POPs on body weight and liver weight

The concentration of each compound in the high dose was generally below the No Observed Adverse Effect Level (NOAEL), where such a level was available (Berntsen et al., 2017). Although we did not observe any clinical signs in the animals during the present study, we did observe apparent adverse effects at the end of the study, indicated by reduced body weight and increased relative liver weight in both experiments. These effects were seemingly unrelated to AOM treatment and may have been caused by additive or synergistic effects between individual POPs in the mixture. In another experiment using the same mixture but a different mouse strain (129:C57BL/6F0), there was no significant effect of the high POPs feed on body weight (Hudecova et al., 2018). This suggests there are mouse strain differences in sensitivity to POPs.

Aberrant *Apc* expression as a consequence of the germline mutation in *Apc* has been shown to affect the ability of the liver to metabolize xenobiotics (Benhamouche et al., 2006), and may lead to degrees of pollutant tolerance. In addition, the large number of intestinal lesions in the mice exposed to the high dose of POPs in our experiment may have contributed to a lower absorption rate of nutrients, which could have reduced the body weight of mice in this group.

Our findings of increased relative liver weights is in line with other studies where animals have been exposed to perfluorinated compounds (Seacat et al., 2003; Tan et al., 2013). These chemicals have been thoroughly investigated for hepatotoxicity, because of their high affinity to serum proteins and subsequent accumulation in the liver (Jones et al., 2003).

The highest concentration of POPs in our study is relatively large, but the low mixture concentration could potentially be considered more relevant for humans when taking life-long exposure and slow pollutant metabolism into account (Martignoni et al., 2006; Hudecova et al., 2018).

The occurrence of high mortality in offspring after neonatal AOM treatment (Experiment 2) was surprising, as the dosage used has not previously been associated with increased mortality. It is therefore unclear whether the lethality observed was caused by an abnormally high sensitivity to AOM, either alone or in combination with stress. However, we can conclude that the event was not caused by dietary POPs, since the AOM injection was given prior to weaning.

4.2. Effects of POPs on intestinal lesions

The process of cancer is divided into three phases; initiation, promotion and progression (Farber and Cameron, 1980). Depending on their mode of action, compounds may interfere with the molecular processes within each of these phases, and ultimately affect the carcinogenic process. Initiation is the irreversible heritable change in DNA, while promotion is the non-genotoxic advantages of mutated cell growth (Ludewig and Robertson, 2013). In the present study, the high mixture concentration of POPs initiated the formation of new lesions in the colon of A/J Min/+ mice, which was reflected by a significantly larger intestinal area covered by flat ACF. The low concentration of POPs did not affect the number of newly formed lesions, but promoted intestinal tumorigenesis by resulting in more colonic tumors of a larger diameter (>30 crypts). This initiating and promoting effect was not visible in the small intestine.

Previous studies have reported that some POPs affect both carcinogenic initiation and promotion *in vivo*. Liver tumorigenesis was initiated by a mixture of PCBs (Kanechlor 500) in mice (Ito et al., 1973). The same study also showed a promotional effect of the PCBs when administered together with hexachlorobenzene (HCB, α or β). Developmental exposure (*in utero* and via lactation) to dieldrin initiated the formation of mammary, ovarian and liver tumors in a transgenic mouse model for mammary tumorigenesis (Cameron and Foster, 2009). *In utero* exposure to perfluorooctanoic acid (PFOA) induced hepatocellular adenomas in CD-1 mice (Filgo et al., 2015). However, PFOA and PFOS did not increase the formation of intestinal lesions (Ngo et al., 2014). HCB was shown to promote mammary, liver and lung tumorigenesis in xenograft mouse models, without having initiating effects (Pontillo et al., 2013). The organochlorine metabolite *p,p'*-DDE has been suggested as a promoting agent in mammary tumorigenesis (Johnson et al., 2012). In addition, its parental compound *p,p'*-DDT (*p,p'*-dichlorodiphenyltrichloroethane) has been shown to promote CRC growth in mice injected with a suspension of the human colorectal adenocarcinoma cell line DLD1 (Song et al., 2014). The study also demonstrated that the CRC promotion by *p,p'*-DDT was achieved through the Wnt/β-catenin signaling pathway mediated by oxidative stress. *p,p'*-DDT elevated the production of reactive oxygen species (ROS), inhibited enzymes and reduced antioxidants levels in intestinal cells. Subsequently, there was an accumulation of β-catenin and the consecutive expression of target genes, which induced the proliferation of colorectal cancer cells and thus promoted CRC growth. The study also demonstrated that an increased production of ROS could affect colorectal carcinogenesis by interacting with specific pathways or by damaging DNA.

