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

Philosophiae Doctor (PhD)
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Exploring the short and long-term effects of developmental exposure in zebrafish exposed to anthropogenic chemicals

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Maria Christou

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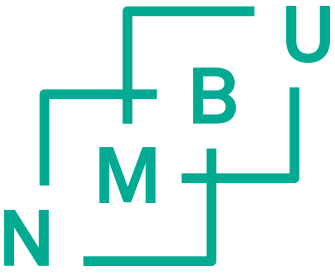
Adamstuen (2020)



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Acknowledgments

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from Greece, **Gianna, Margarita, Anastasia, Lena** and all my friends back in Cyprus, thank you for supporting me from afar, through many hours of videocalls for making me laugh and for reminding me that everything will be ok in the end.

"Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean."

*Ithaka, By **C. P. Cavafy, 1911***
Translated by Edmund Keeley

Abbreviations

ADHD	Attention deficit hyperactivity disorder
Ahr	Aryl hydrocarbon receptor
ASD	Autism spectrum disorder
BFRs	Brominated flame retardants
cDNA	complementary DNA
DDT	Dichlorodiphenyltrichloroethane
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DOHaD	Developmental Origins of Health and Disease
Dpf	Days post fertilization
EDC	Endocrine disrupting chemical
FDR	False discovery rate
FET	Fish Embryo Acute Toxicity
GABA	gamma-Aminobutyric acid
GSI	Gonadosomatic index
HBB	Hexabromobiphenyl
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
Hpf	Hours post fertilization
HTSs	High-throughput screens
IPA	Ingenuity pathway analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LMEs	Linear mixed effects
LOAEL	Lowest observed adverse effect level
MAPK	Mitogen-activated protein kinase
MB	Methylene blue
MMP	Maximal mappable prefixes
OCPs	Organochlorine pesticides
OECD	Organisation for Economic Co-operation and Development
***p,p'*-DDE**	Dichlorodiphenyldichloroethylene
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzodioxin
PCDF	Polychlorinated dibenzofuran
PCN	Polychlorinated naphthalene
PCP	Pentachlorophenol
PeCB	Pentachlorobenzene
PFASs	Per- and polyfluoroalkyl substances

PFBA	Pentafluorobenzoic acid
PFBS	Perfluorobutanesulfonic acid
PFCAs	Perfluorinated carboxylic acids
PFDA	Perfluorodecanoic acid
PFDoDA	Perfluorododecanoic acid
PFDS	Perfluorodecanesulfonate
PFHpA	Perfluoroheptanoic acid
PFHpS	Perfluoroheptanesulfonic acid
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexanesulphonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PFOSA	Perfluorooctanesulfonamide
PFPeA	Perfluoropentanoic acid
PFSAs	Perfluoroalkane sulfonates
PFTA	Perfluorotetradecanoic acid
PFTrDA	Perfluorotridecanoic acid
PFUnDA	Perfluoroundecanoic acid
POP	Persistent organic pollutant
POSF	Perfluorooctanesulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
qPCR	quantitative real-time polymerase chain reaction
RNA-seq	RNA sequencing
RyR	Ryanodine receptor
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
$\mathbf{U_{crit}}$	Critical swimming speed

Summary

Persistent organic pollutants (POPs) are anthropogenic chemicals omnipresent in the environment and animal tissues. Due to their persistency they are rarely found as single compounds within organisms but rather in complex mixtures. Epidemiological and animal studies have associated exposure to POPs with adverse effects spanning the lifetime of an organism. However, as these chemicals might interact in a synergistic or antagonistic manner, predicting their effects from single chemical toxicology studies is challenging. Additionally, due to the lipophilic nature of many of these chemicals, solvents are used in animal studies to accommodate their administration to test tissues although the solvents themselves may also affect endpoints of interest. The aims of the current thesis were to evaluate whether methodological approaches such as the use of solvents and other laboratory practices can affect the outcome of toxicology studies using a behavioral paradigm in zebrafish. Additionally, using zebrafish as an animal model we set to investigate the short- and long-term effects of chemical exposure to a complex mixture of POPs on physiological and behavioral endpoints.

Neurotoxicity effects of chemicals are often evaluated using behavioral tests, and zebrafish larvae have been used regularly in recent years because of their small size that facilitates large screening of multiple chemicals simultaneously. However, many different experimental and methodological parameters can affect the locomotor behavior of larval zebrafish. Using a light-dark transition test we investigated whether different concentrations of common solvents such as dimethyl sulfoxide (DMSO) and methanol can affect basal locomotor behavior. Furthermore, we investigated the effects of other parameters such as the use of the antifungal agent (methylene blue, MB), media volume in wells and different laboratory strains. Finally, we tested the interaction between DMSO or MB with positive controls on behavioral endpoints. DMSO and MB increased basal locomotor activity in a dose dependent manner as did media volume. Furthermore, even if basal locomotor activity was different among zebrafish stains, all strains responded in the same manner to the solvents and MB, therefore the choice of strains should not be of concern when designing behavioral tests using zebrafish. However, results indicated that DMSO and MB could both have additive or interactive effects on behavioral endpoints when combined with positive controls (Paper I).

Taking into consideration the parameters affecting zebrafish larval behavior, we investigated the short and long-term effects of exposure to a complex mixture of POPs or perfluorooctanesulfonic acid (PFOS) on zebrafish larvae and adults. The POP mixture consists of 29 chemicals and was based on the average blood levels in the Scandinavian population. PFOS was identified as a driving agent of behavioral changes in previous studies and was included as a single exposure to test whether responses to the mixture are comparable to PFOS responses. Embryos were exposed to two concentrations of the POP mixture or PFOS alone (10x and 70x what is found on average human blood levels) from 6 – 96 hours post fertilization (hpf) and afterwards reared in clean water until adulthood. In larvae we tested the effects of exposure on behavior, using the light-dark transition and thigmotaxis test and we employed transcriptomics analysis in order to identify possible mechanisms of action. In adults we measured growth, swimming performance, and reproductive output at different life stages. In addition, we assessed anxiety behavior of the adults and their offspring, as well as performing a transcriptomic analysis on the adult zebrafish brain.

Behavioral responses of 96 hpf larvae included hyperactivity and higher thigmotaxis in the exposed individuals. Transcriptome analysis revealed upregulation of transcripts related to muscle contraction and further pathway analysis revealed that one of the most affected pathways was the calcium signaling pathway via the activation of ryanodine receptors (RyR). Mechanistic analyses with RyR inhibitors and behavioral outcomes substantiate these findings. In adults, exposure to the POP mixture and PFOS reduced swimming performance and increased length and weight, compared to controls. No effect of developmental exposure was observed on reproductive output, adult anxiety behavior, or behavior of subsequent offspring. Pathway analysis of the brain transcriptome of adults exposed as larvae to the low concentration of PFOS revealed enrichment in pathways such as calcium, mitogen-activated protein kinase (MAPK), and gamma-Aminobutyric acid (GABA) signaling, all of which are important for learning and memory (Paper II & III)

To conclude, the results obtained in this dissertation increase our understanding of how different methodological approaches can affect the locomotor activity of larval zebrafish and point to the necessity of protocol standardization to increase the

reproducibility and comparability of results among laboratories. Zebrafish proved a useful and practical model to assess the toxicity of complex POP mixtures. Most of the effects were comparable between single and mixture exposures, but some differences were also evident, such as exposure effects on gene expression.

Sammendrag

Persistente organiske miljøgifter (POPs) er menneskeskapte kjemikalier som finnes i levende organismer og i miljøet de lever i. På grunn av sin persistens og tendens til oppkonsentrering i næringskjeder, finnes de sjelden alene, men i komplekse blandinger. Epidemiologiske data og dyrestudier viser at eksponering for POPs kan være forbundet med helseskadelige effekter. POPs vil kunne interagere med hverandre på ulike måter slik at det ikke er mulig å forutsi hva slags effekter de måtte ha basert på kunnskap om enkeltstoffer. I tillegg er det slik at mange POPs er fettløselige. Det gjør at man må bruke løsemidler for å anvende dem i eksperimentelle studier. Løsemidler kan i seg selv ha uønskede effekter og i noen tilfeller påvirke de endepunktene man ønsker å undersøke.

Hensikten med denne avhandlingen var å vurdere om metodisk tilnærming, blant annet bruk av løsemidler, kunne påvirke resultatene i toksikologiske studier knyttet til adferd hos zebrafisk. I tillegg ble zebrafisk brukt som dyremodell for studier av kort- og langsiktige effekter på fysiologiske og adferdsmessige endepunkter etter eksponering med en kompleks blanding av POPs.

Nevrotoksiske effekter av kjemikalier blir ofte undersøkt med adferdstester. Zebrafisklarver er, blant annet på grunn av sin størrelse, godt egnet til adferdsstudier. Modellen muliggjør også screening-undersøkelser av flere kjemikalier samtidig. Det er imidlertid slik at forsøksoppsett og metodiske parametre kan påvirke adferd hos zebrafisk. Ved å bruke en såkalt “light-dark transition test” undersøkte vi hvordan ulike konsentrasjoner av vanlige løsningsmidler som dimethyl sulfoksyd (DMSO) og metanol påvirker basalt bevegelsesmønster. Forsøkene omfattet også effekter knyttet til andre metodiske parametre, herunder bruk av soppmiddel (methylenblått, MB), mediumvolum i brønnene samt forskjeller mellom ulike stammer av zebrafisk. Vi undersøkte også interaksjonseffekter på adferd mellom DMSO, MB og positive kontroller. DMSO, MB og medievolum økte basalt bevegelsesmønster med henholdsvis økende dose eller volum. Resultatene indikerte at DMSO og MB eller kjemikalier kan ha additive effekter eller interaksjonseffekter på adferd. Selv om det i utgangspunktet var forskjell i bevegelsesmønster mellom de ulike stammene av zebrafisk, reagerte de på samme måte på løsemidlene of MB. Valg av stamme skulle derfor ikke ha betydning for design av

adferdstester basert på zebrafisklarver. Resultatene viste imidlertid at både DMSO og MB kunne ha additiv effekt eller interaksjonseffekt når de ble brukt sammen med positive kontroller (Artikkel I).

Med kunnskap om hvilke faktorer som påvirker adferd hos zebrafisklarver (Artikkel I), undersøkte vi kortsiktige og langsiktige effekter av eksponering med en kompleks blanding av POPs i tillegg til enkeltstoffet PFOS (perfluorooctan sulfonat) på zebrafisklarver og voksne zebrafisk. Blandingen av POPs besto av 29 kjemikalier og var laget for å etterlikne et gjennomsnitt av blodkonsentrasjonen av disse stoffene i den skandinaviske befolkningen. PFOS har forårsaket adferdsendringer i tidligere studier og ble tatt med for å vurdere om effekter av POP-blandingen liknet effekter sett med PFOS.

Zebrafisk embryo ble eksponert med to konsentrasjoner POP-blanding eller PFOS alene (10x og 70x gjennomsnittlig human blodkonsentrasjon) fra 6 – 96 timer etter fertilisering (hpf) og etter dette holdt i rent vann inntil voksen alder. Hos larver brukte vi adferdstestene «light-dark transition test» og «thigmotaxis test». Vi brukte transkriptomanalyser for å identifisere virkningsmekanismer. Hos voksne undersøkte vi vekst, svømmeevne og reproduksjon i ulike livsstadier. I tillegg undersøkte vi angstadferd hos voksne zebrafisk og gjorde også transkriptomanalyser av hjernen til voksne zebrafisk.

Eksponerte larver viste hyperaktivitet og høyere thigmotaxis 96 hpf. Transkriptomanalyse viste oppregulering av transkripter knyttet til muskelkontraksjon. Videre analyse viste at kalsiumsignalering var påvirket via ryanodine reseptor (RyR). Adferdsstudier og mekanistiske studier ved bruk av RyR-hemmere støttet disse funnene. Hos voksne førte eksponering med POP-blandingen og PFOS til redusert svømmedyktighet og økt lengde og vekt sammenliknet med kontrollfisk. Det var ikke holdepunkter for at eksponering påvirket verken reproduksjonevne eller angst- og bevegelsesadferd hos avkom. Transriptomanalyse av hjernevev hos voksne som ble eksponert for PFOS som larver viste oppregulering av signalveier knyttet til kalsium, mitogenaktivert protein kinase (MAPK) og gamma-amminobutyric acid (GABA). Alle disse signalveiene er knyttet til læring og hukommelse (Artikkel II og III).

Vi konkluderer med at resultatene i denne avhandlingen har bidratt til å øke forståelsen av hvordan ulike metodiske tilnærminger påvirker bevegelsesadferd hos zebrafisklarver. Økt reproduserbarhet i eksperimentelle studier med denne modellen

forutsetter etablering av standardiserte protokoller. Dette vil gjøre det lettere å sammenlikne resultater oppnådd i ulike laboratorier. Zebrafisk viste seg å være en god modell for å undersøke toksisitet knyttet til komplekse POP-blandinger. De fleste effektene viste stor grad av likhet når POP-blandingen ble sammenliknet med PFOS, men det ble også funnet forskjeller, for eksempel i effektene på genekspresjonen.

List of papers

Paper I

DMSO effects larval zebrafish (*Danio rerio*) behavior, with additive and interaction effects when combined with positive controls.

Maria Christou, Arturas Kavaliauskis, Erik Ropstad, Thomas W. K. Fraser

Science of the Total Environment, Volume 709, 20 March 2020, 134490, doi: *10.1016/j.scitotenv.2019.134490*

Paper II

Calcium signaling as a possible mechanism behind increased locomotor response in zebrafish larvae exposed to a human relevant persistent organic pollutant mixture or PFOS.

Maria Christou, Thomas W. K. Fraser, Vidar Berg, Erik Ropstad, Jorke H. Kamstra

Environmental Research, Volume 187, 1 August 2020, 109702, doi: *10.1016/j.envres.2020.109702*

Paper III

Developmental exposure to a POPs mixture or PFOS increased body weight and reduced swimming ability but had no effect on reproduction or behavior in zebrafish adults.

Maria Christou, Erik Ropstad, Stephen Brown, Jorke H. Kamstra, Thomas W. K. Fraser

Manuscript

1. Introduction

1.1 Environmental toxicology and the emergence of endocrine disrupting chemicals

The rise of anthropogenic chemicals in our environment has increased dramatically in the 20th century due to industrialization and agriculture. For instance, the invention of the insecticidal properties of dichlorodiphenyltrichloroethane (DDT) by Paul Hermann Müller in 1939 led to an increase of its use post the World War II era (Hayes 1991) and by 1948 DDT was detected in human tissues (cited in Loganathan and Kannan 1994). In the mid-1940s, studies on laboratory and field animals provided the first evidence of the acute and chronic toxicity of DDT (Coburn and Treichler 1946). By the 1950s other chemicals were in common use in agriculture e.g. dieldrin, aldrin, chlordane and heptachlor.

With the publication of her book *Silent Spring* in 1962, Rachel Carson introduced the groundbreaking and provocative idea that man was progressively poisoning the planet, with her account on pesticide effects on organisms, the contamination of surface and ground water, the production of ecological imbalance, the persistence of chlorinated hydrocarbons and human safety (Carson 1962). Her book is accredited by many to the rise of the ensuing global environmental movement. Carson was the first to provide the hypothesis that certain pesticides, such as DDT, might be interfering with the hormonal balance of eagles and other avian species and that this may be related to the observed populational declines (Carson 1962). Research ensued in the following years, providing further evidence on the effects of DDT and its metabolite p,p'-DDE. Egg shell thinning, reproductive impairment and declines of avian species were correlated with the levels of these substances in their tissues (Ratcliffe 1967, Hickey and Anderson 1968, Heath *et al.* 1969). During the same period, Swedish scientist Soren Jensen, reported some unknown peaks in gas chromatography from environmental samples which were later identified as polychlorinated biphenyls (PCBs) (Jensen 1966). Subsequently the "Yusho" poisoning in humans (1968) related with ingestion of rice oil contaminated with PCBs raised the

awareness of detrimental effects of PCBs (Kuratsune *et al.* 1972). By the early 1970s, restrictions on the use of DDT and PCBs were starting to be implemented in North America and Europe (Rattner 2009).

During the late 70s and following decades, further effects of pesticides were characterized in animals and humans. In 1977 a study emerged indicating a behavioral effect of pesticides. Hunt and Hunt (1977) noted the presence of female pairs of sea gulls in California, which was later attributed to be a result of both a reduced male population and anomalies in male reproductive structures and behavior due to the possible estrogenic action of DDT and other contaminants (Fry 1995). It wasn't until 1992 when a publication of an article increased the attention about the effects of estrogenic contaminants on human health. Carlsen *et al.* (1992) noted the progressive decrease of semen quality over a span of 50 years, that was attributed by Sharpe and Skakkebaek (1993) to estrogenic chemicals in the environment (Carlsen *et al.* 1992, Sharpe and Skakkebaek 1993).

In the 1990s, epidemiological data of developmental abnormalities in the male reproductive system and an observation of reduced gonadal size of alligators led scientists to formulate the "Endocrine disruptor hypothesis" stating that many chemicals can disturb the development of the endocrine system and systems that respond to the endocrine signal of an organism exposed to chemicals during prenatal and/or postnatal life (Colborn *et al.* 1993, Guillette *et al.* 1994, Moller 1998). Since then the term endocrine disrupting chemical (EDC) was formulated to describe any compound that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body (Vos *et al.* 2000) with DDT and PCBs being some of the first recognized EDCs (Weiss 2011).

1.2 Persistent organic pollutants

"Forever chemicals", or as they are formally known, persistent organic pollutants (POPs), are a group of organic compounds that have long half-lives, as they are resistant to environmental and biological degradation. For example, it has been suggested that the half-lives of PCBs in water can range from 3 – 27 years depending on the congener (Sinkkonen and Paasivirta 2000). Consequently, they persist in the environment for many years even after restrictions or banning of their use. POPs include PCBs, brominated flame retardants (BFRs), per- and polyfluoroalkyl substances (PFASs), organochlorine pesticides (OCPs), and dioxins such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Ritter *et al.* 1998). POPs are additionally semi-volatile which allows them to be present either in the vapor phase or absorbed on atmospheric or organic particles making possible their long-term transport through air and/or water to remote areas, such as the Arctic (Butt *et al.* 2010), the Antarctic (Fuoco *et al.* 2009), and deserts (Garrison *et al.* 2014). As such, the occurrence of POPs in the environment is a global phenomenon.

1.2.1 Chemical composition and uses

Due to the plethora of POPs that were or are still in use, the focus of this section will be on POPs that are included in the studies performed in this thesis.

PCBs are synthetic chlorinated aromatic hydrocarbons that are non-flammable and hydrophobic. Their use and production was banned in the 1980s (Breivik *et al.* 2002). There are 209 identified PCB congeners with 1-10 chlorine atoms on a biphenyl molecule[1]. Based on the chlorine binding position, PCBs are divided into coplanar or non-coplanar isoforms. PCBs have been used as di-electric fluids in electrical capacitors, transformers and hydraulic systems (UNEP 1999).

[1] http://www.caslab.com/PCB-Congeners-Aroclors-Testing/209-PCB-Congeners.php

BFRs include polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD). PBDEs are brominated aromatic hydrocarbons and like PCBs, 209 congeners exist. BFRs were produced and used as mixtures e.g pentaBDE, octa-BDE, as flame retardants in many consumer products such as cell phones, computers and other electronic devices, furniture and textiles (UNEP 2017).

PFASs are organofluoride chemical compounds with either a carboxyl, alcohol or sulfonate terminal. According to the Organization for Economic Co-operation and Development (OECD) there are at least 4730 different PFASs with perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) most commonly found in the environment and animal and human tissues (Lau *et al.* 2007, Kato *et al.* 2015). PFASs are non-flammable, stable at high temperatures and non-volatile. They have been used since the 1950s in fire-fighting foams, as water- and stain-resistant coatings on clothes, furniture and carpets and as surfactants and surface protection products. In the Nordic countries, PFASs are also used in ski products, such as ski waxes (Renner 2001, Grønnestad *et al.* 2019).

DDT and its metabolite *p,p'*-DDE, hexachlorobenzene (HCB), chlordane, lindane (with by-products α-HCH, β-HCH) and dieldrin all belong to the group of chlorinated hydrocarbon derivatives most commonly called OCPs. OCPs were mainly used to control pests in agriculture but also for eliminating insects that transmit infectious pathogens like malaria, from the 1940s until the mid-1970s when most of them were phased out (Ritter *et al.* 1998, Jayaraj *et al.* 2016).

1.2.2 Levels of POPs in the environment

Most POPs are characterized by high lipid solubility and can readily bioaccumulate in tissues and biomagnify in top predators and humans (Ritter *et al.* 1998). Consequently, POPs have been detected in human and animal tissues such as birds, mammals and fish worldwide (Giesy and Kannan 2001, Chen and Hale 2010, Porta *et al.* 2012). For

example, in Figure 1 a recent review by Jian *et al.* (2018) gives an overview of the levels of PFASs found in human blood worldwide with PFOS being the most prevalent. A similar global study found PFOS ranged even higher in animal blood plasma from 1 – 2570 ng/ml (Giesy and Kannan 2001). In contrast, in samples of water worldwide PFOS was found at a range of 0.1 – 100 ng/L (Rahman *et al.* 2014). The most common routes of exposure for humans are through food consumption, drinking water, outdoor and indoor air and from the working environment (EFSA 2008, Guo *et al.* 2019, WHO 2020).

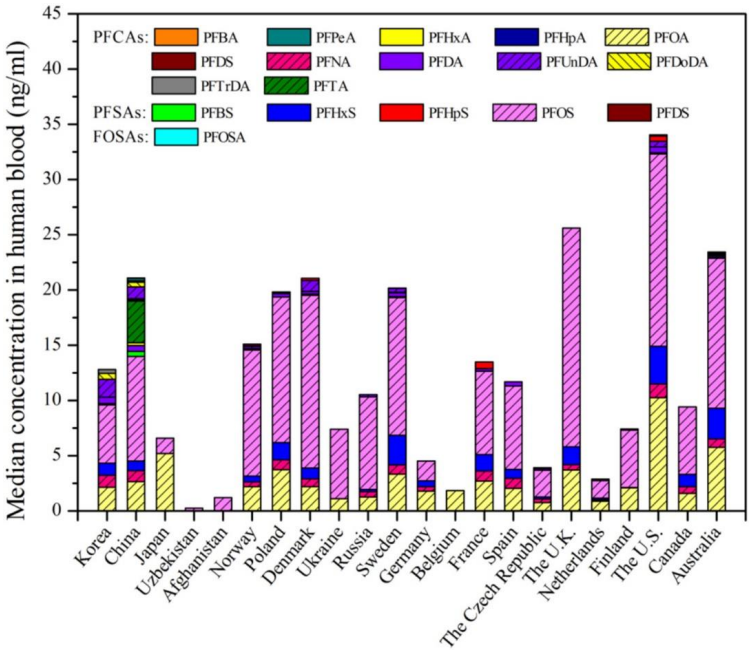

Figure 1. Median concentrations of PFASs in human blood from different countries (from Jian *et al.* (2018), with permission from Elsevier). **Perfluorinated carboxylic acids (PFCAs):** Pentafluorobenzoic acid (PFBA), Perfluoropentanoic acid (PFPeA), Perfluorohexanoic acid (PFHxA), Perfluoroheptanoic acid (PFHpA), Perfluorodecanesulfonate (PFDS), Perfluorodecanoic acid (PFDA), Perfluoroundecanoic acid (PFUnDA), Perfluorododecanoic acid (PFDoDA), Perfluorotridecanoic acid (PFTrDA), Perfluorotetradecanoic acid (PFTA), **Perfluoroalkane sulfonates (PFSAs):** Perfluorobutanesulfonic acid (PFBS), Perfluorohexanesulphonic acid (PFHxS), Perfluoroheptanesulfonic acid (PFHpS), Perfluorooctanesulfonamide (PFOSA).

1.3 Human and Environmental health

1.3.1 POPs as endocrine disruptors

Apart from their persistency in the environment and tissues, some POPs also have endocrine disrupting abilities. An EDC is defined as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” (EC 2019). As EDCs, DDT, *p,p’*-DDE, some compounds belonging to BFRs (PBDEs), PCBs and PFASs act as estrogens, antiestrogens, antiandrogens or disrupt the function of the thyroid hormones. Endocrine activity is mediated by binding on receptors such as the aryl hydrocarbon (Ahr), the peroxisome proliferator-activated receptors (PPAR) and steroid receptors. All hormone-sensitive physiological systems are vulnerable to EDCs (Figure 2) (Diamanti-Kandarakis *et al.* 2009, White *et al.* 2011, WHO/UNEP 2012, Lee and Choi 2017).

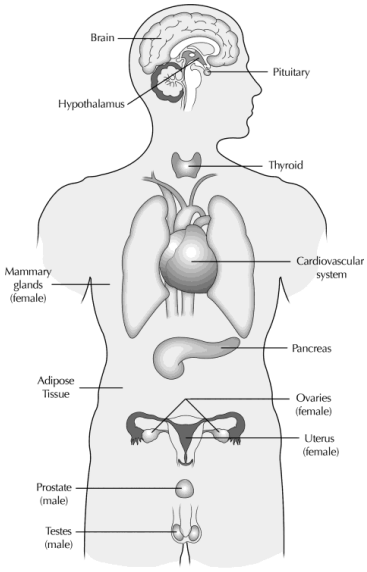

Figure 2. Hormone-sensitive biological systems in humans that are vulnerable to EDCs (from Diamanti-Kandarakis *et al.* (2009), with permission from Oxford University Press).

1.3.2 General human toxicity

Information about the mode of action of POPs, the estimation of tolerable levels of exposure and the implementation of regulation and guidelines for acceptable levels of POPs in water and foodstuff has mainly been established through *in vitro* and *in vivo* experiments in animal models such as primates, rats, mice and fish. The results from *in vivo* studies are extrapolated to humans based on the similarities between humans and laboratory animals in physiological processes and metabolism (Zeng *et al.* 2019). Subsequently, epidemiological studies of the general and occupationally exposed population to POPs have provided some associations between exposure and adverse effects on human health that could be expected based on animal studies.

Adverse effects of exposure to POPs show associations between exposure and the development of multiple types of cancer, such as thyroid, breast, and prostate cancer (Stahl *et al.* 2011, Ennour-Idrissi *et al.* 2019). Obesity and the risk of type II diabetes have also been associated with exposure to POPs in human and animal studies (Yang *et al.* 2017). Additionally, reproductive effects such as infertility, altered sex ratio, abnormalities of female and male reproductive tracts, and lower semen quantity and quality have also been observed (Damstra 2002, Li *et al.* 2006, Mumford *et al.* 2015). Most of the adverse effects of POPs can be attributed to their endocrine activity that leads to hormonal imbalance and dysfunction of physiological systems. For example, activation of PPARa can lead to expression of genes involved in lipid metabolism and energy homeostasis leading to possible metabolic disorders (White *et al.* 2011). Interference with testicular estrogen receptors causing lower testosterone levels, decreased proliferation and increased apoptosis of germ cells has been proposed as a mechanism inhibiting sperm production (Qu *et al.* 2016).

1.3.3 Neurobehavioral toxicity

Behavior represents the unique interaction between internal and external stimuli that determine an individual's health and survival (Little *et al.* 1990). Behavioral endpoints are generally more sensitive than physiological endpoints in assessing toxicity outcomes since effects can be observed at environmentally relevant levels. Studies employing behavioral tests are progressively increasing since the automation and availability of testing methods and equipment can provide faster results and can provide indication of the effects of real-life exposures.

Neurobehavioral effects after exposure to persistent organic pollutants in animal studies include changes in locomotor activity (such as hyperactivity), motor function, habituation, learning, memory and attention as well as attention deficit hyperactivity disorder (ADHD)-like symptoms (Johansson *et al.* 2008, Fonnum and Mariussen 2009, Yang *et al.* 2009, Onishchenko *et al.* 2011). Neurotoxic effects of POPs include oxidative stress in neurons, induced neuroinflammation, influence on the formation and plasticity of synapses, disturbance of calcium ion channel and homeostasis and altering of neurotransmitter levels (Figure 3) (Westerink 2014, Zeng *et al.* 2019).

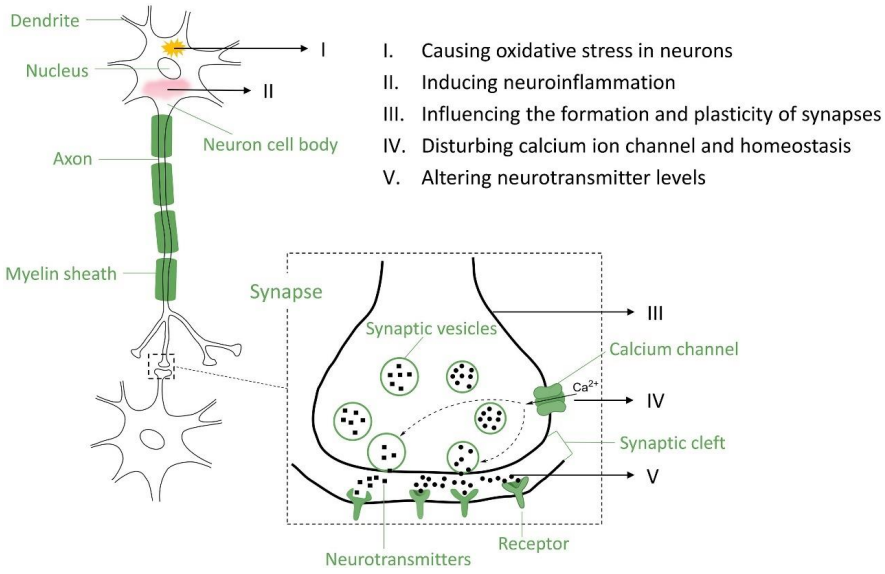

Figure 3. The main neurotoxic effects of POPs (from Zeng *et al.* (2019) with permission from Elsevier).

The neurotoxic effects of chemicals have also been recorded in epidemiological studies. For example, an association between brain levels of OCPs and the development of Parkinson's disease has been reported (Fleming *et al.* 1994, Le Couteur *et al.* 1999). Memory impairment have also been reported in older people eating large amounts of fish from the Great lakes showing high concentrations of PCBs (Schantz *et al.* 2001) and firefighters exposed to PCBs exhibited higher rates of depressive disorders compared to control groups (Kilburn *et al.* 1989)

1.3.4 Effects in wildlife

Many of the effects that have been described in humans and laboratory studies are also found in wildlife species. Among these the most common are reproductive effects. One of the earlier examples comes from alligators in Florida where low hatching

rates, abnormal gonadal morphology, altered gonadal steroidogenesis and sex steroid concentrations have been associated with exposure to *p,p*'-DDE (Guillette *et al.* 1994). In mammals, masculinization, reproductive impairment, reduced testosterone levels, decreased fecundity, sterility and subsequent decreases in population size have been observed. In birds the most common effects of POPs on reproduction include eggshell thinning, decreased hatching success, impairment of reproductive behavior that hinder reproductive success, feminization and the presence of both ovarian and testicular tissue in individuals, deformities and/or increased mortality in chicks (Vos *et al.* 2000, Fox 2001, Sonne *et al.* 2020 and references therein). In fish histopathological changes, reduced gonad size or intersex gonads, altered reproductive behavior, lower egg production and hatching rates have also been observed (Johnson *et al.* 2013 and references therein). Non-reproductive effects include poor overall survival, lower growth, developmental abnormalities, lower immunocompetence, thyroid hormone alterations and skeletal pathologies (Vos *et al.* 2000, Johnson *et al.* 2013, Sonne *et al.* 2020 and references therein).

1.3.5 The Stockholm convention

Due to the increasing evidence of the effects of POPs in human and environmental health the Stockholm Convention was adopted in 2001 and entered into force in 2004[2] with the intention to limit or restrict the use of certain POPs and protect human and environmental health. The Stockholm Convention is administered by the United Nations Environment Program[3]. For a chemical to be included in the Annexes of the Stockholm Convention they need to meet the criteria of persistency, long-range transport and toxicity. As of now, 31 POPs have been listed in the Stockholm Convention (Table 1).

[2] www.pops.int
[3] www.unep.org

Table 1. List of POPs included in the Annexes of the Stockholm Convention

The initial 12 POPs (2004) "Dirty dozen"	POPs included in 2009	POPs included from 2011-2019	Under consideration
Aldrin[1]	α-HCH[3]	Endosulfan[1]	PFHxS and PFHxS-related compounds[2]
Chlordane[1]	β-HCH[3]	HBCD[2]	Dechlorane Plus[2]
DDT[1]	Lindane (γ-HCH)[1]	HCBD[3]	Methoxychlor[1]
Dieldrin[1]	Chlordecone[1]	PCP[1,2]	
Endrin[1]	HBB[2]	PCN[2]	
Heptachlor[1]	Penta- and OctaBDE[2]	DecaBDE[2]	
HCB[1,2,3]	PeCB[1,2,3]	Short chain chlorinated paraffins[2]	
Mirex[1]	PFOS[2]	Dicofol[1]	
Toxaphene[1]	POSF[2]	PFOA and PFOA-related compounds[2]	
PCBs[2,3]			
PCDD[3]			
PCDF[3]			

[1] pesticides, [2] industrial chemicals, [3] by-products. Acronyms are listed in the Abbreviations.

Currently 184 countries have ratified the Stockholm Convention and have committed to implement national regulations to either eliminate (Annex A), restrict (Annex B) or reduce the unintentional release (Annex C) of chemicals listed in Table 1.

Even if a restriction or ban on the use of these chemicals has been implemented, the fact that these POPs can still be detected after having been banned for decades, often 20 to 30 years, in many countries, emphasizes just how persistent they are. A raising concern is how the levels of POPs are going to be affected due to global warming. Global increases in temperature for example might affect the re-release from soils and oceans as well as POPs that are “trapped” in glaciers. Changes in atmospheric circulation and ocean currents will affect the global re-distribution of POPs while extreme weather events such as floods might increase the release of POPs through soil erosion (Wang *et al.* 2016)

1.4 Risk assessment

1.4.1 Limitations to traditional risk assessment

In general, the products of risk assessment have been numerical risk values derived from animal toxicology studies of observable effects at high doses of individual chemicals. These risk assessment strategies rely on the classical dogma of toxicology that "*the dose makes the poison*" and rely on dose response effects. Maximum tolerable levels are estimated based on endpoints such as lethality or malformation prevalence. These studies have been usually applied on one life-stage or one generation, and solvents such DMSO, ethanol or methanol are frequently used to dissolve the predominantly lipophilic POPs. Although this approach has contributed to our understanding of overt health outcomes from chemical exposures, it does not always match consequences of real exposure scenarios, such as low-dose effects, non-linear responses, life stage effects, mixture effects and possible solvent artefacts. Additionally, they fail to assess transgenerational effects of chemical exposure (Gwinn *et al.* 2017).

1.4.2 Low-dose effects

Information of low-dose effects mainly has been provided from EDC studies that have challenged the principle of toxicology that "*the dose makes the poison*". The National Toxicology program of the U.S. Environmental Protection Agency, defined a low-dose effect as any biological change occurring at a concentration 1) that occurs in typical human exposures, 2) lower than those that are typically used in traditional toxicological assessments, 3) which is lower than doses previously reported as lowest observed adverse effect level (LOAEL) or 4) which when administered to an animal, produces a comparable internal concentration as to what is found in blood samples of the general human population (Brucker-Davis *et al.* 2001, Melnick *et al.* 2002, Welshons *et al.* 2006). Importantly, low-dose effects might be observed at an endpoint that was not targeted by

studies implementing higher exposure doses (Vandenberg *et al.* 2012). Doses of PCBs, DDT and BDE 99 that meet the criteria of low-dose concentration have been observed to affect brain sexual dimorphism in rats (Dickerson *et al.* 2011) , neurobehavior in mice (Palanza *et al.* 1999) and alter the thyroid hormone levels in rainbow trout and rats (Buckman *et al.* 2007, Kuriyama *et al.* 2007)

1.4.3 Nonmonotonic responses

Nonmonotonic responses challenge the traditional approach in toxicology, which assumes that a dose-response is monotonic. Responses in monotonic scenarios follow a linear or a nonlinear response curve, but the slope does not change sign, which justifies the use of higher doses in toxicology for evaluation of chemical safety. When this relationship between concentration and response is not followed, testing of higher doses cannot be used to assess the safety of low doses (Figure 4) (Vandenberg *et al.* 2012). Nonmonotonic response curves often have a U- or inverted U-shape also known as a biphasic-response curve because responses show an increase or a decrease in relation to concentration (Kohn and Melnick 2002). For example, gene expression might be downregulated relative to control in low-doses and upregulated in high doses or *vice versa* (Conolly and Lutz 2004, Molina *et al.* 2018). Nonmonotonic responses have been observed in mice after exposure to DDT regarding number of pups, sex ratio and neonatal body weight (Palanza *et al.* 2001) and in birds after exposure to PCBs in regards to corticosterone levels (Love *et al.* 2003, Franceschini *et al.* 2008). However even if nonmonotonic responses do occur, current exposure standards by regulatory agencies have been developed using an assumption of monotonicity (Lucier 1997, Fenner-Crisp 2000).

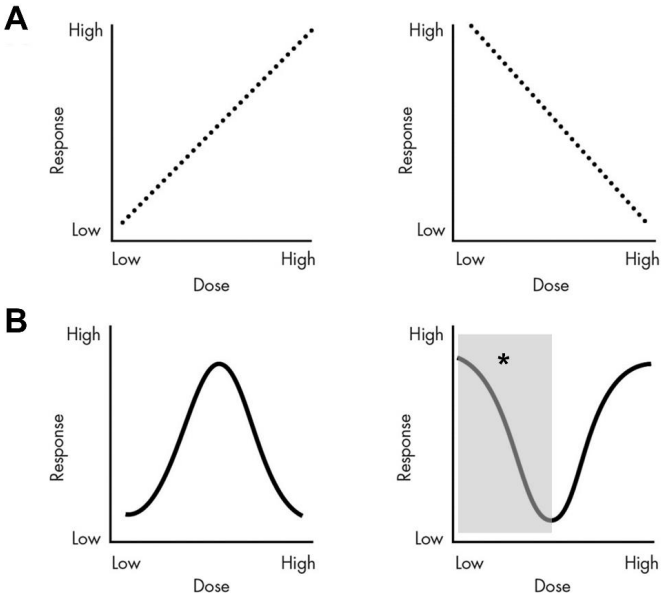

Figure 4. Examples of dose response curves. **A)** Linear responses with either a positive or a negative association between dose and effect, allow for extrapolations from one dose to another. Consequently, knowing the effect at high doses allows for the prediction of the effect at low doses. **B)** Non monotonic response curves, such as an inverted U-shape and a U-shape curve. It is clear from these curves that knowing the effect of dose or multiple doses, does not allow for assumptions to be made about the effects of other doses such e.g. low doses that might lead to a non-monotonic response (*) (adapted from Vandenberg *et al.* (2012), with permission from Oxford University Press).

1.4.4 Mixture effects

Chemicals are ubiquitous in the environment. Due to their application in various sectors, a vast number of different chemicals such as POPs, EDCs, metals, pesticides, surfactants and plasticizers are present in the environment and each environment has its own unique range and concentration of chemicals (Kortenkamp 2014, Mori and Todaka 2017, Thrupp *et al.* 2018). Analysis of human and animal tissues and serum have identified multiple chemicals present in an organism simultaneously. For example the

median number of chemicals detected for OCPs, PBDEs and PFASs, were 6, 6 and 4, respectively from blood and urine samples of pregnant women in US (Woodruff *et al.* 2011). Moreover 15 POPs belonging to OCPs, PFASs, PCBs and PBDEs have been detected in mother and children plasma/serum samples from six European cohorts (Haug *et al.* 2018). In bottlenose dolphins, 20 different PCBs, PBDEs and DDT and its metabolites were found in tissue samples such as blubber, liver and brain (Yordy *et al.* 2010). Although this has been acknowledged for many years, the field of toxicology is mainly dominated by single chemicals exposures.

Certain difficulties arise when trying to assess the effect of a mixture of chemicals. Due to the high numbers of different classes of chemicals present in the environment, the combinations of chemicals in mixtures and the levels an organism can be exposed to are innumerable. To address the issue of mixture effects one has to be realistic. By definition, a mixture is considered to be a combination of 2 or more chemicals. Ideally for each component of the mixture the toxicokinetics, metabolic pathways, mechanisms of action and levels of exposure need to be available. This will allow one to evaluate whether the mixture of chemicals in question have an interactive or non-interactive adverse effect on the exposed organism (IGHRC 2009). Non-interactive effects are described by a dose-addition relationship where the observed effect is the additive effect of all chemicals present in the mixture. Interaction can manifest either as synergism, where the observed effect is higher than what is expected, or antagonism where the observed effect is lower (Reffstrup *et al.* 2010, Kortenkamp 2014). The additive and synergistic effects are brought into perspective, considering their potency to produce adverse effects when chemicals in the mixture are present in low concentrations. These low concentrations on their own might not produce an effect, but when combined can lead to a phenomenon, first described *in vitro* by Silva *et al.* (2002), known as “something from nothing”. Additionally, these low concentrations can lead to a phenomenon termed “a lot from something” that best describes the interactive relations of chemicals and can lead to adverse effects greater than expected (Figure 5) (Thrupp *et al.* 2018).

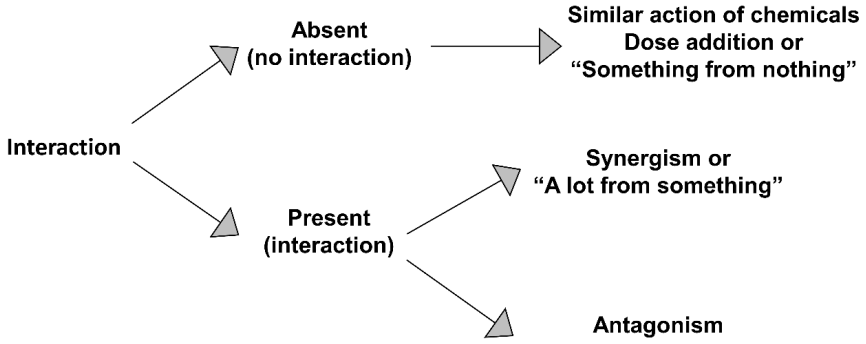

Figure 5. Overview of the possible relationships in a chemical mixture.

Currently, a consistent, common methodology and guidance for assessing risks of combined exposure to multiple chemicals across different regulatory agents is still lacking. Recently efforts are being made by funding agents in that they have launched multiple research projects that aim in addressing mixture knowledge gaps and develop methodologies, such as the development of models for exposure assessment and making available internal and external exposure levels of chemicals. These guidelines can be applied in epidemiological and toxicological approaches for mixture risk assessment and for prioritizing mixtures of concern (Bopp *et al.* 2018)

1.4.5 Solvent effects

Solvents are regularly used in toxicological risk assessment studies to facilitate the administration of lipophilic chemicals. Some of the most common solvents recruited in toxicological assays are DMSO, methanol and ethanol. Even if often overlooked, many solvents have their own effect. Ethanol exposure and locomotion activity for example displays a non-monotonic response where low concentrations (0.5-2%) produce hyperactivity of zebrafish larvae and higher concentrations (4%) induce hypoactive

behavior (de Esch *et al.* 2012). Hyperactive behavior was also recorded in a dose-response manner in zebrafish larvae exposed to DMSO (0.01, 0.1, and 1%) (Chen *et al.* 2011), while whole transcriptome changes have been observed in a concentration as low as 0.01% (Turner *et al.* 2012). DMSO in addition, has been shown to alter membrane permeability, which might affect the accumulation of chemicals in exposed organisms (Kais *et al.* 2013). Finally, all three solvents have been implicated in the development of malformations in a dose-dependent manner (Maes *et al.* 2012). The combination of solvents and chemicals, in binary or complex mixtures might lead to mixture effects. However, whether mixture effects between solvents and test compounds are manifested in toxicology studies remains unknown.

1.4.6 Developmental exposure effects

Another concern is exposures during pre- and post-natal life since POPs can pass the placental barrier and have been detected in human milk. Exposure during these sensitive life periods can have long-lasting effects on normal development and function of organs such as the central nervous system (Lam *et al.* 2017, Guo *et al.* 2019, Pessah *et al.* 2019). For example, maternal exposure to PFASs and exposure during nursing has led to developmental effects in offspring including elevated or decreased body weight depending on the species tested and the dose administered (Christian *et al.* 1999, Hines *et al.* 2009) and reduced viability of offspring (Lau *et al.* 2003). Prenatal and children serum levels of PFOS has also been associated with increased risk of ADHD and behavioral difficulties (Hoffman *et al.* 2010, Oulhote *et al.* 2016, Lenters *et al.* 2019). Additionally, an inverse association between maternal plasma PFOA levels and birth weight was observed in a study within the Danish National Birth Cohort (Fei *et al.* 2007). Epidemiological studies concerning PCBs and PBDEs have suggested an association between prenatal exposure and cognition endpoints such as poorer scores on overall intelligence and reading comprehension (Jacobson and Jacobson 1996, Forns *et al.* 2012, Lam *et al.* 2017). Additionally, a correlation was reported between exposure either

to PCBs, PBDEs or OCPs and ADHD-related traits including impulse control problems, hyperactivity and attention (Sagiv *et al.* 2010, Hoffman *et al.* 2012, Lenters *et al.* 2019).

During early development, organisms exhibit a high level of developmental plasticity in response to the surrounding environment leading to the manifestation of a broad range of adult phenotypes (Feuer *et al.* 2014). Changes in the genetic or epigenetic background are the basis on which these changes occur and are these changes that increase the risk of developing diseases in adulthood. It was in the 1980s that the Developmental Origins of Health and Disease (DOHaD) hypothesis was first formulated by Barker and colleagues after epidemiological studies which found an association between a reduction in fetal growth and the development of cardiovascular and metabolic diseases in later life (Barker and Osmond 1986, Barker *et al.* 1993). Since then this hypothesis has expanded to include early exposure to environmental contaminants and concerns on how this can elicit changes later in life (Barouki *et al.* 2012). For example, neonatal exposure to PFASs, PCBs and PBDEs has been observed to have persistent effects on the neurobehavior of adult mice causing changes in spontaneous behavior, habituation capability, learning and memory and overall activity (Eriksson and Fredriksson 1998, Viberg *et al.* 2003, Johansson *et al.* 2008). An epidemiological study revealed an increased risk of higher body mass index in 20-year-old females that were prenatally exposed to PFASs (Halldorsson *et al.* 2012).

1.4.7 Multi- and transgenerational effects

Multigenerational effects are the result of exposure of developing embryos (F1) to chemicals their parents were exposed to (F0). Chemicals can accumulate into the developing embryos and manifest as early or late-life effects. Additionally, since the germ line that will produce gametes for the F2 generation are present in the soma of developing embryos, multigenerational exposure can extend to the F2 generation. Transgenerational effects involve the manifestation of effects in generations that have not been directly

exposed to chemicals such as the F3 generation when maternal lineage is considered or the F2 generation when paternal lineage is considered (Figure 6) (Siklenka *et al.* 2015).

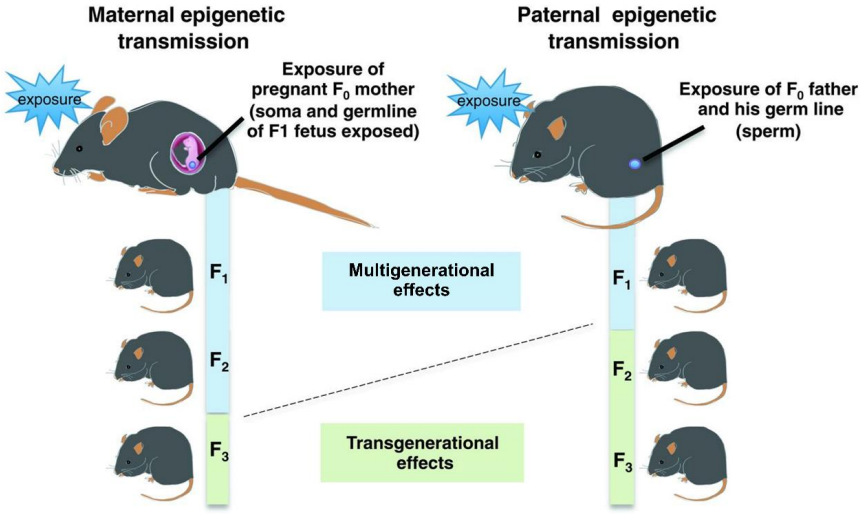

Figure 6. Multigenerational and transgenerational definitions in maternal and paternal epigenetic inheritance (modified from Siklenka *et al.* (2015) with permission from The American Association for the Advancement of Science).

During, fetal gonadal development, the germline epigenome undergoes reprogramming and, chemical exposures can induce germline epigenetic modifications during this DNA demethylation and remethylation period. These modifications can become permanently programmed and can be passed down to subsequent generations (Manikkam *et al.* 2012).

Multi- and transgenerational effects on body weight, gonadal weight, hormone levels and sex ratio have been observed in offspring of pregnant rats exposed to PCBs mixtures. In particular altered progesterone, estradiol and luteinizing hormone levels,

higher body weight, skewed sex ratio towards females, smaller uterine and ovarian weights were observed in F2 and F3 generation (Steinberg *et al.* 2008, Mennigen *et al.* 2018). Epidemiological studies addressing effects of chemicals across multiple generations are scarce but a study of the Seveso Italy population documented altered thyroid function in the children (F2 generation) of women that conceived as long as 25 years after a dioxin exposure accident in 1976 (Baccarelli *et al.* 2008).

1.5 The zebrafish as an experimental model

1.5.1 Practical model

Zebrafish possess many advantages making them a popular model in toxicological studies. Their small size means it is easier to maintain large populations with low cost. Also, their small size reduces the amount of chemical required for dosing solutions and this minimizes the waste disposal volumes. Since they have been used for many years, the optimum maintenance and breeding conditions are well established and their normal development has been characterized (Kimmel *et al.* 1995, Westerfield 1995). Each female can produce 200 – 300 per breeding providing a high number of individuals for toxicological screening. The embryos are transparent which facilitates screening for developmental endpoints of toxicity and non-feeding developmental stages of zebrafish are so far not protected in experimental legislation (Hill *et al.* 2005, Braunbeck *et al.* 2015). Zebrafish reach maturation at 3 – 4 months of age thus allowing easy experimentation for multi- and transgenerational studies evaluating chemical effects. The rapid maturation and availability of protocols also facilitate the establishment of transgenic lines (Hill *et al.* 2005)

1.5.2 Translational relevance

Zebrafish genome sequences and new genome assemblies are released regularly[4] which enables the characterization of toxicant exposure on the molecular level, and 70 % of the zebrafish genome is similar with humans thus making them a powerful tool for translational research (Hill *et al.* 2005, Howe *et al.* 2013). The availability of genomic and bioinformatic resources enables the investigation of mechanisms of action and the abundance of transgenic lines can be used to discover modes of toxicity in target tissues and organs (Hill *et al.* 2005).

Despite being a non-mammalian model, zebrafish brain development and structure share many similarities with that of mammals. Similar structures include the hippocampus, the diencephalon and the cerebellum which are comprised by the same cell types (Figure 7A) (Kozol *et al.* 2016). The development and specification of the central nervous system begins early at gastrulation, around 6 hpf (Woo and Fraser 1995). The major structures of the zebrafish brain develop by 10 hpf and by 24 hpf, the forebrain, midbrain and hindbrain are defined and can be easily distinguished. These embryonic structures will provide the foundation from where the adult brain structures will form. The forebrain will develop into the telencephalon, diencephalon, hypothalamus and retina, responsible for receiving and processing of information and directing behavior. From the midbrain the tectum and tegmentum are formed which are related to vision and hearing. Motor neurons originate from the hindbrain and innervate and control the movement of the eyes, jaw, head, and body (Vaz *et al.* 2019). The cerebellum is also formed from the hindbrain which is responsible among other aspects for motor control, receiving and processing sensory stimuli and learning (Rodríguez *et al.* 2005). Zebrafish neurons and neurotransmitters are classified into 5 subtypes: 1) glutamatergic neurons that express glutamate, 2) glycinergic neurons which have glycine as neurotransmitter, 3) GABAergic neurons expressing GABA, 4) cholinergic neurons expressing acetylcholine and 5) aminergic neurons expressing other modulatory molecules such as dopamine, serotonin,

[4] https://www.ncbi.nlm.nih.gov/grc/zebrafish

noradrenaline or histamine. All aforementioned neuron types are found both in zebrafish and mammals (Panula *et al.* 2010).