Furthermore, the metabolic activation of compounds may create products or intermediates that can interfere directly with DNA. PCBs have been shown to form highly reactive products and by-products that have the ability to mutate DNA, as reviewed by Ludewig and Robertson (2013). PBDEs are structurally similar to PCBs and have been shown to induce ROS formation, leading to chromosomal breakage (Ji et al., 2011). POPs may therefore have the ability to affect DNA and to increase tumorigenesis by inducing mutations in oncogenes or tumor suppressor genes such as *KRAS*, *p53* and *APC*. Changes in these genes are necessary for the development of colorectal cancer (Fodde, 2002). It has also been shown that most intestinal lesions in the Min/+ mouse have lost their remaining functioning *Apc* allele (Luongo et al., 1994). In the present study, mutations in *Apc* might have caused the formation of new lesions and enhanced the growth from flat ACF to tumors in the A/J Min/+ mice. However, this remains to be investigated.

4.3. Effects of AOM and POPs on intestinal lesions

AOM is converted to methylazoxymethanol (MAM) by cytochrome P450 enzymes (CYP450) located in both the liver and the intestines (Sohn et al., 2001). This highly reactive metabolite causes DNA mutations that are thought to initiate colorectal carcinogenesis (Takahashi and Wakabayashi, 2004). Different strains of mice vary in their susceptibility to AOM-induced CRC, and the A/J strain is known to be highly sensitive (Rosenberg et al., 2009). In addition, Min/+ mice exposed to AOM during their first two weeks of life have been shown to be particularly susceptible to induced and spontaneous intestinal carcinogenesis (Paulsen et al., 2003).

In the present study, neonatal mice in Experiment 2 were given one injection of AOM. This treatment did not seem to initiate the formation of new colonic lesions. Instead, it promoted the growth of already existing lesions, as evident from the increased number of tumors and colonic lesions of the larger size classes in mice from the control group. Combined exposure to AOM and POPs both

initiated and promoted colorectal carcinogenesis and resulted in a severe lesion burden, especially in mice exposed to the high mixture concentration of POPs. This large effect on tumorigenesis, compared to the relatively moderate initiation and promotion by POPs alone, indicates a synergistic effect between AOM and POPs. The high group exhibited the most extreme outcomes, which could be explained by the relatively high concentration of pollutants. However, the numerical differences from the control group demonstrate that the low mixture concentration also displayed initiating and promoting effects in the colon, as shown by a 7-fold increase in colonic tumor load. As with AOM, POPs are metabolized by CYP450 (Docea et al., 2017) and CYP450 has been shown to be a strong biomarker for the presence of POPs in animal tissue (Bachman et al., 2015). This similarity could be the origin of the synergistic effect observed between AOM and POPs, but this remains to be investigated. Previous studies in mice (Swiss and B6129SF2/J strains) have shown that PCBs promote carcinogenesis in lung and liver tissues when the tumors were initiated by N-nitrosodimethylamine (Anderson et al., 1994; Strathmann et al., 2006). The same promotional effect of PCBs was seen in A/J mice when given together with 1-Nitropropane to induce lung tumorigenesis (Nakanishi et al., 2001). The synergistic effect seen in the present study emphasizes the importance of anticipating synergistic effects between compounds which individually have the ability to initiate or promote cancer development. In addition, due concern should be given to chemical mixtures that not individually cause cancer, but which are disruptive in a manner that collectively provokes carcinogenesis (Goodson et al., 2015).

4.4. Histopathology

In the present study, the histopathological characterization of intestinal lesions differentiated between preneoplastic lesions, adenomas and carcinomas. The preneoplastic lesions included both hyperplastic and dysplastic cells. Dysplasia is a known hallmark of malignant potential and is closely related to *APC* mutations (Jen et al., 1994). Moderate to severe dysplasia has previously been shown in flat ACF from both traditional (C57BL/6) and A/J Min/+ mice (Paulsen and Alexander, 2001; Paulsen et al., 2006; Sødring et al., 2016a). In addition, flat ACF have been shown to be reliable surface biomarkers of *Apc*-driven colorectal carcinogenesis (Sødring et al., 2016a).

The initiating and promoting effect observed by intestinal scoring was not evident from the histopathological examination of mice only exposed to POPs (Experiment 1). However, carcinomas were observed in both the colon and small intestine, which could be an indication of the promotional effect explained above. In mice from Experiment 2, AOM and the high mixture concentration increased all types of lesion, which further confirms the synergistic effect observed between AOM and POPs. Interestingly, one injection of AOM alone did not cause the formation of colonic carcinomas. Instead, it induced the formation of preneoplastic lesions and adenomas, suggesting that AOM did not act as a tumor promotor in the A/J Min/+ mice after only one injection.

Because of the difference in method of analysis between intestinal scoring and histopathology, identical results could not be expected. However, similar trends were observed and both methods showed the same synergistic effect between AOM and POPs. It is important to emphasize that the histopathological examination was conducted on only three slides from each intestine. Thus, this method only investigates a small part of the area in question, compared to the scoring of the whole surface of the intestine, which could explain the discrepancy in findings between the two different methods.

5. Conclusion

The present study indicates that a mixture of POPs designed on the basis of human exposure, together with an injection of AOM, increased intestinal tumorigenesis in A/J Min/+ mice. Furthermore, a synergistic effect was observed between POP exposure and one injection of AOM. The results emphasize the importance of anticipating synergies when assessing the carcinogenic potential of compound mixtures.

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