1.5.3 Omics technology

The addition of “omics” to a molecular term implies a comprehensive or global assessment of a set of molecules (http://omics.org/). These include among others, genomics, transcriptomics, metabolomics, proteomics and lipidomics. Omics can provide qualitative (which molecules are present) and quantitative (the level of expression of each molecule) information upon the effect of a chemical in question. For example, differential expression of genes across the whole transcriptome can be readily evaluated, uncovering target genes of chemical exposure and providing insight as to which biological pathways or processes are affected (Hasin *et al.* 2017).

The incorporation of omics in toxicology has largely been facilitated by technological advancements coupled with a cost reduction and improved bioinformatic resources. Using these techniques, there is now available information on the mechanistic action of certain chemicals and how these can lead to adverse effect on fish health. For instance, an impairment of gut and swim bladder development were observed in zebrafish upon exposure to 16 μM PFOS from 48 to 96 hpf. Transcriptome analysis suggested that the phenotypic impairment of gut development may be the causative factor for the perturbation of pathways involved in metabolic processes. Additionally, altered morphology of swim bladder was associated with a dysregulation in swim bladder-related genes (Chen *et al.* 2014). Another study employing transcriptome analysis after exposure of zebrafish embryos to PFOS (0.03, 0.3 and 1.0 mg/L) revealed transcriptome dysregulation in myosins, actin and tropomyosin which might be related to spine deformities observed at higher concentration. A significant increase in transcripts related to lipid transport and metabolism was associated with the disruption of yolk-sac absorption which was also supported by changes observed in the metabolism of glycerophospholipids using a metabolomic analysis (Ortiz-Villanueva *et al.* 2018,

Martínez *et al.* 2019). An early life exposure (2-24 hpf) of zebrafish embryos to PCB 126 caused a dysregulation of genes in the adult brain involved in calcium signaling. Altered calcium homeostasis can affect neurobehavior and be responsible for the observed behavioral alterations in adult zebrafish in a related study (Glazer *et al.* 2016, Aluru *et al.* 2017). Zebrafish adults exposed through feed to a complex mixture of POPs for 5 months had a dysregulation of genes involved in weight homeostasis and insulin signaling which might be the causative factor for the increase body weight at 5 months of age (Lyche *et al.* 2011).

1.5.4 Toxicity assessment

The fish embryo acute toxicity (FET) test is used to determine acute toxicity of chemicals and it is a complementary test along with risk assessment tests in rodents, to establish tolerable levels of chemicals (OECD 2013, Nishimura *et al.* 2016). Acute exposure to POPs during embryo and larval stages has led to reduced hatching, mortality, abnormal development and malformation of larvae. Some of the malformations observed include pericardial or yolk sac edema, heart and spinal aberrations, loss of equilibrium, non-inflated swim bladder, craniofacial and jaw deformities and smaller than normal eyes. Additionally, delay of larval growth has also been observed (Lema *et al.* 2007, Foekema *et al.* 2008, Grimes *et al.* 2008, Huang *et al.* 2010, McClain *et al.* 2012, Rigaud *et al.* 2013, Dach *et al.* 2018, Parsons *et al.* 2019). Acute toxicity studies as the ones mentioned above can contribute to the knowledge of exposure tolerable levels for human risk assessment but also provide an indication of affected organs and tissues that can be investigated further as targets of chemical toxicity.

The endocrine disrupting effects of POPs have been a main focus in studies investigating endpoints of reproduction as well as multigenerational and transgenerational effects in zebrafish. For example, chronic dietary exposure to TCDD caused a decrease of the ovosomatic index in female zebrafish and a decreased egg production and spawning success which was associated with histopathological changes

and decreased level of estrogens (King-Heiden *et al.* 2012). Another study also revealed that TCDD exposure in zebrafish caused a skewed sex ratio of the exposed individuals and a reduction of egg release and fertilization of two subsequent generations (Baker *et al.* 2014a, Baker *et al.* 2014b). A five-month exposure of zebrafish to PFOS produced a female dominant sex ratio, impaired male gonad development in a dose dependent manner and severe deformities of F1 offspring that led to 100% mortality due to the maternal transfer of PFOS to the eggs (Wang *et al.* 2011). These studies illustrate the usefulness of zebrafish as a model to study the endocrine effects of POPs on reproductive output since similar effects have been observed in other animal studies.

1.5.5 Neurotoxicity assessment

Chemical pollutants exert neurotoxic effects through the central nervous system, and their effects can be easily studied using behavioral assays. The zebrafish has risen recently as a powerful model for behavioral research of developmental exposure to chemicals and serves as a complementary model to mammals, helping to provide causal links between chemical exposure and human neurodevelopmental disorders such as autism-spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD)(Vaz *et al.* 2019). Zebrafish exhibit a set of behaviors such as fear/anxiety, social behavior and aggression that can be examined by different established behavioral assays. Behavioral responses are similar between zebrafish and mammalian models when they are subjected to known neuroactive drugs supporting the use of zebrafish as a behavioral model in neurotoxicity studies (Figure 7 B-H) (Champagne *et al.* 2010).

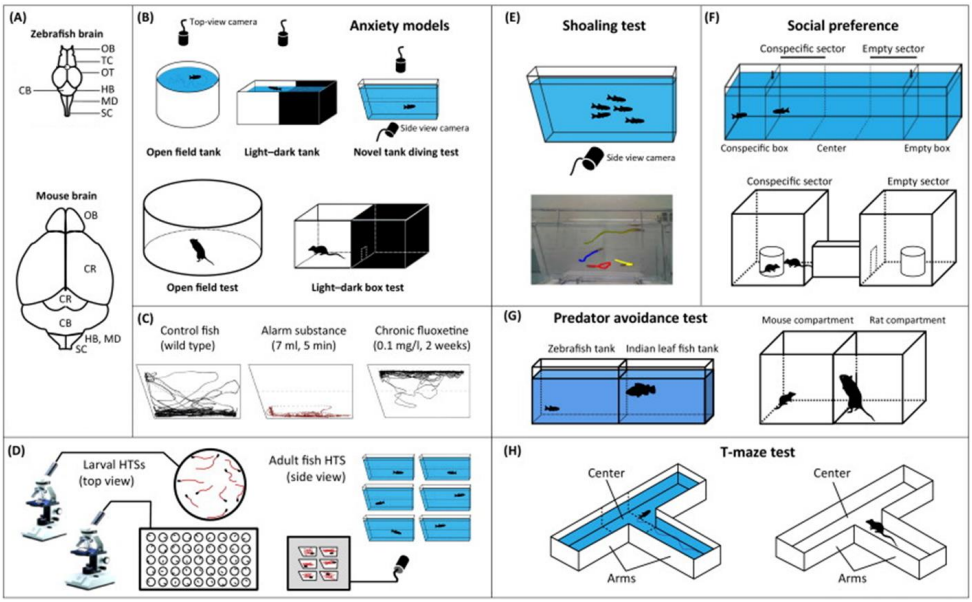

Figure 7. Comparison of zebrafish and mouse experimental models. Panel **A)** Similarities of zebrafish and mouse brain morphology. **OB**, olfactory bulbs; **TC**, telencephalon; **OT**, optic tectum; **CB**, cerebellum; **HB**, hind brain; **MD**, medulla; **SC**, spinal cord; **CR**, cortex. **B)** Parallels of zebrafish neurobehavioral tests of exploration, anxiety and locomotion with those traditionally used in rodents, combined with automated video-tracking systems. **C)** typical anxiety-like behaviors observed in zebrafish in the novel tank test (including anxiety evoked by alarm substance acute 5-min exposure and reduced anxiety produced by a chronic 2-week fluoxetine anxiolytic treatment), an aquatic paradigm of the rodent open field test. **D)** Principles of high-throughput screens (HTSs) using larval and adult zebrafish. **E)** Typical set up for a shoaling test and tracking using behavioral software system (Noldus IT, The Netherlands). **F)** Aquatic social preference test (top) and rodent equivalent (bottom). **G)** Zebrafish predator avoidance test and similarity with the rat exposure mouse test. **H)** Parallels of aquatic and rodent cognitive tests, such as the T-maze test (adapted from Kalueff *et al.* (2014) with permission from Elsevier)

Behavioral tests in larvae can start as early as 17 hpf. Muscle activity in the form of spontaneous coiling reaction is evident and this coiling is thought to help the release of the embryo from the chorion. From 2 days post fertilization (dpf) the larvae can be subjected to a touch response test and from this point on the larvae are able to respond

to external stimuli and control movement. Larval tests that can be used from this stage to assess effects of chemical exposure to anxiety and stress-related behavior include the startle response test (for example to a vibrating stimulus) and the light dark transition test, where alternate periods of light and dark are used to cause a response in zebrafish behavior. During the light dark transition test variables such as swimming speed, distance moved and time spent on the outer walls (thigmotaxis) are measured. An increase in any of these variables is usually considered a sign of anxiety (Saint-Amant and Drapeau 1998, Legradi *et al.* 2015).

Adult zebrafish present a more complex repertoire of behaviors compared to larvae. Tests that are used to assess behavioral defects after chemical exposure include the t-maze test usually with food as a reward, a test used to evaluate learning (Bailey *et al.* 2015) and the novel tank test, open field test or light-dark box tests to assess fear and anxiety related behaviors (Cachat *et al.* 2010, Steenbergen *et al.* 2011). Furthermore, social interactions can be tested in adult zebrafish using the shoaling test or social preference test, a test that assess the preference and interaction of fish that are in the same tank and are separated by a transparent barrier and the mirror test that is usually used to assess aggression (Figure 7 B-H) (Miller and Gerlai 2012).

Due to the availability of a large number of aquatic behavioral tests and the comparability they show with rodent tests, zebrafish has been used extensively to uncover the neurobehavioral effects of POPs. The responses to chemical exposure generally show agreement with what has been observed in mammalian studies (Bailey *et al.* 2013). For example, exposure of larval zebrafish to PFASs has been shown to cause hyperactivity (PFOS and PFOA) or hypoactivity (PFNA). Cell death in tail, eye and brain was suggested to contribute to the observed behavioral disruptions (Huang *et al.* 2010, Spulber *et al.* 2014, Jantzen *et al.* 2016a). Additionally, adult behavioral effects of early life exposure to PFASs has been observed including reduced activity, increased aggression in individuals exposed as larvae to PFNA and reduced aggression in individuals exposed as larvae to PFOS (1 mg/L) (Jantzen *et al.* 2016b). Furthermore in a multigenerational study, F1 offspring of parents exposed chronically to PFOS from 1 – 20

or 21 – 120 dpf exhibited higher swimming speed than control larvae (Chen *et al.* 2013). Exposure to PBDEs caused a decrease in touch response and free-swimming speed in a dose dependent manner in larval zebrafish of different ages. The motor deficits were suggested to be associated with observed inhibition of axonal growth of primary and secondary motor neurons and cardiac abnormalities (Chen *et al.* 2012, McClain *et al.* 2012). Long-term effects in adults exposed early in life to PBDEs included a reduction in anxiety-like behaviors using the novel tank and tap test (Glazer *et al.* 2018). Lastly, a parental exposure to PBDEs caused a slower motor neuron development, loose muscle fiber, slower locomotion behavior under normal conditions and hyperactivity under a light-dark stimulation test of their offspring (He *et al.* 2011).

1.5.6 Current status of POP mixtures in zebrafish

In recent years, more studies have started to focus on the use of realistic mixtures of POPs in zebrafish studies. Some of these studies employ long-term dietary exposures and other studies use the water exposure routes either short or long-term. Dietary studies to POPs such as the one by Alfonso *et al.* (2019) tested the effects on the behavior of the adults of F0 generation and 4 offspring generations. No effects were observed in the behavior of F0, F1 and F3 adults but F2 adults displayed anxiety-like behavior. Larvae of F1 generation showed hyperactivity during a light-dark transition test whereas F2, F3 and F4 larvae displayed hypoactivity. Chronic exposure to mixtures of PCBs either orally or in the water led to reduced ovary weight, egg production and egg fertilization (Örn *et al.* 1998, Daouk *et al.* 2011). Long-term exposure of zebrafish from post-hatching to adulthood to a POP mixture containing PCBs, PBDEs, DDTs and other OCPs caused a male skewed sex ratio, advanced sexual maturation and reduced egg production and survival of offspring originating from exposed adults (Nourizadeh-Lillabadi *et al.* 2009, Lyche *et al.* 2013). Another study, testing the interactions between chemicals exposed male zebrafish to either a PCB mixture (Aroclor 1254) or DDT and their binary mixture. A decrease in sperm count and activity was recorded after 1 month of exposure to either the PCBs or DDT and after 2 weeks to their binary mixture suggesting that these two

classes of chemicals might act synergistically impairing male reproductive output (Njiwa *et al.* 2004). Finally, a recent study tested the effect of 9 different PFASs and their mixture. The authors observed diverse responses in locomotion and burst activity during a light-dark transition test depending on the compound tested, while the combination of the compounds in the mixture seemed to decrease the potencies of individual PFASs (Menger *et al.* 2020).

In this thesis, a mixture of POPs containing PFASs, PCBs, BFRs and OCPs was used. This mixture was designed and produced in the Norwegian University of Life Sciences and it is based on the average levels of chemicals found in human blood of the general Scandinavian population (Berntsen *et al.* 2017). Previous work with this mixture focused on the acute effects after a 90h exposure in larval zebrafish (6 – 96 hpf). An increase in the locomotor activity in terms of swimming speed was observed, following a light-dark transition test. The behavioral effects were related to PFOS incorporated in the mixture. However, a disagreement in the expression of neurodevelopment and behavior related genes was observed between the POP mixture and the single PFOS exposure (Khezri *et al.* 2017).

2. Aims of the study

From the introduction it is clear that some knowledge gaps remain in toxicological research. These mainly concern the lack of report on whether methodological parameters can interact with chemicals affecting the outcome of toxicological assays and whether developmental exposure to chemicals can produce adverse effects early or later in life under the DOHaD hypothesis. Therefore, this thesis aims to investigate the effects of anthropogenic chemicals such as solvents and/or pollutants on behavior and physiology in zebrafish. Our specific goals were to:

- Identify whether commonly used solvents for toxicity testing, such as DMSO and methanol or substances that control fungal pathogens such as methylene blue (MB) can influence behavioral outcomes of different zebrafish strains. (**Paper I**)
- Investigate possible underlying mechanisms of a POP mixture or single PFOS exposure on the transcriptome level, responsible for the observed behavioral toxicity previously reported by Khezri *et al.* (2017) (**Paper II**)
- Determine whether short-term exposure of larval zebrafish can have latent effects on adult zebrafish health supporting the DOHaD hypothesis (**Paper III**)

3. Materials and Methods

In this section an overview of some of the methods used in this thesis will be presented. Details about the protocols are given in the corresponding papers.

3.1 Experimental setup

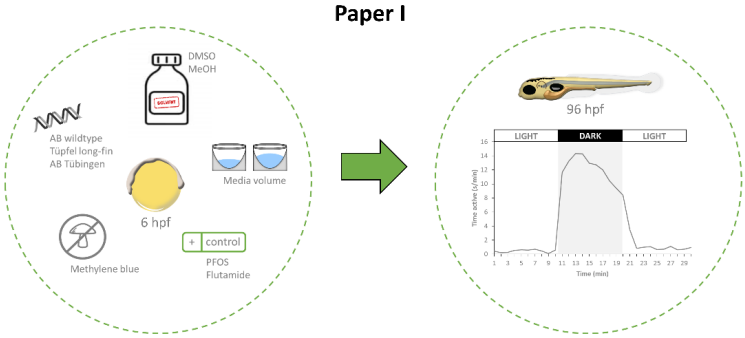

Figure 8. Overview of experimental setup of parameters tested in Paper I. Figure adapted from Christou *et al.* (2020).

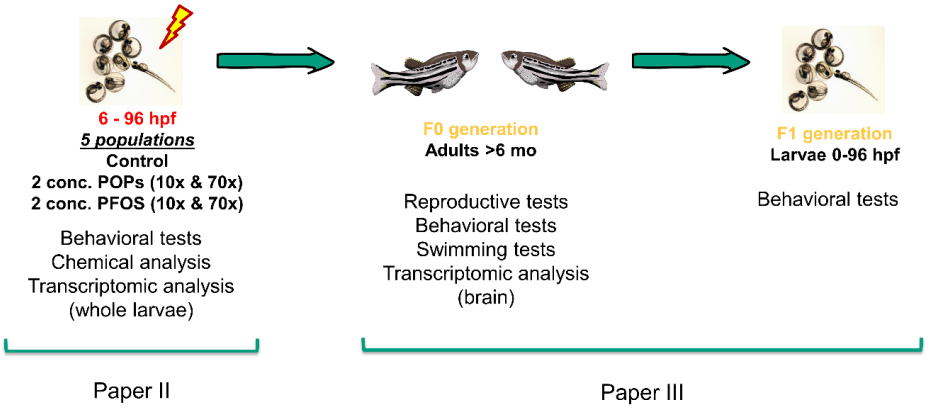

Figure 9. Overview of experimental design and tests performed in larvae and adult zebrafish in Paper II & III.

The studies were carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway (application ID: FOTS 13094).

3.2 The POP mixture

The mixture used in Paper II & III was developed at NMBU based on studies reporting mean values of different compounds/congeners in human blood originating form Scandinavian populations. It consists of 29 chemicals belonging to PCBs, BFRs, PFASs, OCPs and their metabolites (Berntsen *et al.* 2017). The composition and concentration of compounds in the stock mixture is presented in Table 2.

3.3 Exposures

Zebrafish embryos were exposed to either x10 or x70 the human blood concentration of the POP mixture (POP10 and POP70) or the equivalent concentration of PFOS only (0.55 and 3.83 µM, PFOS10 and PFOS70) in 0.1% DMSO from 6 to 96 hpf. The controls were exposed to 0.1% DMSO only. The concentrations were chosen based on previous studies performed in our group where the lower concentration with no observable behavioral effects was equal to x10 and the lower concentration with an observed behavioral effect was equal to x70 (Khezri *et al.* 2017). Following these exposures, the larvae were transferred to clean water and reared in tanks until adulthood under standard conditions (Paper III).

Table 2. The composition and concentration of the stock POP mixture used in the present study.

Chemicals	Nominal concentration of stock solution (µM)	Nominal concentration of stock solution (mg/mL)
	1 000 000x (stock mixture)	1 000 000x (stock mixture)
PFASs		
PFOA	10923	4.523
PFOS	54801	29.425
PFDA	962	0.495
PFNA	1723	0.800
PFHxS	7873	3.450
PFUnDA	990	0.560
BFRs		
BDE-47	18	0.009
BDE-99	7	0.004
BDE-100	3	0.003
BDE-153	1	0.001
BDE-154	3	0.002
BDE-209	11	0.011
HBCD	38	0.025
PCBs		
PCB 28	50	0.013
PCB 52	34	0.010
PCB 101	24	0.008
PCB 118	196	0.064
PCB 138	615	0.222
PCB 153	1003	0.362
PCB 180	490	0.194
Other organochlorines		
p,p'-DDE	1578	0.502
HCB	410	0.117
α-chlordane	26	0.011
Oxy-chlordane	51	0.022
Trans-nonachlor	92	0.041
α-HCH	20	0.006
β-HCH	182	0.053
γ-HCH (lindane)	20	0.006
Dieldrin	63	0.024

3.4 Behavioral assays

3.4.1 Larval behavior

We carried out two larval behavior tests, the light-dark transition test in 96 well plates (Nunc™ MicroWell™) and the thigmotaxis test, also known as “wall hugging”, in 24 well plates on 96 hpf larvae zebrafish. Behavioral tests were conducted using a ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France) (Figure 10). During both tests the larvae are submitted to alternate periods of light and dark periods. Zebrafish larvae usually show a freezing behavior during light periods followed by a high activity during the dark periods. For the light dark-transition assay different activities can be measured such as distance moved, time the larvae are active and the swimming speed. A hyperactive behavior is usually a sign of anxiety (MacPhail *et al.* 2009). For the thigmotaxis assay, the arena (well) is split into two zones, a center zone and an outer zone, to assess thigmotaxis. Work has demonstrated that larvae treated with anxiolytic compounds are more active in the inner zone, whereas anxiogenic compounds increase the amount of movement in the outer zone (Richendrfer *et al.* 2012, Schnörr *et al.* 2012).

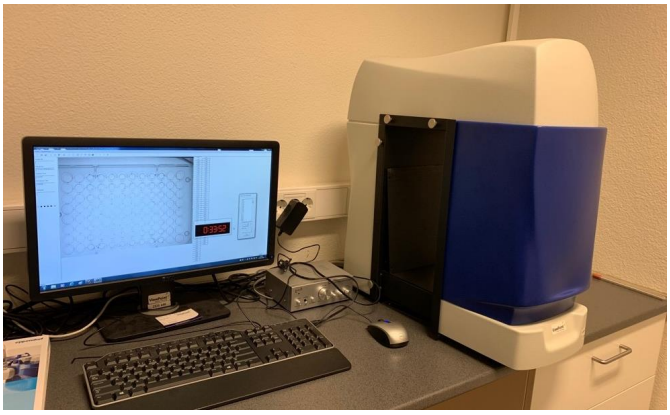

Figure 10. Behavioral apparatus used in behavioral assay of zebrafish larvae.

3.4.2 Adult behavior

Following several months rearing in clean water, adult zebrafish were assessed for behavioral responses in the novel tank diving test using the Ethovision XT13 software. Here, fish are introduced to a novel tank and their swimming pattern is recorded for 5 minutes. The tank is divided into two zones, the top and bottom zones. Initially when fish are introduced in the novel tank, they tend to spend more time in the bottom zone while as the test progresses, they tend to start exploring and entering the top zone of the tank. Variables than can be measured include time spent in each zone, the number of transitions between zones and the latency to enter for the first time the top zone. The amount of time spent in the top and bottom zones can be used to assess an individual's anxiety (Cachat *et al.* 2010).

3.5 Chemical analysis

Larval zebrafish exposed to either POP70 or PFOS70 were sampled at 96 hpf to estimate the accumulation of chemicals in their tissues. The exposure media was also collected for chemical analysis. Different methods were used for the quantification of different groups of chemicals using internal controls.

3.6 Swimming test

Swimming tests were performed in a swim tunnel (Loligo® Systems) using a U_{crit} (critical swimming speed) protocol in adult male zebrafish (Figure 11). A U_{crit} test consist of incremental changes in water velocity until exhaustion of the fish. The water velocity where the fish fatigues is considered the sustained U_{crit} (Brett 1964).

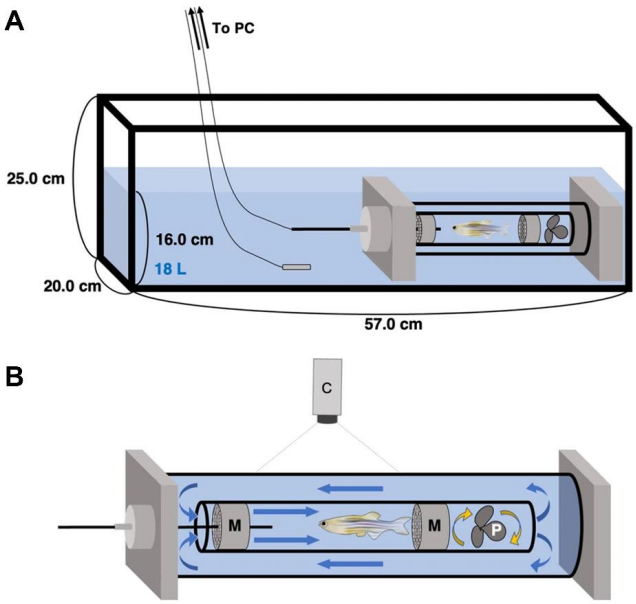

Figure 11. Overview of the swimming test. **A)** Whole view of the swim tunnel equipment used in this study. **B)** Enlarged view of the swimming chamber. Zebrafish swim in the water flow to stay at the same position. Two plastic meshes were put in the small cylinder to avoid the fatigued fish hitting the spinning propeller and to evenly distribute the water flow. **M**: mesh; **P**: propeller; **C**: high speed camera; **Blue arrows**: water flow (modified from Wakamatsu *et al.* (2019).

3.7 Reproductive tests

The reproductive outcome of adult zebrafish, exposed as larvae to either the POP mixture or PFOS, in terms of egg production, mortality rate and hatching rate was investigated. The experimental protocol was based on a paper published by Uusi-Heikkila *et al.* (2010). Fish from exposed and control populations were placed in breeding tanks for 5 days with a ratio of 1 female to 2 males per tank. Seven breeding pairs were set for each condition per replicate. Each morning, eggs were collected from the tanks. The number of eggs was estimated and 100 fertilized eggs from each condition were chosen

for the examination of mortality at 24 hpf and hatching rates at 72 hpf. The procedure was repeated for each of the five days. At the end the reproductive experiment, fish were euthanized and weighted both for total and gonadal weight. The gonadosomatic index (GSI = [gonad weight / total tissue weight] × 100) was calculated for the male and female zebrafish.

3.8 Transcriptomic analyses

Transcriptomic analysis was performed from samples of whole larvae exposed to either the POP mixture or PFOS both at the low and high concentration and a control population. A second transcriptomic analysis was performed in adult zebrafish brain samples from all 5 conditions (Control, POP10, POP70, PFOS10, PFOS70). Transcriptomic analysis was carried out using RNA sequencing technology followed by bioinformatics analysis and quantitative real-time polymerase chain reaction (qPCR) for gene validation of differentially expressed genes (DEGs).

3.8.1 RNA sequencing

RNA sequencing (RNA-seq) is a technique that can detect and quantify gene transcripts in biological samples. The method allows the quantification of virtually all expressed mRNAs given the sequencing read depth is high enough. Good quality RNA is essential for a successful analysis, therefore in our studies we used Agilent 2100 Bioanalyzer (Agilent Technologies, Ca, USA) RNA Nano LabChip Kit (Agilent Technologies, Ca, USA) to measure the quality and integrity of RNA prior to sending the samples for RNA-seq at Novogene (Hong Kong). At Novogene, libraries were made of the RNA using the Tru seq protocol, using poly A purification enabling the analysis of solely mature mRNAs. RNA-seq was performed on the Illumina Hiseq 4000 platform with 20 million reads/sample,150 bp paired end reads.

3.8.2 Bioinformatics analysis

After obtaining the short reads from RNA-seq, bioinformatics analysis was performed consisting of trimming the adaptors attached on each read using trim_galore under standard parameters. Using STAR aligner, the reads were aligned to the zebrafish reference genome. For every read that STAR aligns, STAR will search for the longest sequence that exactly matches one or more locations on the reference genome. These longest matching sequences are called the Maximal Mappable Prefixes (MMPs). The different parts of the read that are mapped separately are called 'seeds'. The first MMP that is mapped to the genome is called *seed1*. STAR will then search again for only the unmapped portion of the read to find the next longest sequence that exactly matches the reference genome, or the next MMP, which will be *seed2* and proceeds until a read is uniquely aligned to a single site of the reference genome (Dobin *et al.* 2012). Quantification of differential expression was performed in SeqMonk using the Deseq2 filter, which is based on the negative binomial distribution, and uses Benjamini Hochberg false discovery rate (FDR), to correct for multiple comparisons (Love *et al.* 2014). Data were normalized by reads per million and the fold change was calculated to determine DEGs. DEGs were then imported to pathway analysis software for the investigation of possible mechanisms of action of chemical exposure. An overview of the bioinformatics methods and other tools used in this study is presented in Figure 12.

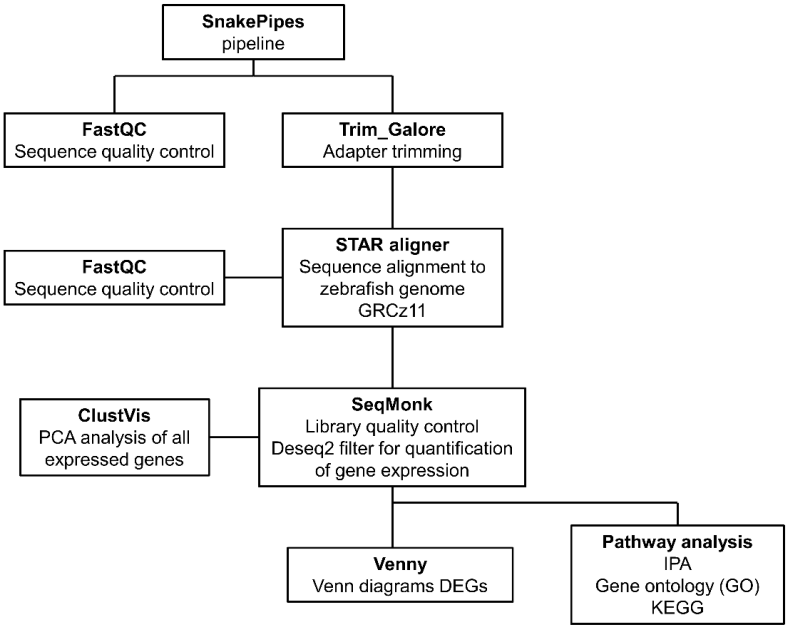

Figure 12. Overview of bioinformatics analysis process performed in this study. **IPA** Ingenuity pathway analysis, **KEGG** Kyoto Encyclopedia of Genes and Genomes

3.8.3 qPCR for gene validation

qPCR is a methodology that allows the quantification of specific DNA molecules and can be used to quantify the expression of targeted mRNA fragments derived from the tissue of interest (Bustin 2002). For this purpose, total RNA is extracted from a biological sample and after genomic DNA removal, cDNA is synthesized from the isolated RNA using reverse transcription. Primer pairs are designed to specifically target the genes of interest, and preferably span exon-exon junction, to avoid the amplification of any remaining genomic contaminants. These primers bind to a targeted gene's cDNA and create multiple copies through consecutive amplification cycles. These copies are labelled with a fluorescent signal, and the intensity of fluorescent is measured after each

cycle. The measure of fluorescent is then translated into quantitative gene expression value by comparing the signal to a standard curve. Gene expression values are normalized based on the expression of reference genes. Primer validation and specificity is essential to avoid primer dimerization and that only target genes are amplified (Bustin and Huggett 2017).

4. Results: Summary of papers

4.1 Paper I - DMSO effects larval zebrafish (*Danio rerio*) behavior, with additive and interaction effects when combined with positive controls.

Alterations in larval locomotor activity of zebrafish are commonly used to identify neurotoxic compounds. In this paper we investigated whether different concentrations of common solvents dimethyl sulfoxide (DMSO, 0.01-1%) and methanol (0.01-1%), or the fungicide agent MB (MB, 0.0001 – 0.0005%), can influence larval behavior in a light/dark paradigm conducted in 96-well plates. In addition, we tested whether the media volume within the behavioral arena or the zebrafish strain, AB wild type, AB Tübingen (AB/TU), or Tüpfel long-fin (TL), could also influence larval behavior. Furthermore, we tested whether DMSO and MB in different concentration have a potency to interact with other compounds with known behavioral effects in larval zebrafish, flutamide and PFOS. Our results revealed that concentrations of DMSO and MB equal or exceeding 0.55% and 0.0005% respectively significantly affect the basal locomotion activity of zebrafish whereas methanol did not produce a significant effect in all concentrations tested. The TL strain showed less movement compared to the other 2 strains tested and there was a positive correlation between media volume and larval movement, but all strains showed the same response to DMSO and MB. In the co-exposure studies, we found interaction effects between DMSO and either PFOS or MB, but no interaction was observed after exposure to flutamide. In addition, media volume had no effect on the DMSO concentration response curve, but additive effects between DMSO and media volume were observed on behavior. In conclusion, methodology can lead to alterations in baseline locomotor activity and compounds can have additive or interaction effects on behavioral endpoints. However, we found no evidence that strain effects should be a concern when deciding on solvents for a light/dark behavioral test in larval zebrafish.

4.2 Paper II - Calcium signaling as a possible mechanism behind increased locomotor response in zebrafish larvae exposed to a human relevant persistent organic pollutant mixture or PFOS.

Previously, using zebrafish as a model vertebrate, our scientific group found that larvae exposed to a mixture of 29 POPs based on average blood levels from the Scandinavian population showed hyperactivity, and identified PFOS as the driving agent for the behavioral changes. In this publication, we exposed zebrafish larvae from 6 to 96 hpf to the same mixture of POPs in two concentrations or a single PFOS exposure (0.55 and 3.83 μM) and performed behavioral tests and transcriptomics analysis in order to identify possible modes of action. Behavioral alterations of exposed zebrafish larvae included hyperactivity and confirmed previously reported results. Transcriptomics analysis revealed an upregulation of transcripts related to muscle contraction which is highly regulated by the availability of calcium in the sarcoplasmic reticulum. Ingenuity pathway analysis showed that one of the affected pathways in larvae exposed to the POP mixture and PFOS was calcium signaling via the activation of the ryanodine receptors (RyR). Functional analyses with RyR inhibitors and behavioral outcomes substantiate these findings. Additional pathways affected were related to lipid metabolism in larvae exposed to the lower concentration of PFOS. By using omics technology, we propose that the altered behavioral pattern in exposed zebrafish larvae may be controlled directly by mechanisms affecting muscle function rather than via mechanisms connected to neurotoxicity.

4.3 Paper III - Developmental exposure to a POPs mixture or PFOS increased body weight and reduced swimming ability but had no effect on reproduction or behavior in zebrafish adults

Complex mixtures of persistent organic pollutants (POPs) are regularly detected in the environment and animal tissues. Often these chemicals are associated with latent effects following early-life exposures, following the developmental origin of health and disease paradigm. We investigated the long-term effects of a human relevant mixture of 29 POPs on adult zebrafish following a developmental exposure, in addition to a single PFOS exposure for comparison, as it was the compound with the highest concentration within the mixture. Zebrafish embryos were exposed from 6 to 96 hours post fertilization to x10 and x70 the level of POP mixture or PFOS found in human blood before being transferred to clean water. We measured growth, swimming performance, and reproductive output at different life stages. In addition, we assessed anxiety behavior of the adults and their offspring, as well as performing a transcriptomic analysis on the adult zebrafish brain, as the POP mixture and PFOS concentrations used are known to affect larval behavior. Exposure to POP mixture and PFOS reduced swimming performance and increased length and weight, compared to controls. No effect of developmental exposure was observed on reproductive output or anxiety behavior. Additionally, RNA-seq did not reveal pathways related to anxiety although pathways related to synapse biology were affected at the x10 PFOS level. Furthermore, pathway analysis of the brain transcriptome of adults exposed as larvae to the low concentration of PFOS revealed enrichment in pathways such as calcium, MAPK, and GABA signaling, all of which are important for learning and memory. Based on our results we can conclude that some mild effects on the endpoints measured were apparent, but if these effects lead to adversities at population levels remains elusive.

5. General discussion

5.1 Methodological considerations

5.1.1 Behavioral experimental considerations and choice of behavioral tests

The intention of Paper I was to investigate whether the common solvents DMSO and methanol, used in toxicological studies, can influence the locomotor activity of zebrafish larvae using the light/dark transition assay. Additionally, MB was included in the study because it is commonly used to suppress fungal infection in zebrafish embryos. Zebrafish larvae from 3 different strains, the AB wild type, AB Tübingen (AB/TU), or Tüpfel long-fin (TL) were examined in 96 well-plates. The choice of 96 well-plates was based on the fact that more concentrations can be tested simultaneously and the test can be performed at specific time points since the time of day has been shown to affect basal locomotion activity (MacPhail *et al.* 2009). There was no effect of methanol at any concentration tested and all strains responded similarly to DMSO and MB exhibiting an increase in swimming speed. Concentrations with observable effects were 0.55% and 0.0005% for DMSO and MB respectively. We concluded that the choice of strains should not be a concern when deciding which solvents to use in a simple light-dark paradigm. Consequently, we chose the AB strain to test for interaction effects between solvents or MB and the positive controls PFOS and flutamide. These two controls were chosen based on consistent effects (hyperactivity) reported in previous studies (Fraser *et al.* 2017, Khezri *et al.* 2017). We observed an interaction effect between DMSO (1%) and PFOS (0 – 4 µM) as well as between MB (0.0005%) and DMSO (0 – 1%).

Based on these observations and to avoid any confounding effects, in Paper II and III zebrafish embryos belonging to AB wild type were used. The embryos were not treated with MB and a concentration of DMSO equal to 0.1% was used as solvent for the chemicals tested since it was proven not to interact PFOS nor MB, with the exception of

one experiment in Paper II where, due to low solubility of the tested substance, 0.68% DMSO was used. In this experiment the behavioral endpoints were compared to a relevant control population that was treated also with 0.68% DMSO. We were not able to choose methanol as a solvent because the stock of the POP mixture was prepared in DMSO.

Since the light/dark test was used in a previous study to test the effects of the POP mixture on anxiety-like behavior of zebrafish larvae in our group, we also employed the same test in Paper II to verify previous findings (Khezri *et al.* 2017). We also expanded our research on the possible anxiogenic effects of the POP mixture or single PFOS using the thigmotaxis assay, another indicator of increased anxiety (Schnörr *et al.* 2012). In view of the anxiogenic effects observed by both tests in larvae zebrafish we set to test whether anxiety-like behaviors could also be detected in adult zebrafish after the early developmental exposure to POPs using the novel tank test (Paper III). The novel tank test as well as the light/dark tank preference test are validated as indicators of anxiety (Cachat *et al.* 2010, Kalueff *et al.* 2014). Due to limited resources, we were only able to employ the novel tank test to evaluate anxiety levels in adult zebrafish.

5.1.2 Exposure window

During this study, our goal was to assess how acute exposure to chemicals can affect zebrafish individuals both short-term, following the exposure period, as well as long-term during juvenile and adult stages. To achieve this goal in Paper II, we exposed zebrafish embryos from 6 – 96 hpf to the POP mixture or single PFOS. This exposure window follows the consensus established by the Fish Embryo Acute Toxicity (FET) test (OECD 2013), where zebrafish embryos are exposed to different concentrations of chemicals until 96 hpf and tolerable levels of exposure are calculated. However, this guideline does include 80% daily refreshment of medium and exposure in 24 well plates, which increases the wasted disposal volumes and can stress the embryos and larvae. This exposure window encompasses the major stages of development, including post-

hatching stages, since the chorion has been shown to provide some protection against adsorption of chemicals in the developing embryos (Kimmel *et al.* 1995, Kais *et al.* 2013). Additionally, this gave us a maximal chance to detect an effect, in the case that a particular chemical has a narrow time window or 'critical period' of effect. There is generally a lack of standardization of exposure windows in toxicological studies with developmental exposure typically from 0 – 96/120 hpf (Huang *et al.* 2010, Ali *et al.* 2012, Chen *et al.* 2012, Chen *et al.* 2014, Jantzen *et al.* 2016a, Blanc *et al.* 2017, Dach *et al.* 2018, Martínez *et al.* 2019, Parsons *et al.* 2019). Furthermore, as aforementioned, the selected exposure window in this study follows the common practice in our group, which makes our previous results comparable (Khezri *et al.* 2017), since an extension of the exposure period could potentially influence measured endpoints, especially behavior which has been shown to differ across ages (de Esch *et al.* 2012).

Since long-term effects were also included in this study, and endpoints such as reproductive traits were evaluated in adult zebrafish, we could have extended the exposure window in Paper III, to include the period of sexual differentiation in zebrafish (21 – 23 dpf) (Uchida *et al.* 2002) since it is likely that chemicals with endocrine activity would likely manifest their effect until this stage. However, this would produce a very large volume of waste and would require special toxicology racks that would enable different exposure conditions across different tanks, that were not available during these experiments. Additionally, it was the aim of the current thesis to address the DOHaD hypothesis upon early exposure to chemicals.

5.1.3 Transcriptomic analysis

We used RNA-seq technology to analyze the changes in gene expression in our samples in Paper II & III upon chemical exposure to the POP mixture or single PFOS. Microarrays and RNA-seq are common methods for performing transcriptomic analysis with RNA-seq gaining ground over microarrays in the last years. Microarrays were and are still used because of several advantages such as the fact that it is a robust, reliable,

short turn-around time, low cost method with straightforward data analysis. However, it is dependent on a prior sequence knowledge, it produces relative expression levels and their hybridization strategy limits their sensitivity which means they cannot detect the differences in expression between very similar sequences such as isoforms. The reason we chose to use RNA-seq technology is the fact that unlike microarray analysis, which measures probe intensity, sequencing provides absolute quantification of digital reads aligned to a particular sequence. Additionally, since every single transcript in the samples is sequenced this enables the identification of structural variations such as gene fusion and alternative splicing events that can provide insight on whether chemicals are mutagenic. Sequencing data can finally be stored and re-analyzed when new discoveries are made, whereas with microarrays, one would need to re-run in order to take advantage of new sequencing information (Rao *et al.* 2019).

We chose to employ a transcriptomic analysis to examine the effects of chemicals exposure on the molecular level, because studies that examine gene expression after chemical exposure are more abundant than studies examining protein or metabolite levels hence making the comparison of results between our study and others more feasible.

5.1.4 Choice of statistical analyses

In this study we used linear mixed effects (LMEs) models to analyze our behavioral, reproductive, swimming and growth (final weight, length and condition factor) data. The term “mixed-effects” refers to the partition of the statistical model into fixed-effects and random-effects. The linear mixed-effects model assumes that the observations follow a linear regression where some of the regression parameters are fixed or the same for all subjects, while other parameters are random, or specific to each subject (Verbeke and Molenberghs 2013). The fixed effects in our data can be defined as the condition in which each population was exposed, the sex of individuals, or the length for example whereas random effects can include biological replicates. Additionally, the

advantage of this statistical test is the fact that interaction between multiple fixed effects can be tested.

In the case of our behavioral data in Paper II and III, missing data due to non-moving individuals, or larvae that had to be excluded due to mortality or malformations can lead to non-homogenized variance and unequal sample sizes that are not well fitted for other analyses such as ANOVA. Using the LMEs model, we could also include the biological replicate as a random effect as it may affect the final outcome (Liu *et al.* 2015).

For the reproductive data, we performed a longitudinal analysis where we measured the same parameters over a 5 days period (fertilized and unfertilized eggs, mortality at 24 hpf, hatching at 72 hpf). The same units of fish were used for the duration of 5 days. This study design makes the repeated measures non-independent because they derive from the same units each day and this dependency of the data could be added as a random effect along with the biological replicate using the LME model (Pinheiro 2014).

5.2 General findings

5.2.1 Solvents in toxicological research

In Paper I the implications of the use of solvents in toxicological research was examined using the light-dark transition test, a standard behavioral assay that is complementing the more traditional FET test (OECD 2013, Legradi *et al.* 2015). The reason behind this is that behavioral endpoints can be more sensitive indicators when testing sublethal concentrations of chemicals, since organisms are not regularly exposed to lethal concentrations of chemicals but rather are exposed to low concentrations over time. Hence, safety concentrations based on lethality or abnormalities as endpoints may not reflect a real-life exposure scenario (Hellou 2011). Sometimes the use of solvents in

toxicology is unavoidable due to the low water solubility of chemicals. The most commonly used solvents include ethanol, methanol and DMSO.

Focusing on DMSO as a solvent, Hallare *et al.* (2006) using a zebrafish bioassay with survival as endpoint concluded that a concentration ≤ 1.5% is appropriate for use in toxicity studies. However, using the light-dark transition test in Paper I we concluded that a DMSO concentration ≥ 0.55% can significantly affect larvae behavior causing an increase in swimming speed. Another study using the transcriptional changes as an endpoint has found that concentrations of DMSO as low as 0.01% can affect the expression of genes (Turner *et al.* 2012). This shows that DMSO can have a range of "acceptable" concentrations depending on the endpoint examined which further complicates the standardization of DMSO usage in toxicological research. A range of 0.001% - 0.5% DMSO has been used in studies as vehicle and control, that examined the effect of chemical exposures on behavioral, physiological and molecular endpoints with 0.1% being the most commonly used (Shi *et al.* 2008, Shi *et al.* 2009, Huang *et al.* 2010, Chen *et al.* 2012, Chen *et al.* 2014, Zhao *et al.* 2014, Glazer *et al.* 2016, Lovato *et al.* 2016, Blanc *et al.* 2017, Dach *et al.* 2018, Martínez *et al.* 2019, Parsons *et al.* 2019). Another fact that needs to be taken in consideration is that DMSO in concentrations ≥ 0.1% increasingly facilitated the uptake of chemicals in the perivitelline space of 48 hpf zebrafish embryos, which means that chemical accumulation and exposure levels might be higher in studies employing higher concentrations of DMSO (Kais *et al.* 2013).

In conclusion, and even if recommendations exist on the use of DMSO by OECD (0.01%) (OECD 2002) there is still a lack of agreement in the concentrations used in different studies. The concentration of solvents should be as low as possible to rule out confounding effects.

5.2.2 Non-monotonic response of chemical exposure

In Paper II & III we employed RNA-seq technology to explore changes in the transcriptome level after acute exposure to the POP mixture or single PFOS in whole larvae, and adult brains after 7 months raised in clean water. In both analyses a non-monotonic response became evident especially for PFOS.

Transcriptome analysis of zebrafish larvae in Paper II revealed a higher number of DEGs in the lower exposure of the POP mixture or PFOS that was equal to x10 human blood level than the high concentrations equal to x70 the human blood levels. In the case of PFOS particularly, 879 DEGs (95.8% downregulated) were identified following PFOS10 exposure and 162 (1.2% downregulated) for PFOS70. In Paper III the same pattern was observed in brains of adults exposed as larvae to PFOS10. These individuals had the highest number of DEGs (466, 99.3% upregulated). The earlier effects on the transcriptome of zebrafish larvae exposed to PFOS70 did not seem to persist in adults where only 2 DEGs were identified in the brains. Furthermore, and based on pathway analysis, there is a disagreement in pathways affected in the low and high exposure to PFOS in zebrafish larvae in Paper II.

Non-monotonic responses to PFOS (0.1 and 1 mg/L) have been recorded before, in respect to proteome changes in the gills of *Cottus gobio* (Dorts *et al.* 2011). It is therefore possible that lower exposures may have a more disruptive effect than high exposure depending on the outcome examined. In Paper II for instance we did not observe any effect on zebrafish larvae exposed to PFOS10 using the light-dark transition test. The consequence of non-monotonic dose response curves for toxicity testing is that a safe dose established from higher doses does not guarantee safety to lower, untested doses that may be closer to human exposures. In a system that is responding nonmonotonically, it is not appropriate therefore to use a high dose test to predict low-dose effects (Vandenberg *et al.* 2012). Further studies dealing with a large number of concentrations to adequately characterize the concentration–response relationship are needed to strengthen the non-monotonic response to PFOS.

5.2.3 Exposure levels

In the experiments performed in Paper II & III, zebrafish larvae were exposed from 6 – 96 hpf to the POP mixture or single PFOS at concentrations representing x10 and x70 higher than what is found on average in human blood levels of the Scandinavian population. Although these concentrations might not represent real-life exposure scenarios, chemical analysis performed in POP70 and PFOS70 exposures on the uptake of chemicals in larval tissue and total concentration recovered from the larva and exposure media revealed a much lower actual exposure level (Tables 3 & 4). With the chemical analyses the recovery of the chemicals did not reach 100 %. It is not clear why the recovery of chemicals was lower than expected. Reasons behind this might be unknown interactions between the chemicals integrated in the mixture, adsorption on the wells, metabolism of chemicals by the larvae and/or the concentrations of chemicals might be affected by practical steps during the preparation of the exposure media.

In Table 3 an estimation of the internal levels in larvae in ng/g of lipid is presented. This assessment is based on parameters from previous publications (Falcinelli *et al.* 2015, Hachicho *et al.* 2015), enabling the comparison between zebrafish and human internal levels. The lipid concentration (ng/g) of PCBs, BFRs and other organochlorines in larval zebrafish is comparable and sometimes lower than what has been measured in human serum of the general population. For example, lipid accumulation in zebrafish for PCB 153 was equal to 11.2 ng/g of lipid (Table 3), whereas it ranged from 10.2 to 320 ng/g lipid in human serum from multiple countries such as the UK, USA, Greece, Canada, Norway, China and Japan (Kalantzi *et al.* 2011, Berntsen *et al.* 2017, Haines *et al.* 2017). For p,p'-DDE, average human levels ranged from 53 – 1975 ng/g lipid with Norway presenting some of the lowest values and Canada and Romania having some of the highest, whereas accumulation in the larvae reached 28.2 ng/g lipid (Kalantzi *et al.* 2011, Berntsen *et al.* 2017, Haines *et al.* 2017). Persistent organic pollutants are known to bioaccumulate and values reported from human populations were obtained from individuals that were 20 years or older, giving the chemicals many years to accumulate. We only exposed larvae to POPs for 4 days, and longer exposure might lead to levels

similar to what is detected in humans. Nevertheless, this proves that even if exposure levels were somewhat high, the internal levels, that can produce adverse effects, were similar with what was detected in the general population. Also, chemical analysis was only performed in the high exposure (x70) thus it can be assumed that the accumulation in x10 exposure is even lower but can still lead to adverse effects.

Actual exposure levels were also calculated based on the percentage of chemicals recovered from larva tissue and exposure media (Table 4). The highest recovery rate was 50.2% for β-HCH incorporated in the POP mixture (detection limit was equal to 0.002 per larva and 0.0068 ng/mL in exposure media) and 35.5% for the single PFOS exposure (detection limit was equal to 0.03 per larva and 0.02 ng/mL in exposure media). The actual exposure levels that larvae were exposed to, with the exception of PFOS, are comparable to concentrations of POPs in human umbilical cord blood, making this study highly relevant to the effects of POPs on early life development (Cabrera-Rodríguez *et al.* 2020). PFOS was the only chemical that was detected at a high concentration both in the POP mixture and the single PFOS exposure. These values are not comparable to levels found in general population but they are comparable to human serum levels from highly exposed populations (100 – 1000 ng/mL) (Olsen 2015). Relatively few zebrafish studies have assessed internal concentrations of chemicals, which is key in interpreting findings generated in such models, and in relevance to human exposure levels.

Table 3. Accumulation of chemicals in the POP mixture or PFOS in the tissue of 96 hpf zebrafish larva after exposure to 70x higher than human blood levels.

Chemical	*Nominal concentration (ng/mL)*	*Concentration per larva (ng)*	*Concentration per larva (ng/mg)*[a]	*Lipid concentration (ng/g lipid)*[b]
PFASs				
PFOA	316.61	0.83	1.92	
PFOS	2059.75	52.27	120.21	
PFDA	34.65	1.14	2.63	
PFNA	56.00	0.51	1.16	
PFHxS	241.50	0.84	1.94	
PFUnDA	39.20	1.65	3.80	
Σ PFASs	2747.71	57.24	131.66	
BFRs				
BDE-47	0.63	0.00	0.01	0.41
BDE-99	0.28	0.00	0.00	0.01
BDE-100	0.21	0.00	0.00	0.09
BDE-153	0.07	0.00	0.00	0.00
BDE-154	0.14	0.00	0.00	0.00
BDE-209	0.77	0.01	0.02	0.79
HBCD	1.75	0.04	0.10	4.77
Σ BFRs	3.85	0.05	0.12	6.08
PCBs				
PCB 28	0.91	0.00	0.00	0.00
PCB 52	0.70	0.00	0.00	0.00
PCB 101	0.56	0.00	0.00	0.00
PCB 118	4.48	0.02	0.05	2.62
PCB 138	15.54	0.15	0.34	17.03
PCB 153	25.34	0.10	0.22	11.18
PCB 180	13.58	0.05	0.12	6.22
Σ PCBs	61.11	0.32	0.74	37.06
Other organochlorines				
p,p'-DDE	35.14	0.25	0.56	28.20
HCB	8.19	0.03	0.08	3.91
α-chlordane	0.77	0.02	0.04	1.92
Oxy-chlordane	1.54	0.03	0.06	3.05
Trans-nonachlor	2.87	0.05	0.10	5.22
α-HCH	0.42	0.01	0.02	1.05
β-HCH	3.71	0.11	0.25	12.58
γ-HCH (lindane)	0.42	0.01	0.03	1.55
Dieldrin	1.68	0.02	0.05	2.67
Σ Organochlorines	54.74	0.52	1.20	60.16
Single PFOS	2059.75	129.67	298.25	

[a] *The ng/mg concentration was calculated based on the study by Falcinelli et al. (2015) where the weight of an individual larva was equal to 0.43 mg*
[b] *The lipid concentration is based on the study by Hachicho et al. (2015) where the lipid percentage of a 96 hpf zebrafish larva was equal to 5%*

Table 4. Total exposure levels of the POP mixture and single PFOS in (ng/mL) based on the recovered percentage in zebrafish larvae and exposure media after exposure to 70x higher than human blood levels.

Chemical	*Nominal concentration (ng/mL)*	*% in larva*	*% in media*	*Total % recovered*	*Exposure level (ng/mL)*
PFASs					
PFOA	316.61	1.32	5.16	6.48	20.52
PFOS	2059.75	12.69	5.75	18.43	379.71
PFDA	34.65	16.48	3.90	20.37	7.06
PFNA	56.00	4.52	5.19	9.70	5.43
PFHxS	241.50	1.75	8.73	10.48	25.30
PFUnDA	39.20	21.05	1.20	22.25	8.72
Σ PFASs	2747.71				446.76
BFRs					
BDE-47	0.63	2.85	0.00	2.85	0.02
BDE-99	0.28	0.18	0.00	0.18	0.00
BDE-100	0.21	1.89	0.00	1.89	0.00
BDE-153	0.07	0.00	0.00	0.00	0.00
BDE-154	0.14	0.00	0.00	0.00	0.00
BDE-209	0.77	4.45	5.25	9.70	0.07
HBCD	1.75	11.86	0.00	11.86	0.21
Σ BFRs	3.85				0.30
PCBs					
PCB 28	0.91	0.00	0.00	0.00	0.00
PCB 52	0.70	0.00	0.00	0.00	0.00
PCB 101	0.56	0.00	0.00	0.00	0.00
PCB 118	4.48	2.54	0.03	2.57	0.12
PCB 138	15.54	4.77	0.12	4.89	0.76
PCB 153	25.34	1.92	0.00	1.92	0.49
PCB 180	13.58	1.99	0.02	2.01	0.27
Σ PCBs	61.11				1.63
Other organochlorines					
p,p'-DDE	35.14	3.49	0.40	3.89	1.37
HCB	8.19	2.07	0.00	2.07	0.17
α-chlordane	0.77	10.85	0.00	10.85	0.08
Oxy-chlordane	1.54	8.62	0.00	8.62	0.13
Trans-nonachlor	2.87	7.91	0.12	8.02	0.23
α-HCH	0.42	10.91	13.64	24.55	0.10
β-HCH	3.71	14.74	35.42	50.16	1.86
γ-HCH (lindane)	0.42	16.05	27.28	43.33	0.18
Dieldrin	1.68	6.91	0.00	6.91	0.12
Σ Organochlorines	54.74				4.24
Single PFOS	2059.75	31.5	4.1	35.53	731.76

5.2.4 Mixture effects

In this study we focused on the effects of defined mixtures of chemicals either in binary combinations (Paper I) or as a complex mixture of 29 chemicals that were detected in the human blood of the Scandinavian population (Paper II & III). In addition, a single PFOS exposure was included (Paper II & III), since it is the most abundant chemical in the POP mixture, and it was previously found to be the chemical in the POP mixture causing increased swimming speed (Khezri *et al.* 2017).

In Paper I substances that increased swimming speed in a dose dependent manner such as DMSO, PFOS and MB, created an additive effect when combined (i.e. DMSO + PFOS or DMSO + MB). Therefore, when it comes to binary mixtures of chemicals it is somewhat easier to establish whether an interaction exists on a certain endpoint compared to complex mixtures. However, these same chemical combinations had an interactive effect in distance moved and time spent active in the same behavioral test. The mixture effect of these binary mixtures therefore cannot be considered only based on one endpoint, but an array of tests should be performed before establishing a relationship between chemicals.

In Paper II and III, we aimed to characterize whether the effects of a single PFOS exposure can explain the effects of the POP mixture. As it was shown before, there seems to be no interaction of the different chemicals in the POP mixture on the behavioral outcome, with PFOS being the driving factor of larval hyperactivity. However, the same relationship was not observed on the transcriptional level of neurobehavioral genes where the POP70 exposure had more DEGs than the PFOS exposure (Khezri *et al.* 2017). In Paper II, our results on the behavioral alterations seem to agree with previously reported results. In contrast, transcriptomic analysis in Paper II and III revealed that single PFOS exposures has the highest numbers of DEGs with some overlap in common genes between the POP mixture and single PFOS exposures. This might suggest underlying interactive or antagonistic effects of the chemicals on the transcriptome level, not evident on the behavioral level. An *in vitro* study using the same POPs mixture, revealed that 4

compounds in the mixture exerted AhR agonistic activities and 16 compounds exerted antagonistic activities, and the total POP mixture was found to be AhR antagonistic (Doan *et al.* 2019). Therefore, the differences between the exposures in the gene expression levels observed in Paper II & III might be due to antagonistic interactions on different key genes between the chemicals comprising the POP mixture.

In conclusion, to establish the relationships between chemicals in a mixture either binary, or complex, the effects on multiple endpoints and biological levels should be studied. Additionally, mathematical models using the information of components to predict combined effect can also be incorporated in studies assessing mixture toxicity.

5.2.5 Behavioral studies as indicators of anxiety-like effects of chemicals

Increased locomotor activity, thigmotaxis and bottom-dwelling during the novel tank test are all considered indicators of anxiety in zebrafish (Cachat *et al.* 2010, Irons *et al.* 2010, Schnörr *et al.* 2012). In this context, zebrafish larvae and adults have been used to uncover the possible effects of chemicals on anxiety-like behavior in Papers I-III. Zebrafish are established as models in neurotoxicity research because they are easy to handle, inexpensive to keep and high-throughput assays exist to assess behavioral endpoints. Additionally, they have an homologous central nervous system function and anatomy as humans and other mammalian models and share the same neurotransmitter systems as higher mammals (Fontana *et al.* 2019).

Thigmotaxis and bottom dwelling behaviors are more straightforward in establishing a link between exposure and anxiety effect. Locomotor activity however is not consistently defined. During a light-dark transition test the distance moved, the time spent active and the swimming speed can be calculated during dark and light periods. These three measurements can all be used to describe locomotor activity but sometimes they do not have the same response. For example, in Paper II, in our mechanistic work

with RyR agonist, caffeine caused a decrease in distance moved and time spent active but a general increase in swimming speed. We can conclude from this that larvae showed either hypoactivity or hyperactivity regarding different endpoints. Furthermore, the behavioral phenotype might not be related to changes in the brain but on the effect of chemical on the locomotor output alone. Another aspect complicating the translational potency of zebrafish behavioral tests is that anxiety-like behavior frequently overlaps with fear-related behavior and further studies are needed to characterize these two domains (Maximino *et al.* 2010, Kalueff *et al.* 2013). Additionally, mechanisms of anxiety in zebrafish and humans are still not fully understood, so it is difficult to provide a causal link between changes in the brain and observed behavioral phenotype (Gerlai 2010)

In conclusion, when evaluating anxiety-like behaviors using behavioral tests, further studies are needed to decipher which parameters are actually measures of anxiety and until then the term anxiety-like behavior might include both anxiety- and fear-related behaviors.

5.2.6 Zebrafish as a model – results, relevance and limitations

This thesis revealed that a developmental exposure to a complex mixture of POPs or PFOS influenced the behavior of zebrafish larvae, causing hyperactivity, and produced changes on the transcriptome level. Investigation of the DOHaD in adult zebrafish after a period reared in clean water revealed effects on fitness in terms of decreased swimming performance, and increased body weight, along with persistent brain transcriptome changes. No adverse effects were observed in the reproductive output and behavior of adults as well as the survival, hatching and behavior of their offspring.

Results on larval behavior in terms of hyperactivity have been previously observed in other studies exploring the effects of persistent organic pollutants in mice (Johansson *et al.* 2009). Additionally, the increase of weight in adult zebrafish in Paper III seems to agree with the notion that exposure to POPs in humans and animals is associated with

the development of obesity (Reaves *et al.* 2015). However, some considerations need to be taken when trying to infer results from animal studies on humans and wildlife. Firstly, even if hyperactivity is one of the symptoms of exposures to POPs in animals that does not prove a direct causation of exposure to POPs and hyperactive related behaviors such as ADHD. Epidemiological studies rely on correlation and other confounding factors such as diet, income, smoking or education of mothers need to be taken into consideration when trying to link chemical exposures and adverse effects, since these factors have been shown to affect the levels of chemicals found in humans (Tyrrell *et al.* 2013). The same goes for extrapolating effects observed in laboratory fish and wildlife populations. Fish in the laboratory are raised under controlled conditions and have a limited gene pool, which cannot be said for their wildlife counterparts which are faced with changing environments in terms of temperature, diet or even predation threats. Under these conditions the response to chemicals might be different since the organism is exposed to multiple stressors not only chemicals (Vasseur and Cossu-Leguille 2006). Nonetheless, groups of laboratory fish can be administered measured doses of chemical(s) and under these well controlled conditions, adverse effects to a specific chemical exposure can be attributed with greater certainty. Additionally, zebrafish can be utilized for short, intermediate, and chronic exposure studies that can mimic realistic exposure scenarios. Through these studies, scientists can characterize the spectrum of adverse effects of chemicals. Finally, even if the toxicokinetics of chemicals are quite different in zebrafish and humans, zebrafish larvae can be considered an analogous to the human embryo when it comes to route of exposure, where both are exposed through their surrounding environment (such as the exposure media and amniotic fluid for zebrafish larvae and human embryo respectively).

Transcriptome analysis is a helpful tool in investigating the effects of chemicals. Zebrafish and mammals share many physiological and molecular similarities in xenobiotic metabolism and adaptive response to toxicant insults making the zebrafish an ideal toxicology model (Garcia *et al.* 2016). The effects of exposure to PFOS during early development on lipid metabolism and PPAR signaling pathways seem to agree with what is generally reported from other toxicological studies (Stahl *et al.* 2011, White *et al.* 2011).

Pathway analysis furthermore uncovered effects on other pathways such as the calcium signaling pathway both in brain and whole larva tissue, and perturbations of GABA and MAPK signaling pathways which agrees with the mode of action of the examined chemicals (Westerink 2014).

Although the small size of zebrafish makes it ideal in research for some aspects, it also presents some limitations. RNA-seq in Paper II was performed in whole larval tissue to secure adequate amount of RNA, hence effects on pathways cannot be directly associated with a particular system but rather a whole-body effect. Therefore, the lack of a specific neurotoxic effect after exposure to POPs and PFOS may be due to masking effects of neural related genes being differentially expressed at different parts of the nervous system. In this thesis, we aimed to assess this by isolating neural cells from the zebrafish transgenic line *elavl3* (*elavl3* is only expressed in neural related cells) (Figure 13). However, even if the isolation of these cells was successful, we failed to obtain an adequate amount of RNA to perform a transcriptome analysis. Additionally, in Paper III, our initial goal was to isolate only the telencephalon of adults, since it is the structure of the brain that mainly orchestrates behavior (Lau *et al.* 2011). The RNA amount extracted from this tissue, however, was not enough to meet the criteria of RNA-seq analysis provided by the company that performed the library preparation and analysis of our samples. Even so, brain structures are not comprised by a uniform cell population but rather a combination of different neuronal populations that do not uniformly express genes or neurotransmitters (Lau *et al.* 2011, Mes *et al.* 2018). This might be the reason why we failed to identify many pathways directly related to behavior in whole brain RNA-seq in Paper III. The lack of behavioral effects might be also attributed for the absence of behavior-enriched pathways. Furthermore, even if transcriptome analysis is a powerful tool in establishing the mechanisms involved in chemical exposure there is still a shortage of studies providing phenotypic anchoring of molecular effects. Further efforts can be conducted in future experiments, also including powerful single cell sequencing technology for more in depth mechanistic assessments.

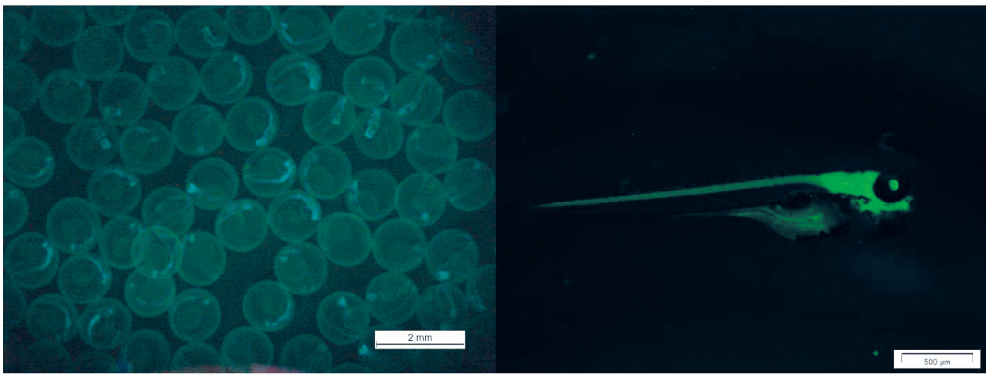

Figure 13. Embryo and larva of the zebrafish transgenic *elavl3* line expressing the *elav3* gene in neural cells under fluorescent signal (Image credits: Dinh Duy Thanh, Laboratory for Organogenesis and Regeneration, GIGA Institute, University of Liege).

This dissertation builds on the current knowledge on the effects of chemical exposure. By utilizing the zebrafish as a model, we have uncovered that a developmental exposure to a human relevant mixture can have effects short-term. Transcriptomics has provided further understanding of the mechanisms of chemical toxicity and how some of these changes might be correlated to behavioral endpoints. The observation of long-term effects even after a short exposure window (6 – 96 hpf) heeds to the necessity of more studies, including the use of multiple doses and longer exposure windows that include life-specific events such as sexual determination.

6. General conclusions

This thesis increases our understanding on how methodological approaches can influence the outcome in behavioral toxicity studies. In Paper I we proved that solvents can interact with chemicals and can differentially affect behavioral endpoints such as distance moved and swimming speed. Furthermore, parameters such as media volume or the use of antifungal agents should also be taken into consideration when performing a light-dark behavioral test.

The zebrafish proved to be a useful model in examining the short- and long-term effects of a complex mixture of persistent organic pollutants or a single PFOS exposure. Short-term exposure in Paper II caused a hyperactive response and a thigmotactic behavior in zebrafish larvae. Using transcriptome analyses we identified possible pathways involved in the observed behavioral responses. The calcium signaling pathway via the activation of ryanodine receptors in muscle cells was identified as a likely pathway and further mechanistic work, with a RyR antagonist provided further support on this hypothesis. Transcriptome analysis additionally revealed a disruption of lipid metabolic pathways after exposure to PFOS. Chemical accumulation within the larval tissue was comparable to levels found in human and animal tissues.

Long-term effects of early exposure to the POP mixture or PFOS were also investigated (Paper III). Chemical exposures had a persistent effect reducing swimming performance of adult zebrafish. Effects were also observed on the body parameters of adult zebrafish. Adults exposed as larvae had higher weight and condition factor relative to controls supporting the obesogenic effects of POPs. Short-term exposure did not affect the reproductive output of adult zebrafish. No behavioral effects were observed in zebrafish adults or their offspring. Transcriptome changes in adults' brains and further pathway analysis showed enrichment of calcium, MAPK and GABA signaling which might be involved in learning and memory processes.

7. Future perspectives

Zebrafish use in neurotoxicity studies has increased in recent years. Studies employing behavioral tests to rapidly screen for the effects of chemicals are now widely used. The presence of interactions between solvents and chemicals might hinder the comparability of the effects of chemicals across laboratories. Therefore, in the future more studies should be conducted, testing possible interactions between other frequently used solvents such as ethanol and methanol and POPs.

Concerning chemical exposure to the POP mixture it will be interesting to expand the exposure period to include other sensitive developmental periods, such as the period of sexual determination in zebrafish, due to the endocrine disrupting potency of many of the studied chemicals. Regarding the translatability of zebrafish as a model to test the effects of chemical exposure in wildlife populations and taking into account the unpredictability of the natural environment, more stressors should be tested simultaneously such as food deprivation or different temperature regimes in addition to chemical exposure.

Including more behavioral tests in the larval and adult stage will be useful, to investigate whether chemical exposure can affect other components of the behavioral repertoire such as learning, memory and prey capture. The reduction in swimming ability of adult zebrafish after developmental exposure to chemicals can be further investigated to uncover potential physiological changes, that might be involved in the observed effect.

On the molecular level, transcriptome changes in larval zebrafish and adults' brains can be further explored with the implementation of other omics analyses, such as lipidomic analysis, to investigate the changes in lipid profiles of exposed individuals. Additionally, single-cell RNA-seq can be performed to identify patterns of gene expression in different cell types, to avoid possible masking effects of differential expression when using whole tissues such as whole larvae or brain tissue. Finally, transgenic lines or whole-mount in situ hybridization can be employed to identify localized expression of genes of interest brought into focus from our transcriptome analyses such as the expression of muscle-related genes.

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9. Appendix: Papers I-III

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DMSO effects larval zebrafish (*Danio rerio*) behavior, with additive and interaction effects when combined with positive controls

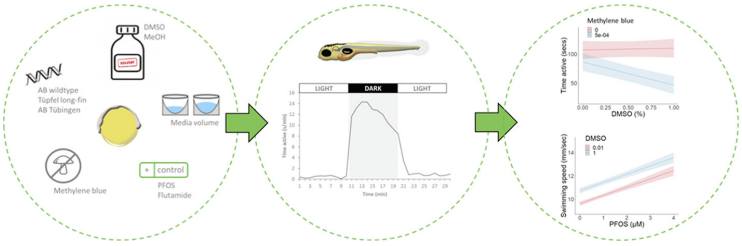
Maria Christou, Arturas Kavaliauskis, Erik Ropstad, Thomas William Kenneth Fraser *

Norwegian University of Life Sciences, Department of Production Animal Clinical Sciences, Oslo, Norway

HIGHLIGHTS

- Solvents are frequently used during zebrafish toxicity testing but their effects are unknown.
- DMSO affected behavior at a concentration of ≥0.55%
- Different zebrafish strains showed different basal activity, but the same behavioral response to DMSO.
- DMSO had an additive and interaction effects on behavior when co-exposed with positive controls.

GRAPHICAL ABSTRACT



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ABSTRACT

Embryonic and larval zebrafish (*Danio rerio*) behavior is commonly used to identify neurotoxic compounds. Here, we investigated whether sub-lethal exposures to the common solvents dimethyl sulfoxide (DMSO, 0.01–1%) and methanol (MeOH, 0.01–1%), or the anti-fungal agent methylene blue (MB, 0.0001–0.0005%), can influence larval behavior in a simple light/dark paradigm conducted in 96-well plates. In addition, we tested whether the media volume within the behavioral arena or the zebrafish strain, AB wild type, AB Tübingen (AB/TU), or Tüpfel long-fin (TL), could also influence larval behavior. Following the single exposures, we co-exposed larvae to DMSO and either MB or two other compounds with known behavioral effects in larval zebrafish, flutamide and perfluorooctanesulfonic acid (PFOS). We found ≥0.55% DMSO and 0.0005% MB significantly affected larval behavior, but there was no effect of MeOH. Similarly, TL showed less movement compared to AB and AB/TU strains, whereas lower media volumes also significantly reduced larval movement. However, all strains responded similarly to DMSO and MB. In the co-exposure studies, we found either additive or interaction effects between DMSO and either MB, flutamide, or PFOS, depending on the behavioral endpoint measured. In addition, media volume had no effect on the DMSO concentration response curve, but again we observed additive effects on behavior. In conclusion, methodology can lead to alterations in baseline locomotor activity and compounds can have additive or interaction effects on behavioral endpoints. However, we found no evidence that strain effects should be a concern when deciding on solvents for a simple light/dark behavioral test in larval zebrafish.

1. Introduction

Since the establishment of the fish acute toxicity test (AFT) and the fish embryo acute toxicity test (FET) (OECD, 1992, OECD, 2013), multiple studies have been conducted using the zebrafish as a

* Corresponding author at: Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Ullevålsveien 72, 0033 Oslo, Norway.
E-mail address: thomas.fraser@nmbu.no (T.W.K. Fraser).

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model organism. These studies have not only helped to determine the maximum tolerable concentrations of a broad spectrum of chemicals and other agents, but have also established guidelines for the testing of these substances (Belanger et al. 2013, Dang et al. 2017, Lammer et al. 2009, Scholz et al., 2014). In more recent years, behavioral endpoints are increasingly used as sub-lethal alternative endpoints to the traditional fish embryo toxicity test in ecotoxicology and chemical screening (Hellou 2011, Legradi et al. 2015). Of particular interest is the translational aspect of these studies, as zebrafish share a similar genome, brain structure, and neurochemical system as mammals (Best et al. 2008, Gerlai 2010, Kokel and Peterson 2008, Levin et al. 2007). Furthermore, zebrafish larvae show behavioral profiles similar to mammalian models following exposure to neuroactive drugs (Irons et al. 2010) making zebrafish ideal for comparative studies of neurotoxic effects (Legradi et al. 2018). The stereotypical behavior of zebrafish is well described, and many behavioral tests have been developed to evaluate the effects on sensory, motor, and cognitive behavior (Egan et al. 2009, Gerlai 2003, Miklosi and Andrew 2006). Behavioral tests that have been employed in zebrafish larvae include the thigmotaxis test (preference for the outer limits of a defined arena, Schnörr et al., 2012), the escape or avoidance test (Pelkowski et al., 2011), the acoustic test (Burgess and Granato 2007), the locomotor assay (Giacomini et al. 2006, Legradi et al. 2015), and the light/dark transition test. The light/dark transition assay is characterized by alternating dark and light periods and it has been established that during the dark periods zebrafish larvae move more than during the light periods (MacPhail et al. 2009).

Due to the increased use of zebrafish embryos and larvae in behavioral assays in toxicology and pharmacology, more attention is been given to the effect of solvents that are used during experimental procedures. Zebrafish embryos and larvae are primarily exposed to an aqueous solution containing the test compounds. However, the low water solubility of many compounds requires the use of solvents to accelerate the dilution process (Hutchinson et al. 2006). The most commonly used solvents in toxicology for the administration of chemicals are dimethyl sulfoxide (DMSO), ethanol, and methanol (MeOH). Studies have shown that different concentrations of the solvents can have an effect on their own on the behavior of zebrafish. For example, high concentrations of ethanol can cause hypoactivity whereas lower concentrations cause hyperactivity in larval zebrafish (de Esch et al. 2012, Lockwood et al. 2004) and DMSO at concentrations between 0.01 and 0.1% can have observable locomotor effects (Chen et al. 2011). The Organization of Economic Co-operation and Development (OECD) has specified a maximum solvent concentration of 0.1 ml/L (0.01% v/v) for aquatic tests (OECD, 1992, OECD, 2013), but standardization of this concentration in toxicity tests is still lacking.

Further to the use of solvents in toxicity testing, another common aspect of using zebrafish is the control of fungal pathogens. Here, laboratories may use the antifungal agent methylene blue (MB) to clean fertilized embryos and/or prevent fungal outbreaks during larval production. Common laboratory techniques for zebrafish rearing suggest that embryo and larvae are kept in embryo medium with MB at a concentration of 0.0002% for the first four days (Westerfield, 2007). To date, there is no information on the effects of MB on the behavioral response in larval zebrafish.

In addition to the solvent and/or use of antifungal agents, other aspects of larval zebrafish behavioral testing are not standardized. For example, a number of different strains are reported within the toxicology literature and these can exhibit different behavioral responses either at baseline levels or when exposed to different chemicals. For instance, larvae belonging to the AB strain were more active during a light/dark assay when compared to larvae of the Tüpfel long fin (TL) strain at days 5 and 6 post fertilization whereas this activity was reversed at day 7 (de Esch et al. 2012). Regarding strain effects on toxic responses, Pannia et al. (2014) observed that zebrafish adults belonging to the TU strain appeared to be more tolerant to ethanol treatment since only the WIK strain showed a dose- and time- dependent decrease in swimming duration following exposure. Furthermore, the physiology of zebrafish strains differ, as AB and TL larvae have differences in hypothalamus-pituitary-interrenal axis activity, expression of neurodevelopment and immune system related genes, and baseline levels of cortisol (van den Bos et al., 2017). In addition, various aspects of the larval behavioral test are known to influence baseline behavior, such as age, size of well, light conditions (Emran et al. 2008, Padilla et al., 2011), rearing conditions (Zellner et al., 2011), and the time of the day the assay took place (MacPhail et al. 2009). Differences in some of these variables can also lead to differences in behavioral outcomes during toxicity testing. For example, 10 μM bisphenol A was found to induce either hyper- or hypo-activity in larval zebrafish depending on the arena size used during testing (Fraser et al. 2017a). One aspect that has yet to be investigated is the volume of the media within a given behavioral arena. For example, zebrafish are commonly tested in 96 well plates, but the media volume can vary between 100 (Noyes et al. 2015) to 500 μl (de Esch et al. 2012).

Following the need for standardization of experimental procedures, we carried out a behavioral assay with zebrafish larvae using two of the most common solvents, DMSO and MeOH, as well as the antifungal agent MB. These compounds were evaluated at sub-teratogenic concentrations for effects on behavior applying a commonly used larval behavior test with three strains of zebrafish. In addition, we also evaluated the use of different media volumes for effects on behavior. Following this, we investigated whether DMSO and media volume could have interaction effects on behavior, as well as co-exposures between DMSO and two positive controls for larval locomotion, flutamide and perfluorooctanesulfonic acid (PFOS).

2. Materials and methods

2.1. Chemicals

DMSO (purity, >99.7%, CAS number 67-68-5), MeOH (purity, ≥99.9%, CAS number 67-56-1), MB (dye content, ≥82%, CAS number 122965-43-9), flutamide (purity, ≥99%, CAS number 13311-84-7) and PFOS (purity, ≥98%, CAS number 2795-39-3), were purchased from Sigma-Aldrich. Stock solutions of flutamide and PFOS were prepared in DMSO. Fresh stock solutions of flutamide were made on the day of testing whereas the PFOS stock solution was stored at − 20 °C.

2.2. Fish husbandry

The study was performed at The Norwegian University of Life Sciences (NMBU), Oslo, Norway, that is licensed by the Norwegian Animal Research Authority (NARA) (www.mattilsynet.no) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway.

AB wild-type (AB), Tüpfel long fin (TL), and AB/Tübingen (AB/TU) zebrafish were maintained at 28 ± 1 °C under a 14:10 light/-dark photoperiod. Animal care was done in accordance with the local protocols. To generate embryos, adults were placed in spawning tanks in the afternoon and spawning occurred the following

morning when the lights turned on (08:00). The embryos were collected (09:00) and maintained in sterile embryo media (60 μg/ml Instant Ocean® sea salts) until the time of exposure.

2.3. Exposures

The exposure concentrations and the strains used to test certain compounds can be found in Table 1. Fertilized embryos were transferred into clear polystyrene 96-well plates (Nunc™ MicroWell™) and continuously exposed under static conditions from 6 hpf until the time of testing at 98–102 hpf (between 11:00–15:00). For DMSO and MeOH, five nominal concentrations ranging from 0.01 to 1% (1.41–141 mM for DMSO and 2.47–247 mM for MeOH) were tested. These concentrations are below the minimum effect concentrations for teratogenicity which are 2.0–2.5% for 24–168 hpf larvae (Maes et al. 2012). For MB, three nominal concentrations of 0.0001, 0.0002, and 0.0005% (3.1, 6.3, and 15.6 μM, respectively) were chosen based on general guidelines for zebrafish (Westerfield, 2007). For media volume, four volumes ranging between 50 and 200 μl were chosen based on volumes frequently used within the literature (Khezri et al. 2017, MacPhail et al. 2009, Noyes et al. 2015). For mixture experiments, we compared the concentration response to flutamide (FLU) between 1 and 10 μM and PFOS between 0 and 4 μM, the range in which we previously found FLU (Fraser et al. 2017b) and PFOS (Khezri et al. 2017) to increase swimming speeds, when co-exposed to 0.01 or 1% DMSO. We compared the concentration response of DMSO between 0.1 and 1% when using only 50 or 200 μl of media. Finally, we compared the concentration response to DMSO between 0.1 and 1% when in the presence or absence of 0.0005% MB. Each scenario described above was repeated three to four times using independent batches of larvae on different days. For DMSO, MeOH, and MB, each of the three strains of zebrafish were assessed separately. Prior to and following exposure, embryos were reared in an incubator at 28 ± 1 °C. The light cycle within the incubator was 14:10 light/dark (lights on 07:30/lights off 21:30). For the single exposures, all groups were spread equally on each row and column over one 96 plate/replicate. For the co-exposures, all groups were spread equally over each row and column in two 96 well plates/replicate.

2.4. Larval behavior

Behavioral tests were conducted using a ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France). Behavioral screening was undertaken at 98–102 hpf that was between 11:00 and 15:00. Previously, we have found this age/time period to produce repeatable behavioral effects with many compounds (i.e. Fraser et al. 2017ab). Larval locomotion behavior, the cumulative distance travelled and the time spent active, were simultaneously measured for all larvae on a given well-plate during a light–dark cycle that lasted for a total of 30 min and consisted of 20 min of light and 10 min of darkness. This protocol has been used in our laboratory (Fraser et al. 2017a) and others (Fetter et al. 2015), but there is no standard protocol for the length or number of cycles (e.g. Noyes et al. 2015). The mean swimming speed was calculated by dividing the cumulated distance travelled by the total time spent active. The light level was set to 100% on the ViewPoint software (7.45 Klux, TES 1337 light meter). Infrared light (850 nm) tracks larval activity during the "dark" periods. The threshold for determining movement was set at 5 mm/sec. The larvae were inspected under a stereomicroscope immediately after behavioral testing in order to identify dead or deformed (coagulated, unhatched, spinal aberrations, yolk sac or cardiac edema, aberrations in pigmentation, swim bladder development, and/or loss of equilibrium) larvae. The maximum % of a batch discounted for behavioral analysis based on these criteria was 16% (Table 1). For FLU and PFOS, the number of larvae excluded from the behavior analysis due to these criteria ranged between 0 and 4% and 5–9% depending on concentration, respectively, confirming no signs of teratogenicity for either compound.

2.5. Statistical analysis

Behavioral data were transferred to R version 3.5.3 (R Development Core Team 2018, http://www.r-project.org). All dead and deformed larvae were discounted for behavioral analyses. For all test scenarios, only motility during the dark phase was analyzed as movement was minimal during the light periods. We used linear mixed effect (LME) models within the "nlme" package of R to assess behavior. The dependent variable was either the cumulative time spent active (seconds), the cumulative distance travelled (mm), or average swimming speed (calculated as the cumulated distance travelled/cumulated time spent active), with concentration set as a continuous variable, strain as a categorical independent variable, and replicate as a random effect. To compare whether strain influenced the behavioral response to DMSO, MeOH, or MB, we compared two models using the Bayesian Information Criteria (BIC) to identify the model with the lowest BIC score and considered this the "true" model (Aho et al. 2014). One model allowed for an interaction between strain and the tested compound (i.e. strain×DMSO), evidence of an interaction, the other allowed no interaction (i.e. strain+DMSO), evidence for no

Table 1
Overview of the experimental design. *Those individuals excluded from the statistical analysis were either dead or deformed according to the criteria detailed in the methods.

Dose response	Concentrations	Co-exposure scenario	Strains tested	Individuals/group (replicates)	Excluded individuals*/replicate (%)
–	–	–	AB, AB/TU, TL	16 (3)	AB, 6, 0, 0: AB/TU, 6, 12, 0: TL, 6, 0, 6
Dimethyl sulfoxide (DMSO)	0.00, 0.01, 0.10, 1.00%	–	AB, AB/TU, TL	16 (3)	AB, 0, 0, 9: AB/TU, 2, 9, 0: TL, 2, 3, 0
Methanol	0.00, 0.01, 0.10, 1.00%	–	AB, AB/TU, TL	16 (3)	AB, 0, 0, 3: AB/TU, 2, 8, 3: TL, 0, 0, 0
Methylene blue (MB)	0.0000, 0.0001, 0.0002, 0.0005%	–	AB, AB/TU, TL	16 (3)	AB, 2, 5, 0: AB/TU, 8, 12, 0: TL, 9, 6, 16
Media volume	50, 100, 150, 200 μl	–	AB	16 (4)	3, 2, 9, 2
Flutamide	0.0, 1.0, 3.2, 5.5, 7.8, 10.0 μM	0.1 or 1.0% DMSO	AB	12 (3)	1, 1, 3
DMSO	0.00, 0.10, 0.32, 0.55,0.78, 1.00%	0 or 0.0005% MB	AB	12 (3)	3, 1, 2
DMSO	0.00, 0.10, 0.32, 0.55,0.78, 1.00%	50 or 200 μl media volume	AB	12 (3)	2, 4, 2
Perfluorooctanesulfonic acid	0, 0.25, 0.50, 1.00, 2.00, 4.00 μM	0.1 or 1.0% DMSO	AB	12 (4)	6, 6, 8, 7

interaction. A null model that included the random effect was included as a third model. The model with the lowest BIC score was run. A final model used those larvae exposed to 0–1% DMSO in the co-exposure studies and 200 μl of media volume (i.e. pooled data from Fig. 1C–D), to determine the lowest effect concentration for DMSO. The "Anova" command within the "car" library was used to extract the results for the main effects whereas the "lsmeans" command within the "emmeans" library was used as a post-hoc test to compare groups against one another while adjusting for the means of other factors within the model (Lenth, 2016). Type II sum of squares were used for models without interactions, whereas main effects were calculated using type III sum of squares when interactions were present within the final model. The R^2 of the model was determined using the command "r.squaredGLMM" that returns the marginal and conditional R^2 that represent the variance explained by the fixed actors alone excluding the random effect and the variance of the entire model including the random effect, respectively (Nakagawa and Schielzeth 2013). The raw data (Behaviour.csv) can be found within the supplementary material as can plots of the raw data (Supplementary figures.pdf). For all models, examination of the residual plots verified that no systematic patterns occurred in the errors (e.g. standardized residuals vs fitted values). Five models were corrected for heteroscedasticity using the command "weights = varPower()", DMSO and strain (distance

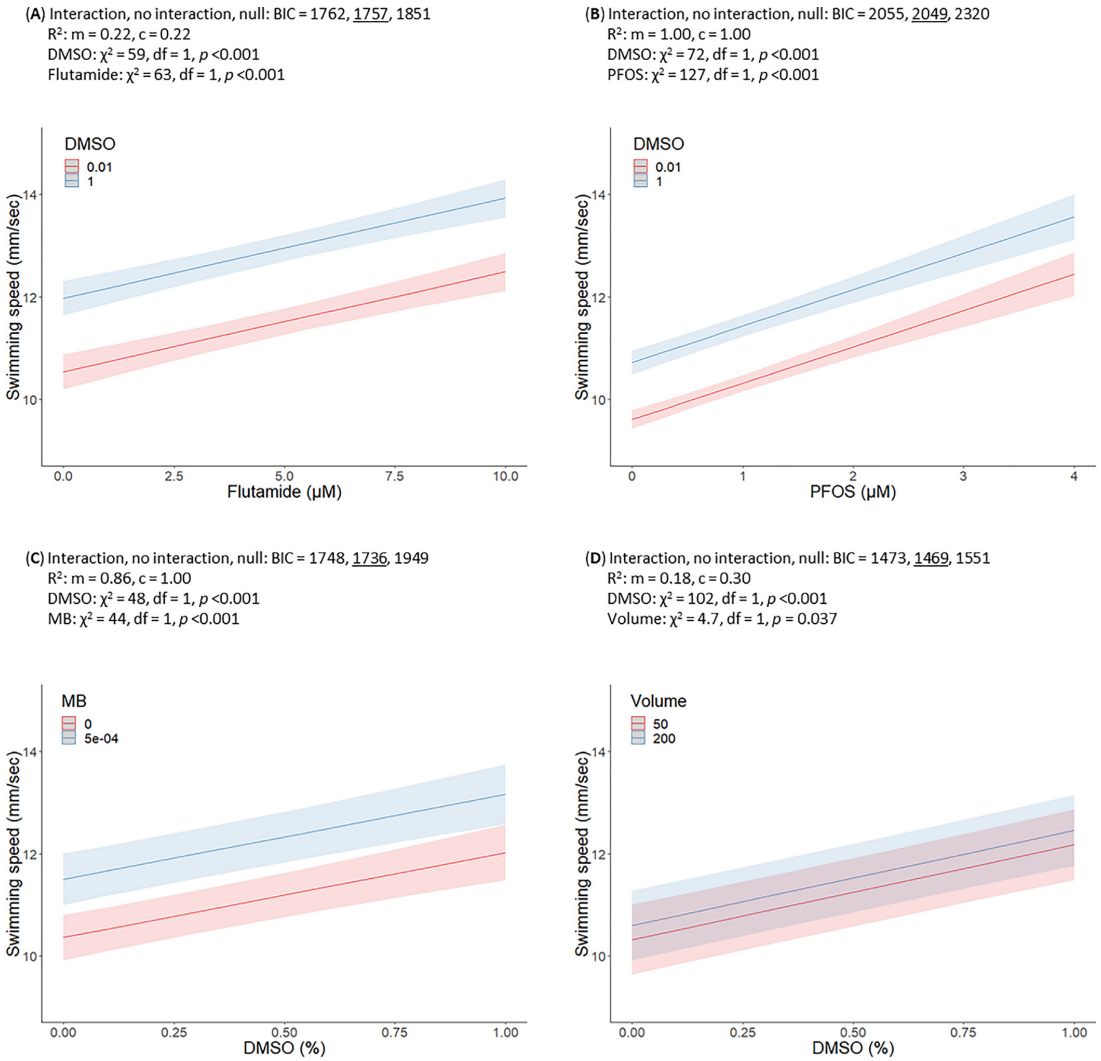

Fig. 1. Dose responses following co-exposure studies in larval zebrafish. (A) Dimethyl sulfoxide (DMSO) co-exposed with flutamide. (B) DMSO co-exposed with perfluorooctanesulfonic acid (PFOS). (C) DMSO co-exposed with methylene blue (MB). (D) DMSO and media volume. The model with the lowest BIC score (underlined) is presented along with the magrinal (m) and conditional (c) R^2. Results are those of linear mixed effect models and include regression lines ± 95% CI. N = 32–47 group^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

moved and time active), DMSO co-exposed with MB (time active and swimming speed), and PFOS co-exposed with DMSO (swimming speed). Significance was assigned at $p = < 0.05$.

3. Results

All results are summarized in Table 2

3.1. Strain and single exposures

Strain effects were apparent with TL showing significantly less locomotion compared to AB and AB/TU. However, there were no interactions between strain and any of DMSO, MeOH, or MB, on behavioral endpoints as all models without the interaction had a lower BIC score than those with the interaction.

There was a significant reduction in the time spent active, but an increase in swimming speed, in those larvae exposed to 1% DMSO compared to the control. When using the data from the co-exposure studies, the lowest observed effect concentration was 0.55% DMSO (lsmean post hoc, df = 412, $t = -4.3$, $p = 0.003$). MeOH had no effect on any behavioral endpoint. The distance moved and time active were significantly reduced by 0.0005% MB, but swimming speed was significantly increased, compared to lower concentrations. Greater media volume led to significant increases in the distance moved and time spent active, but there was no effect on swimming speed.

3.2. Interactions between toxins and methods

Although we saw the expected dose dependent increase in swimming speed with FLU, PFOS, DMSO, and MB, there were no interactions between flutamide and DMSO (Fig. 1A), PFOS and DMSO (Fig. 1B), DMSO and MB (Fig. 1C), or DMSO and media volume (Fig. 1D).

For the distance moved and the time active there was no interaction or addition between DMSO and either FLU (Distance moved: BIC scores, 6421 [interaction], 6418 [no interaction], 6429 [null], $R^2 = 0.05$ [marginal] and 0.08 [conditional], DMSO $\chi^2 = 23$, df = 1, $p < 0.001$, FLU $\chi^2 = <1$, df = 1, p = 0.967; Time active: BIC score, 4337 [interaction], 4334 [no interaction], 4333 [null]) or media volume (Distance moved: BIC score, 6190 [interaction], 6192 [no interaction], 6182 [null]; Time active: BIC scores, 4099 [interaction], 4102 [no interaction], 4094 [null]). In contrast, DMSO had an interaction effect with PFOS (Fig. 2AB) and MB (Fig. 2CD). Here, the distance moved and time active tended to be positively associated with PFOS when combined with 0.01% DMSO, but there as a negative association when combined with 1% DMSO. Similarly, the distance moved and time active tended to show a slight positive association with DMSO concentration in the absence of MB, but a negative association with DMSO concentration when in the presence of 0.0005% MB.

4. Discussion

We assessed various aspects of methodology relevant to toxicity testing on zebrafish locomotor activity using a high-throughput methodology. We found that DMSO, MB, media volume, and strain all had an effect on the behavioral response of larval zebrafish. In co-exposures between DMSO and MB, flutamide, or PFOS, these compounds either acted independently of one another, or interacted with one another, depending on the locomotor endpoint measured. Similarly, DMSO and media volume had additive effects. These results have important implications when trying to translate larval behavioral studies or comparing studies between laboratories, regarding toxicity testing.

Table 2
Behavioral data and statistical results for larval zebrafish of different strains following exposures to carrier solvents and/or media volume. The data are means with the upper and lower ranges based on lsmeans in parentheses, $N = 41–48$ group^{-1}. For model comparison, the BIC score is reported. The model with the lowest BIC score is underlined, and the statisitics are from lme models, unless the null model had the lowest BIC score, and include the marginal (m) and conditional (c) R^2 of the chosen model. Different subscript letters within rows indicate significant group effects (lsmean post hoc).

Parameter	Strain/Concentration/Volume				Model comparison (BIC score)	R^2 (m, c)	Model results (χ^2, df, p)
Strain	AB	AB/TU	TL		Strain, null		Strain
Distance moved (mm)	951 (468–1435)^a	853 (367–1339)^a	628 (143–1113)^b		2029, 2040	0.12, 0.30	23, 2, <0.001***
Time active (seconds)	99 (52–145)^a	87 (40–133)^a	64 (17–110)^b		1381, 1397	0.14, 0.32	28, 2, <0.001***
Swimming speed (mm/s)	9.54 (8.85–10.23)	9.71 (9.00–10.41)	9.86 (9.16–10.56)		389, 382	–	–
Dimethylsulfoxide (DMSO)	0.00%	0.01%	0.10%	1.00%	Strain×DMSO, Strain+DMSO, null		Strain DMSO
Distance moved (mm)	845 (744–946)	892 (788–996)	908 (803–1014)	835 (734–935)	8241, 8229, 8212	–	–
Time active (seconds)	81 (70–92)	83 (72–94)	84 (74–95)	69 (59–80)	5550, 5540, 5538	–	– –
Swimming speed (mm/s)	10.54 (9.81–11.27)^a	10.66 (9.93–11.39)^a	10.75 (10.02–11.48)^a	12.55 (11.82–13.28)^b	2165, 2154, 2274	0.20, 0.37	1, 2, 0.480 155, 1, <0.001***
Methanol (MeOH)	0.00%	0.01%	0.10%	1.00%	Strain×MeOH, Strain+DMSO, null		Strain MeOH
Distance moved (mm)	1162 (1039–1285)	1064 (940–1188)	1077 (954–1200)	1062 (939–1185)	8433, 8421, 8411	–	– –
Time active (seconds)	111 (98–124)	102 (89–115)	103 (90–116)	102 (89–115)	5729, 5717, 5704	–	– –
Swimming speed (mm/s)	10.37 (9.69–11.05)	10.54 (9.86–11.22)	10.49 (9.82–11.17)	10.49 (9.81–11.17)	1970, 1965, 1949	–	– –
Methylene blue (MB)	0.0000%	0.0001%	0.0002%	0.0005%	Strain×MB, Strain+MB, null		Strain MB
Distance moved (mm)	903 (778–1029)	864 (738–989)	897 (771–1022)	739 (613–865)	7948, 7936, 7934	–	– –
Time active (seconds)	91 (79–103)^a	88 (76–100)^a	90 (78–101)^a	70 (58–82)^b	5430, 5417, 5427	0.06, 0.15	2, 2, 0.406 28, 1, 0.001***
Swimming speed (mm/s)	9.75 (9.41–10.09)^a	9.74 (9.41–10.08)^a	10.00 (9.66–10.34)^a	10.53 (10.19–10.87)^b	1865, 1856, 1875	0.11, 0.14	13, 2, 0.001*** 33, 3, 0.001***
Media volume	50 µl	100 µl	150 µl	200 µl	Volume, null		Volume
Distance moved (mm)	669 (415–920)^a	851 (601–1100)^b	952 (701–1202)^{bc}	1025 (774–1276)^c	3623, 3648	0.10, 0.20	34, 3, 0.001***
Time active (seconds)	63 (44–82)^a	80 (61–98)^b	91 (72–110)^{bc}	100 (81–118)^c	2427, 2461	0.14, 0.20	44, 3, 0.001***
Swimming speed (mm/s)	10.30 (9.20–11.41)	10.48 (9.39–11.57)	10.23 (9.13–11.32)	10.30 (9.20–11.41)	828, 823	–	–

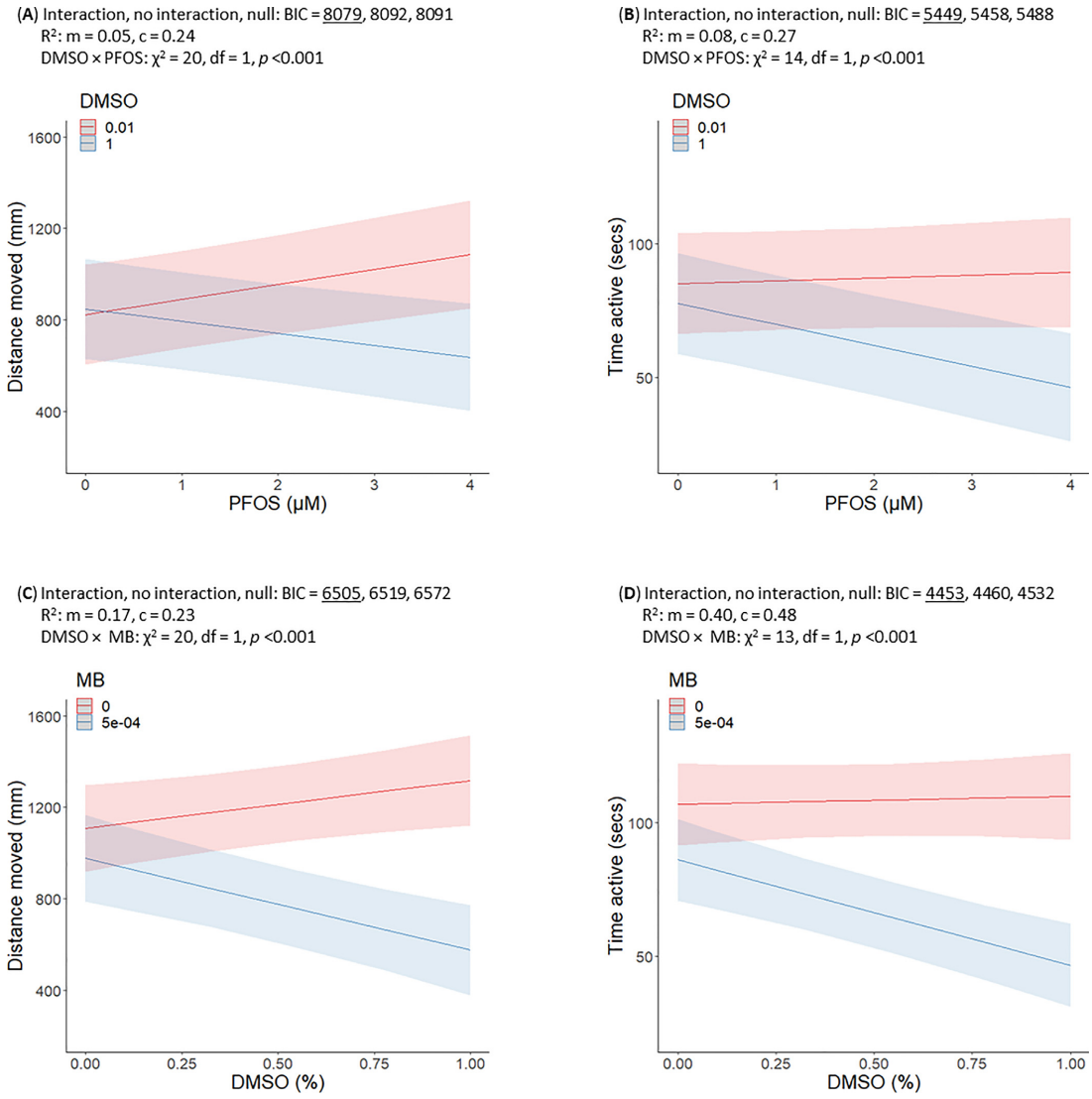

Fig. 2. Dose responses following co-exposure studies in larval zebrafish. The distance moved (A) and the time active (B) for dimethyl sulfoxide (DMSO) co-exposed with perfluorooctanesulfonic acid (PFOS). The distance moved (C) and the time acitve (D) for DMSO co-exposed with methylene blue (MB). The model with the lowest BIC score (underlined) is presented along with the magrinal (m) and conditional (c) R^2. Results are those of linear mixed effect models and include regression lines ±95% CI. *N* = 32–47 $group^{-1}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We found DMSO, but not MeOH, had sub-lethal effects on larval behavior, irrespective of the strain tested. Our lowest observed effect concentration of DMSO was 0.55%. Previous studies have also demonstrated that exposure to ≥0.01% DMSO led to hyperactivity in 144 hpf larvae (de Esch et al. 2012), but also adult zebrafish exposed to 0.05% DMSO for 3–4 min (Sackerman et al., 2010). The differences observed in the lowest effect concentrations of DMSO might be due to the different behavioral assays or age of the fish. Previous studies have shown that behavioral outcomes vary depending on larval age (de Esch et al. 2012, Fraser et al. 2017a, Padilla et al., 2011), and we previously found larval behaviour was less sensitive to endocrine disrupting compounds compared to the literature on adult behaviour and/or molecular endpoints (Fraser et al. 2017b). At the molecular and protein level, it has also been shown that DMSO at concentrations as low as 0.01% can affect the expression of genes that are related to metabolic, developmental, and other biological processes (Turner et al., 2012), and the expression of heat shock proteins (Hallare et al. 2006). In contrast, we found no behavioral alterations in zebrafish larvae exposed to MeOH up to a concentration of 1%. This agrees with a previous study by (Lockwood et al. 2004) which showed exposure to methanol up to 7 dpf at a concentration of

1.5% had no significant effect on swimming speed. However, at the physiological level, larval zebrafish may be more sensitive since 0.05% methanol significantly reduced the expression of CYP1A and inhibited EROD activity (David et al. 2012). A comparison of the teratogenic effects of DMSO and MeOH showed that for both solvents larvae were quite tolerant, exhibiting malformations at concentrations between 2 and 2.5% (Maes et al. 2012). Based on our current results, when testing the behavioral effect of substances using our protocol, we recommend DMSO not be used >0.3% whereas MeOH can be used at concentrations as high as 1%. However, it is clear from the available literature that other endpoints and behavioral protocols maybe more sensitive to the concentrations of DMSO and MeOH recommended for use with our behavioral paradigm.

We found behavioral effects of MB, with a lowest effect concentration of 0.0005% (15.6 μM). It is generally recommended that for zebrafish larvae rearing the concentration of MB should be equal to 0.0002% (6.3 μM, Westerfield, 2007) for which we found no behavioral effects. In a study conducted by Hedge et al. (2017), zebrafish larvae treated for 6 dpf with MB at concentrations up to 10 μM were not affected in terms of their locomotor activity or any of the developmental aspects examined (death, hatching rate, swim bladder inflation, or deformities). Another study found MB led to a hermetic response on memory retention in adult zebrafish tested in a t-maze (Echevarria et al. 2016). Compared to controls, fish exposed to 0.5 μM MB performed significantly better, fish that received 5 μM did not exhibit any differences, whereas fish exposed to 10 μM performed worst (Echevarria et al. 2016). Based on the results of the above-mentioned studies and our observations, we recommend using concentrations of ≤0.0002% MB (6.3 μM) for raising larvae destined for behavioral testing. However, it is noted we continuously exposed larvae to MB from 6 hpf until testing (98–102 hpf) whereas others may only briefly wash larvae in MB immediately after fertilization. Therefore, future work should address toxicity thresholds for shorter exposure periods.

Having observed effects of DMSO and MB alone, we expanded our study to evaluate whether these compounds would interact with one another or other positive controls. Here, we found our results depended on the endpoint measured. When assessing swimming speed, we found no interactions between DMSO and MB, or between DMSO and two positive controls, PFOS or flutamide. Instead, we found these compounds had additive effects when used in combination. In contrast, the distance moved and time active showed interaction effects with PFOS and MB, but not FLU. For example, the distance moved was negatively associated with increased DMSO in the presence of MB, but showed a slight positive association with DMSO in the absence of MB. Therefore, further tests are required to understand whether other endpoints, such as molecular pathways, protein expression, neuroanatomy, may be influenced by solvents or MB during testing.

As expected, we found strain effects on behavioral profiles as the TLs exhibited a general decrease in activity in relation to both the AB and the AB/TU strains. Our results agree with previous studies showing that the AB strain at ages 5 and 6 dpf show higher activity than larvae belonging to the TL strain (de Esch et al. 2012), but are in contrast to another study that reported higher activity in the TL strain compared to AB (van den Bos et al., 2017). However, these studies cannot be directly compared due to differences in methodology. For example, van den Bos et al. (2017) raised the larvae together in petri dishes, which is reported to increase activity compared to those larvae raised in isolation (Zellner et al., 2011), the latter being the method we employed. In addition, van den Bos et al. (2017) used 24 well plates compared to our study that used 96 well plates, and larger arena have been reported to generally increase locomotor activity (Fraser et al. 2017a). In addition, the same strain of fish coming from different laboratories may differ in their levels of genetic variation that may also influence behavior (Coe et al. 2009). Nevertheless, van den Bos et al. (2017) also reported elevated gene expression of several markers related to neurodevelopment in AB larvae compared to TL larvae suggesting AB larvae may develop faster, which may translate to a stronger increase in activity in response to changes between light and dark conditions. The same authors also showed that AB larvae have a higher baseline level of cortisol that is commonly associated with behavioral differences (van den Bos et al., 2017). Therefore, physiological differences in strains exist that could influence behavior.

We observed no interaction between DMSO and different strains of zebrafish. This means that for larval behavioral studies, DMSO may have little influence on different zebrafish strains. However, the behavioral outcome of the AB and TU strains were found to be differentially affected when zebrafish larvae were exposed to the NMDA receptor antagonist MK-801 (Liu et al. 2014). Similarly, Loucks and Carvan (2004) found the response of three zebrafish strains (EK, AB, TU) to different concentrations of ethanol during the first 6 days of development varied in terms of survival, neurocranial and craniofacial skeletal development, and cell death (Loucks and Carvan 2004). Similarly, strain effects on reproductive endpoints have been recorded in zebrafish exposed to endocrine disruptors (Brown et al. 2011, Söffker et al., 2012). Therefore, although there is evidence strain effects exist, they appear to be endpoint sensitive.

In accordance with previous work on arena size, we found the amount of media within a given arena effected baseline behavior. In general, locomotor activity decreased with decreasing media volume. Similarly, Padilla et al. (2011) found zebrafish larvae kept in a 24 well plate moved more than the larvae kept in 48 and 96 well-plates, although the level of activity did not differ between larvae kept in 96 and 48 well plates. Based on their results the authors hypothesized that the activity of larvae is related more to the circumference of the arena rather than the area of the well (Padilla et al., 2011). In our study, we kept the circumference of the arena consistent by using 96 well plates throughout, but changed the area available for larvae to move in by altering media volumes. As activity increased with increasing media volume, it may be that larvae move more due to the increase in available space. Our results agree with a previous study that larvae kept in deep wells were more motile than larvae kept in shallow wells (Ingebretson and Masino 2013). This should be taken into account when comparing behavioral outcomes from different studies that use different testing volumes. However, we observed no interaction between media volume and DMSO concentration, suggesting that for behavioral studies at least, shallower wells may not influence toxicity results.

In conclusion, locomotor activity was shown to be influenced by various aspects of methodology, such as solvent, the use of MB, media volume, and strain. These results show that basal locomotor activity can be influenced by methodology making the standardization of experimental parameters in behavioral testing essential in order for direct comparisons between laboratories. We found both additive and interaction effects between methodologies depending on the behavioral endpoint measured in response to positive controls. Following the identification of sources of variability in this study, but also those preceding it, these parameters should be tested within different laboratories and behavioral tests in order to work towards the standardization of protocols.

CRediT authorship contribution statement

Maria Christou: Investigation, Writing - original draft, Visualization. **Arturas Kavaliauskis:** Investigation, Writing - review &

editing. **Erik Ropstad:** Resources, Funding acquisition, Writing - review & editing, Supervision, Project administration. **Thomas William Kenneth Fraser:** Conceptualization, Investigation, Formal analysis, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134490.

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Supplementary material

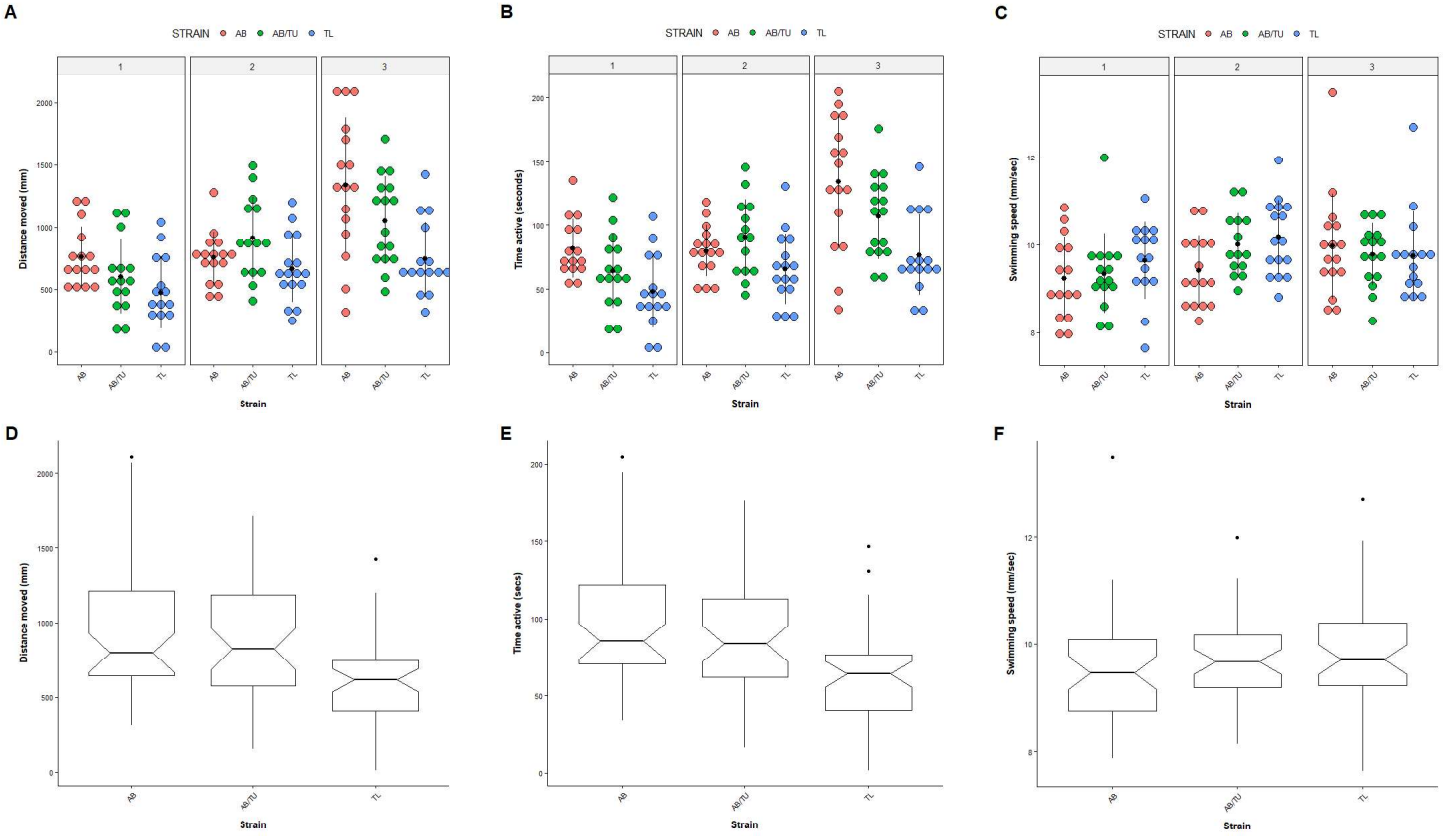


Figure S1 Raw data from studies on strain effects on larval locomotion. Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate. Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates. The notch is based on the median ± 1.58 × interquartile range (IQR)/ square root (n). The box represents the IQR (25t and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

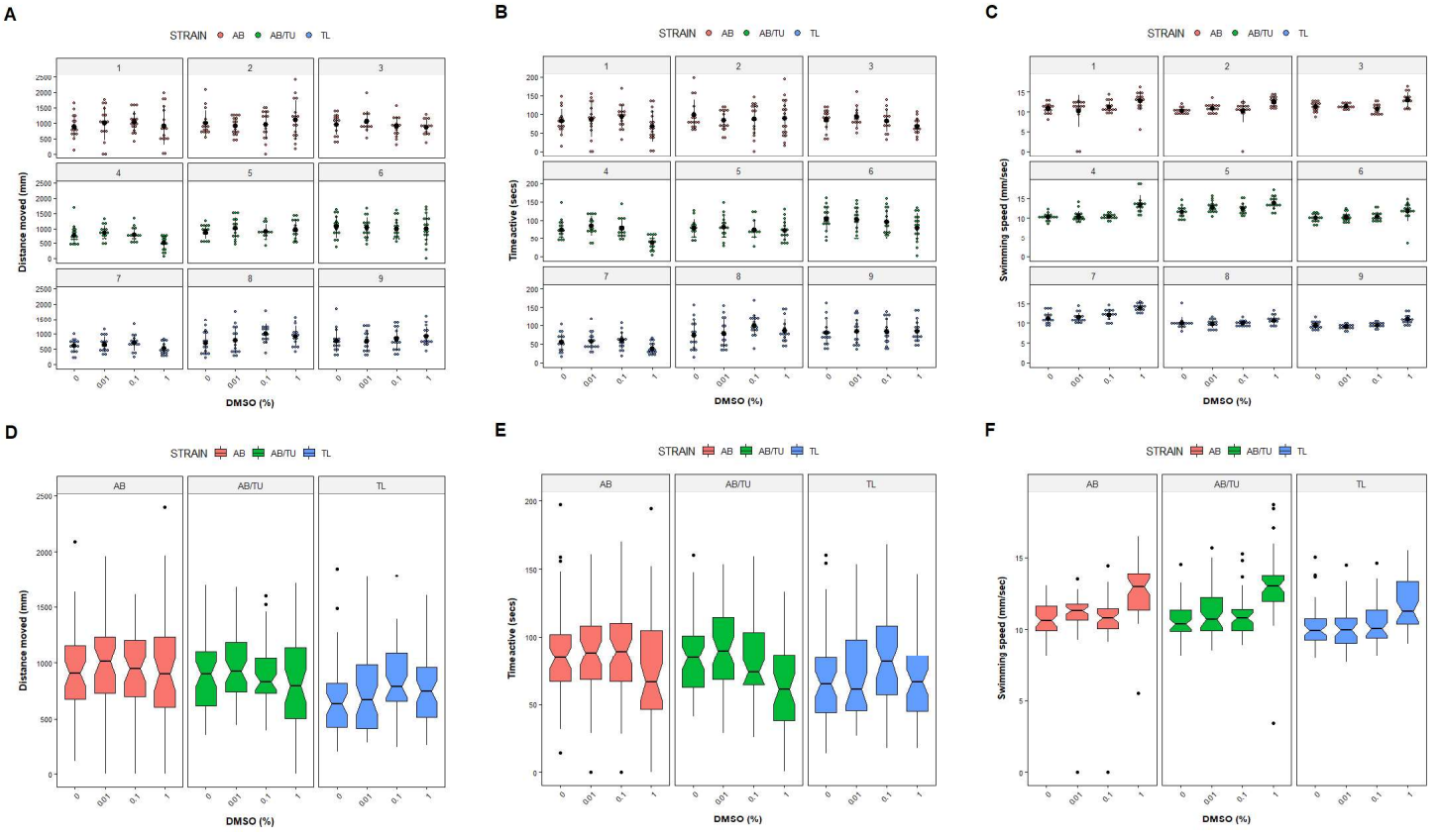


Figure S2 Raw data from studies on strain effects and dimethyl sulfoxide (DMSO) on larval locomotion. Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate and strain. Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates. The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (*n*). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

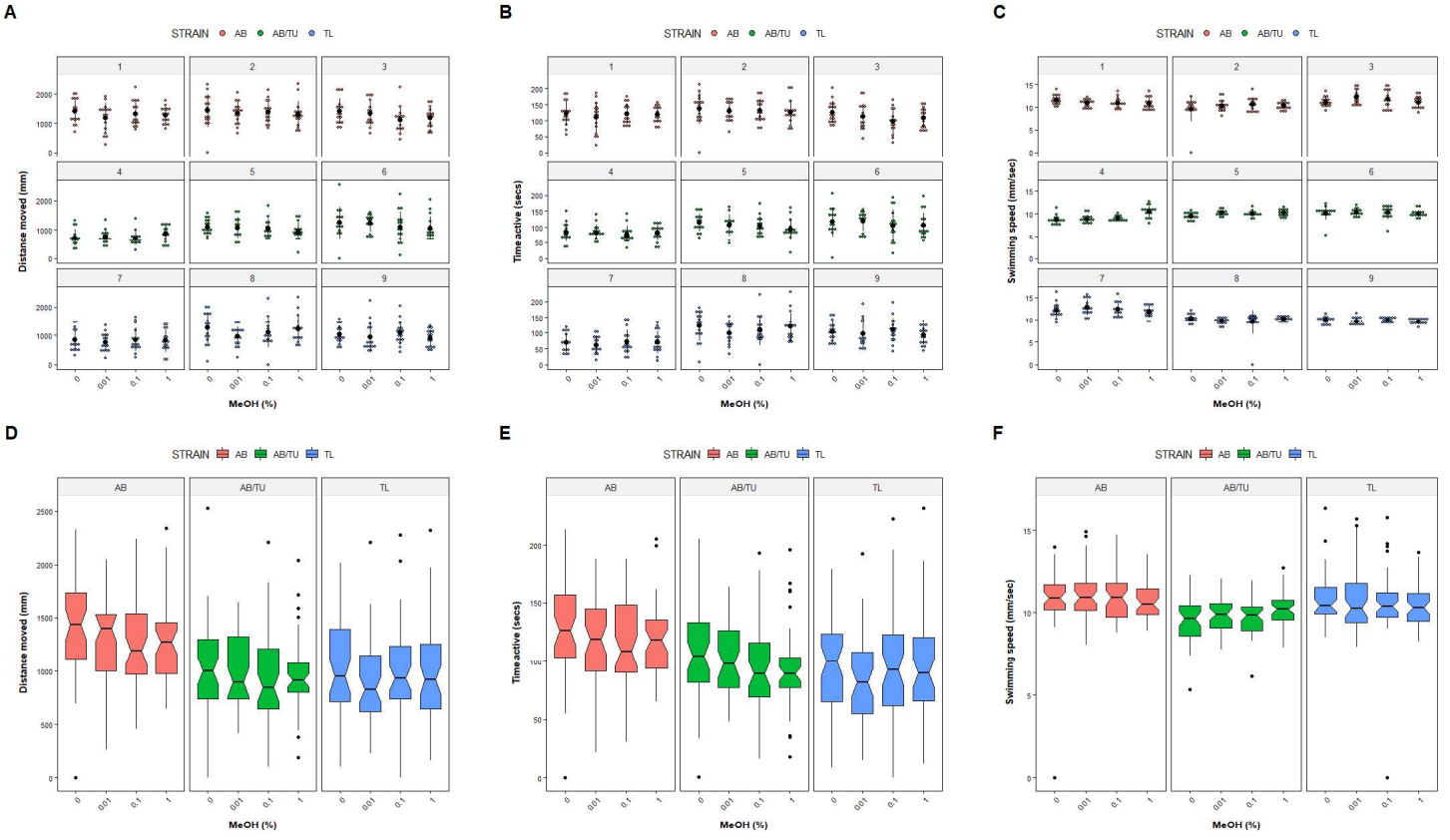


Figure S3 Raw data from studies on strain effects and methanol (MeOH) on larval locomotion. Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate and strain. Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates. The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (n). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

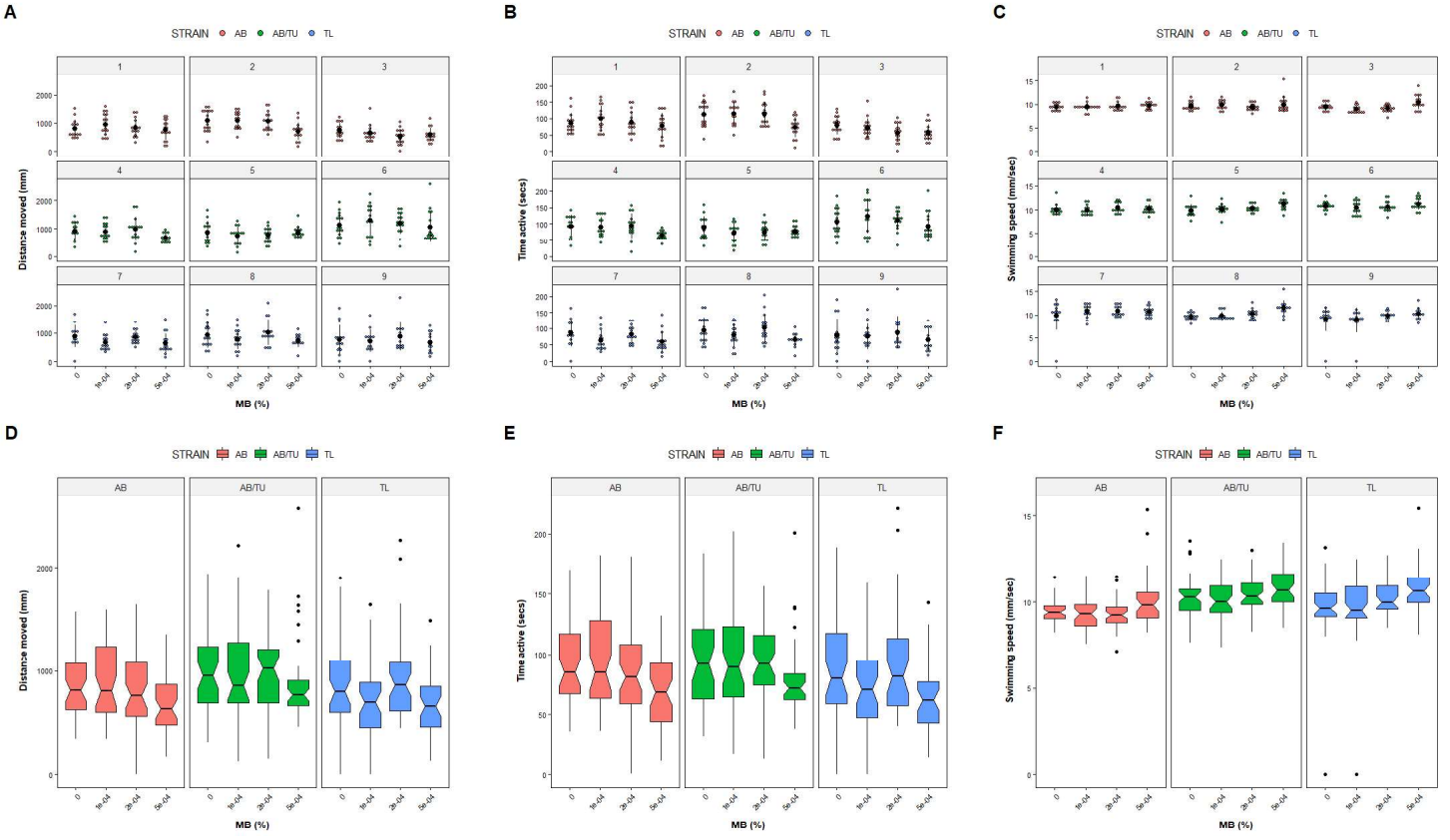


Figure S4 Raw data from studies on strain effects and methylene blue (MB) on larval locomotion. Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate and strain. Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates. The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (n). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

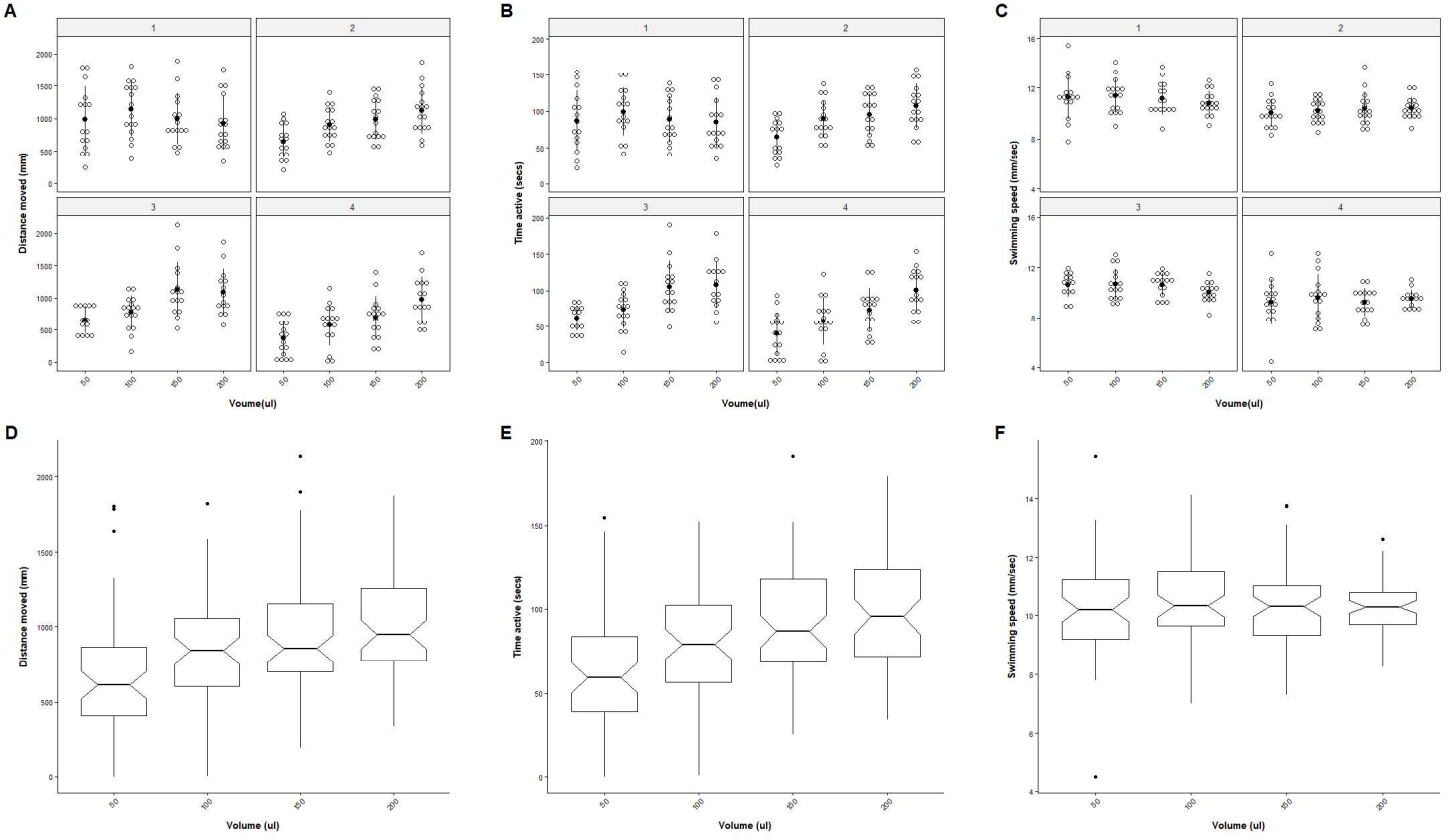


Figure S5 Raw data from studies on media volume on larval locomotion. Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate. Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates. The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (n). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

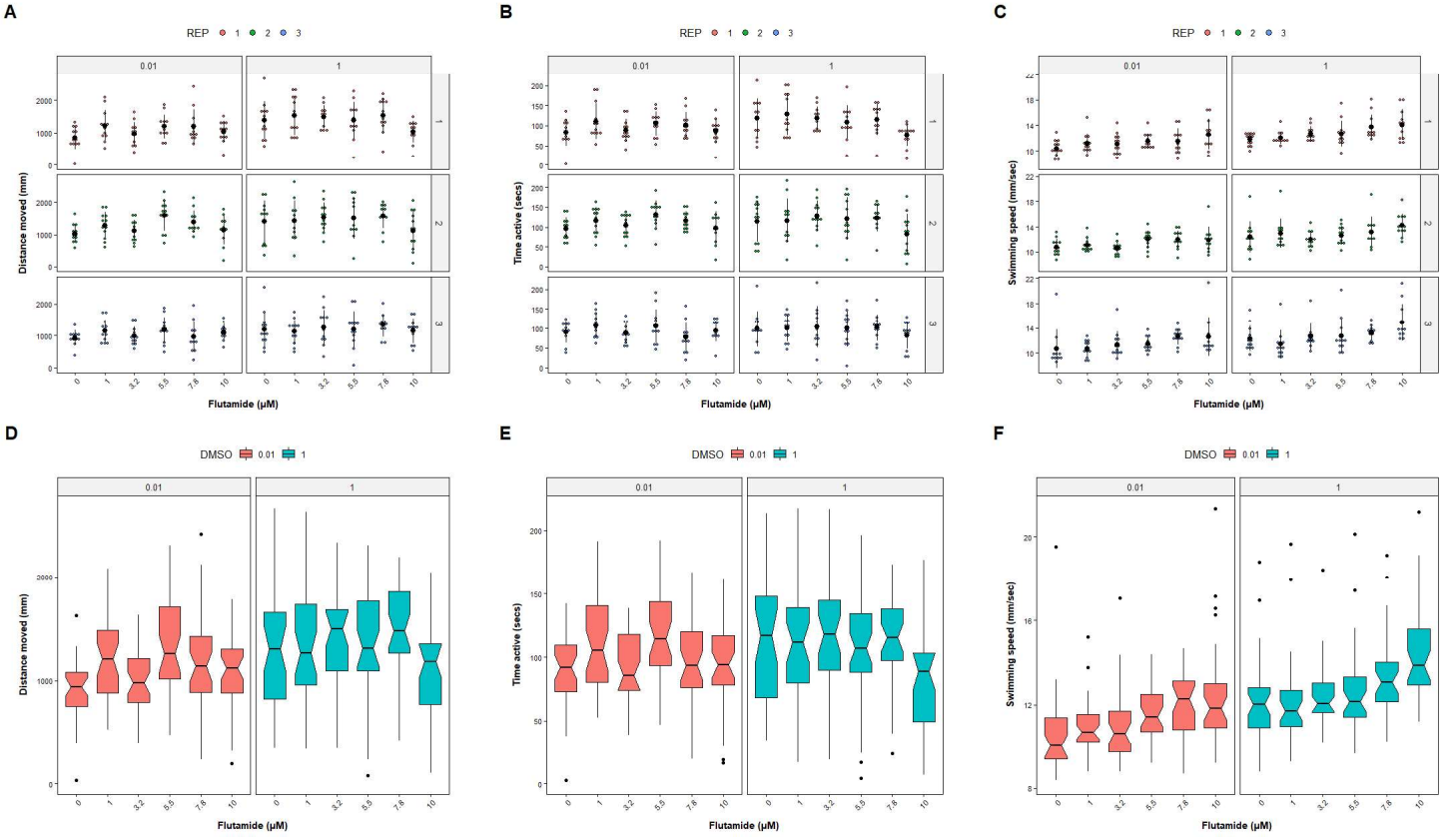


Figure S6 Raw data from studies on flutamide and dimethyl sulfoxide (DMSO). Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate and DMSO concentration (0.01 and 1.00 %). Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates for each DMSO concentration (0.01 and 1.00 %). The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (n). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

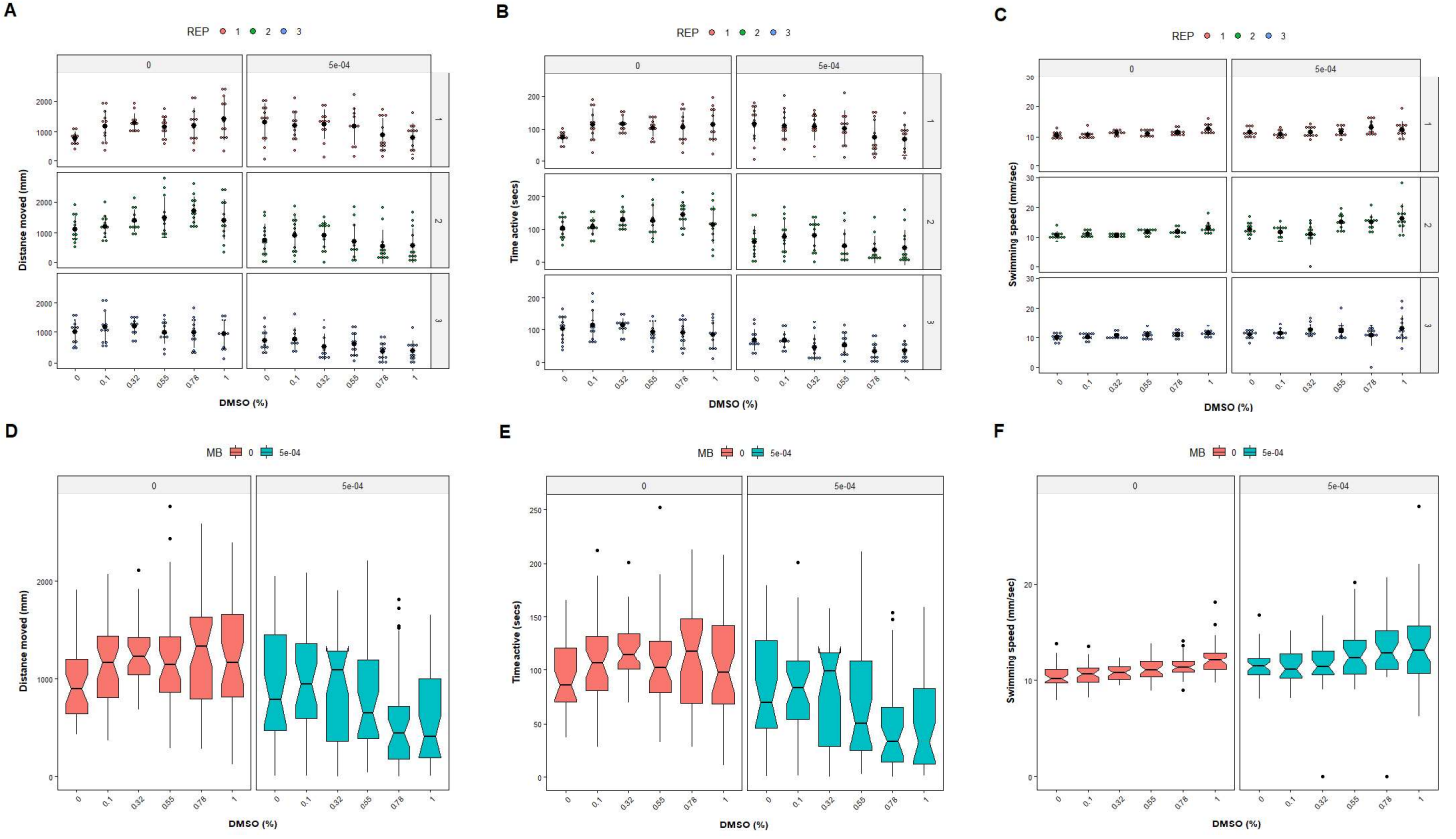


Figure S7 Raw data from studies on methylene blue (MB) and dimethyl sulfoxide (DMSO). Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate and MB concentration (0.0000 and 0.0005 %). Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates for each MB concentration (0.0000 and 0.0005 %). The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (n). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

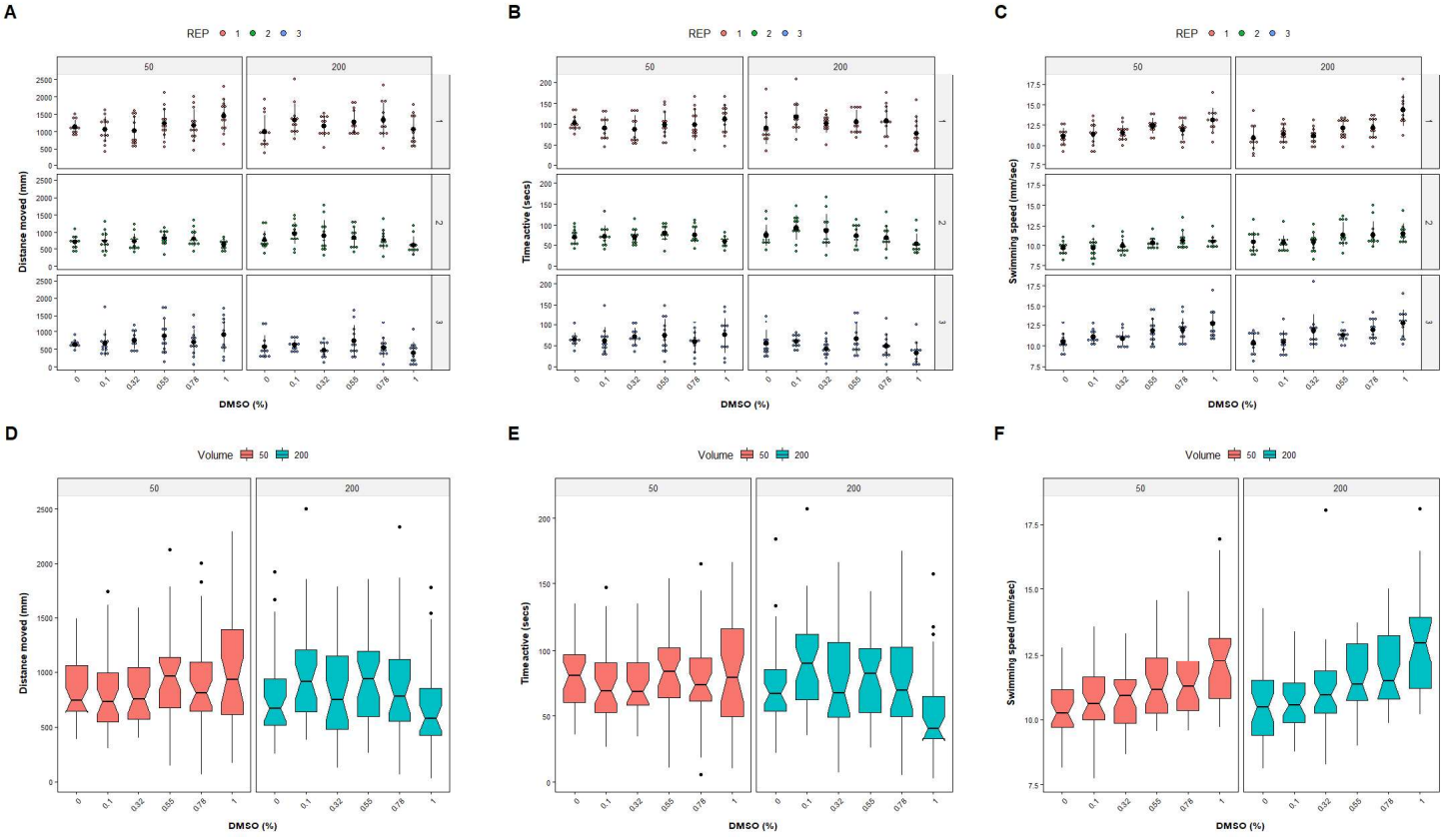


Figure S8 Raw data from studies on media volume and dimethyl sulfoxide (DMSO). Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate and media volume (50 and 200 µl). Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates for each media volume (50 and 200 µl). The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (n). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.

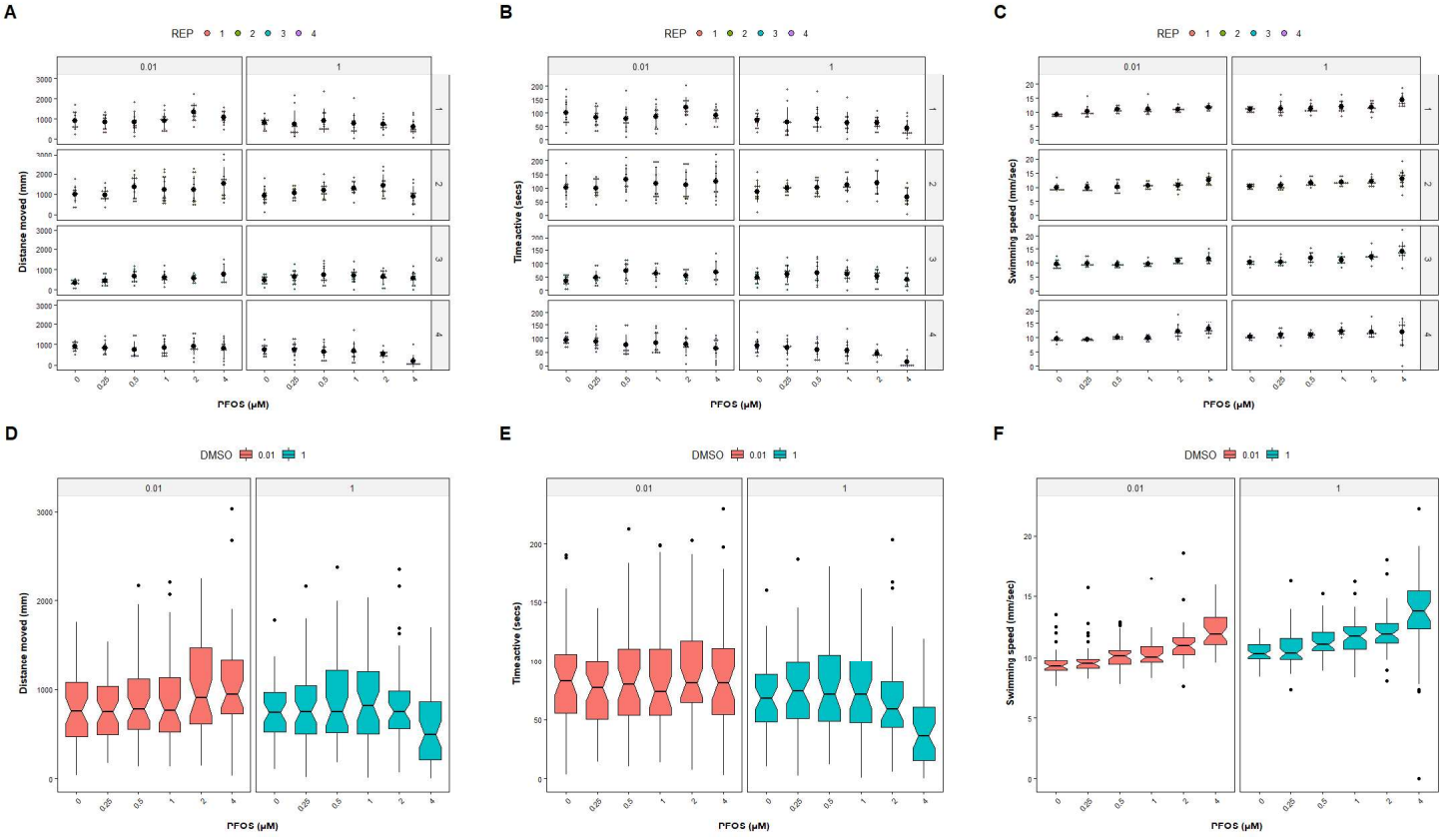
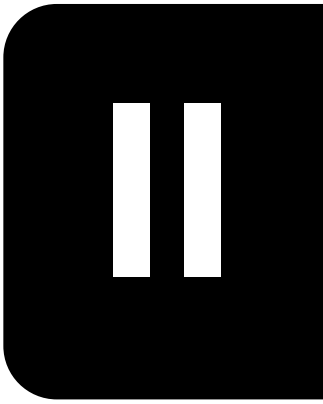


Figure S9 Raw data from studies on perfluorooctanesulfonic acid (PFOS) and dimethyl sulfoxide (DMSO). Dot plots with the mean ± SD for the (**A**) distance moved, (**B**) time active, and (**C**) swimming speed for each replicate and DMSO concentration (0.01 and 1.00 %). Notched Tukey box and whisker plots for (**D**) distance moved, (**E**) time active, and (**F**) swimming speed for pooled data from all replicates for each DMSO concentration (0.01 and 1.00 %). The notch is based on the median ± 1.58 × interquartile (IQR) range / square root (n). The box represents the IQR ranges (25th and 75th), the line represents the 50th percentile, and the whiskers show the smallest and largest value within 1.5× the IQR of the 25th and 75th percentiles, respectively. The dots represent values above and below 1.5× the IQR.



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Calcium signaling as a possible mechanism behind increased locomotor response in zebrafish larvae exposed to a human relevant persistent organic pollutant mixture or PFOS

Maria Christou[a,*], Thomas W.K. Fraser[a], Vidar Berg[b], Erik Ropstad[a], Jorke H. Kamstra[c]

[a] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, P.O Box 369 Sentrum, 0102, Oslo, Norway
[b] Department of Food Safety and Infection Biology, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, P.O Box 369 Sentrum, 0102, Oslo, Norway
[c] Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences, Utrecht University, 3584, CM Utrecht, the Netherlands

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ABSTRACT

Persistent organic pollutants (POPs) are widespread in the environment and their bioaccumulation can lead to adverse health effects in many organisms. Previously, using zebrafish as a model vertebrate, we found larvae exposed to a mixture of 29 POPs based on average blood levels from the Scandinavian population showed hyperactivity, and identified perfluorooctanesulfonic acid (PFOS) as the driving agent for the behavioral changes. In order to identify possible mechanisms, we exposed zebrafish larvae from 6 to 96 h post fertilization to the same mixture of POPs in two concentrations or a single PFOS exposure (0.55 and 3.83 μM) and performed behavioral tests and transcriptomics analysis. Behavioral alterations of exposed zebrafish larvae included hyperactivity and confirmed previously reported results. Transcriptomics analysis showed upregulation of transcripts related to muscle contraction that is highly regulated by the availability of calcium in the sarcoplasmic reticulum. Ingenuity pathway analysis showed that one of the affected pathways in larvae exposed to the POP mixture and PFOS was calcium signaling via the activation of the ryanodine receptors (RyR). Functional analyses with RyR inhibitors and behavioral outcomes substantiate these findings. Additional pathways affected were related to lipid metabolism in larvae exposed to the lower concentration of PFOS. By using omics technology, we observed that the altered behavioral pattern in exposed zebrafish larvae may be controlled directly by mechanisms affecting muscle function rather than via mechanisms connected to neurotoxicity.

1. Introduction

Persistent organic pollutants (POPs) are organic compounds that are resistant to environmental degradation. POPs include polychlorinated biphenyls (PCBs), dichlorodiphenylsichloroethanes (DDTs), brominated flame retardants (BFRs), dioxins and *per-* and poly-fluoroalkylated substances (PFASs) (UNEP, 2005) with perfluorooctanesulfonic acid (PFOS) being the one most frequently detected in the environment (Paul et al., 2009). Many of the POPs were used, or are still used today, as pesticides, industrial chemicals, solvents, and pharmaceuticals, and most of them are man-made. Their persistence in the environment and high lipid solubility means that POPs can readily bioaccumulate in fatty tissues (Ritter et al., 1998). As such, POPs are detected at high concentrations in wildlife, such as birds, fish, and marine mammals, but also humans (Giesy and Kannan, 2001; Boon et al., 2002; Chen and Hale, 2010; Porta et al., 2012; Kato et al., 2015; Olsen, 2015; Jepson et al., 2016). In humans, POP exposures mainly occur through food consumption, but also via drinking water, outdoor and indoor air, and from the working environment (EFSA, 2008; Guo et al., 2019; WHO, 2020).

Exposures to POPs have been found to lead to diverse effects on health. For example, POP exposures have been linked to the rise of type 2 diabetes and obesity (Ruzzin, 2012; Thayer et al., 2012; Guo et al., 2019), endocrine disruption (Li et al., 2008; Gregoraszczuk and Ptak, 2013), hormone-related cancers (Brody et al., 2007), the disruption of sexual development (Vallack et al., 1998; Sanderson, 2006), and cardiovascular disease (Lind et al., 2012; Ljunggren et al., 2014). Of more recent concern are early life exposure to POPs in utero or during breast feeding, since exposure during this sensitive period of development can have persistent effects on different life traits such as neurodevelopment. Early life exposures to POPs for instance have been associated with effects on cognitive functions such as poorer performance in intelligence tests, language, processing speed, short term memory, and association with traits relating to ADHD including problems in impulse

* Corresponding author.
E-mail address: maria.christou@nmbu.no (M. Christou).

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control, hyperactivity, and attention (Hoffman et al., 2010; Lam et al., 2017; Pessah et al., 2019).

Due to their persistent nature, POPs are rarely found as single elements within individuals, but rather occur in mixtures and can be passed on from mothers to developing embryos (Porta et al., 2012; Pumarega et al., 2016; Berntsen et al., 2017; Haug et al., 2018). This information makes the study of mixture effects of high importance since it is hard to predict toxic effects based on single chemical exposure as some of these chemicals may interact in a synergistic, additive, or antagonistic manner (Bopp et al., 2018).

Risk associations of exposure to POPs and related adverse health effects have not only been obtained from epidemiological studies, but also from *in vitro* and *in vivo* testing (Brody et al., 2007; Ruzzin, 2012; Guo et al., 2019; Pessah et al., 2019). Animal testing in particular has provided a plethora of evidence of the effects of chemicals on the physiological and molecular level (Parasuraman, 2011). Zebrafish (*Danio rerio*) are a freshwater fish that has been used extensively as a model organism in human and environmental toxicology due to its many advantages such as rapid development, small size, embryonic transparency, short generation time, and fully sequenced genome (Hill et al., 2005). POP exposures in zebrafish show similarity to effects in mammals (Hallgren et al., 2001; Keil et al., 2008; DeWitt et al., 2009; Chan and Chan, 2012; Martínez et al., 2019; Parsons et al., 2019), including effects on behavior (Johansson et al., 2008; Huang et al., 2010; Jantzen et al., 2016). As the fundamental processes of neurodevelopment are conserved among species (Lein et al., 2005), this makes zebrafish a powerful translational model for human health (Kalueff et al., 2016). Previous work on POPs has shown that larval zebrafish exposed to PCBs displayed a decreased avoidance behavior when presented with a visual stimulus (Lovato et al., 2016). In other studies, exposure to PFASs led to either hyperactive or hypoactive locomotor activity, burst activity and startle response depending on the concentration and age of testing in zebrafish larvae (Huang et al., 2010; Spulber et al., 2014; Jantzen et al., 2016; Khezri et al., 2017; Menger et al., 2020). Exposure to BDE-47 led to an increase in spontaneous movement, a decrease in touch response, and a decrease in swimming speed, in a dose-dependent manner (Chen et al., 2012). In a large-scale study by the National Toxicology Program, behavioral screening of BFRs revealed mainly hypoactivity of zebrafish larvae whereas exposure to DDT and other organochlorine pesticides causes abnormal behavior i.e. hyperactivity or constant movement (Dach et al., 2018).

Previously we observed behavioral effects on zebrafish larvae that were exposed from 6 to 96 h post fertilization (hpf) to a mixture of POPs (Khezri et al., 2017). This POP mixture is based on the average levels of chemicals found in human blood of the Scandinavian population and consists of 29 chemicals including PCBs, BFRs, organochlorine pesticides and PFASs (Berntsen et al., 2017). The aim of this study was to employ zebrafish as a model organism to confirm the behavioral effect observed during developmental exposure to POPs previously observed in our group and employ another behavioral test, the thigmotaxis assay, to evaluate the effect of the POP mixture on anxiety levels of zebrafish larvae. We also included a single PFOS exposure since this chemical is suspected to be solely responsible for the behavioral effect attributed to the mixture exposure (Khezri et al., 2017). Furthermore, we performed RNA-seq analysis on exposed larvae with the aim of elucidating possible modes of action for PFOS and the POP mixture. Finally, chemical analysis was performed on exposed larvae for the evaluation of the uptake and accumulation of each chemical within the POP mixture.

2. Materials and methods

2.1. Fish maintenance and breeding

The experiments were performed at the Norwegian University of Life Sciences (NMBU), Oslo, Norway, which is licensed by the Norwegian Animal Research Authority (NARA) (www.mattilsynet.no). The experiments were carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway.

AB wild-type (AB) zebrafish were kept at 28 ± 1 °C under a 14:10 light/dark photoperiod. Animal care was performed in accordance with local protocols and more information can be found in Supplementary data. For embryo production, adults were placed in breeding tanks in the afternoon with a divider separating males and females. The next morning the separator was removed as soon as the lights turned on (08:00) and embryos were collected 1 h later. Embryos were maintained in sterile embryo media (60 μg/mL Instant Ocean® sea salts) until the time of exposure.

2.2. POP mixture and chemicals

Dimethyl sulfoxide (DMSO, > 99.7%, CAS number 67-68-5), PFOS (≥98%, CAS number 2795-29-3), dantrolene (≥95%, CAS number 14663-23-1) and caffeine (ReagentPlus®, CAS number 58-08-2), were purchased from Sigma-Aldrich. The composition of the POP mixture is described in Supplementary data Table S1 and further details of its preparation can be found in Berntsen et al. (2017).

Stock solutions of PFOS, dantrolene and the POP mixture were prepared in DMSO. A fresh stock solution of caffeine was made the day of the testing (diluted in embryo media) whereas stock solutions of PFOS, dantrolene, and the POP mixture were stored at −20 °C.

2.3. Solutions preparation

For all experiments two concentrations of the POP mixture were used. The low concentration was equal to the levels of chemicals that are 10 times higher than what is found in average Scandinavian human blood levels and the high concentration corresponds to levels 70 times higher than the average (exposures will be referred to as POP10 and POP70 in the remainder of the manuscript). The solutions were prepared on the day of the experiments by diluting the stock solution (1,000,000x) in sterile embryo media and adjusting the DMSO concentration to 0.1%. The concentrations of the POP mixtures were chosen based on previous studies performed in our group where the 70x concentration was the lowest concentration with an observable behavioral effect whereas the 10x concentration was the highest concentration with no observed behavioral effect (Khezri et al., 2017). Concentrations of PFOS were based on the nominal concentration of PFOS in the POP mixture. The low concentration of PFOS corresponded to 0.3 mg/L (0.55 μM, will be referred from now on as PFOS10) and the high concentration was equal to 2.06 mg/L (3.83 μM, will be referred to as PFOS70). PFOS solutions were prepared the day of the experiments by diluting the stock solution of PFOS (54.8 mM) in sterile embryo media and adjusting the DMSO concentration to 0.1%. The dantrolene concentration was chosen based on the current literature (Brennan et al., 2005; Yuen et al., 2013). Working solutions of dantrolene (50 μM) were prepared the day of the experiment by diluting the stock solution (7.4 mM) in sterile embryo media. Because of low solubility of dantrolene in DMSO, the final concentration of DMSO in the working solution was 0.68%. All exposures (Control, POP10, POP70, PFOS10, PFOS70) therefore for the dantrolene experiments were adjusted to a DMSO concentration of 0.68%. Caffeine concentration was selected initially at 250 μM based on Schnörr et al. (2012) but produced an overt toxic effect on larval zebrafish following chronic exposures (see Supplementary data Table S2), hence the concentration was adjusted to 50 μM. Working solutions of caffeine were prepared on the day of experiment in sterile embryo media and the concentration of DMSO was adjusted to 0.1%. Before and after all the experiments, the embryos were reared in an incubator at 28 ± 1 °C. The light cycle in the incubator was set at 14:10 light/dark (lights on 07:30/lights off 21:30).

2.4. Exposures for behavioral tests

For the light-dark transition test, fertilized embryos were transferred into clear polystyrene 96 well plates (Nunc™ MicroWell™) with one embryo per well and exposed under static conditions from 6 hpf until the time of testing at 96–100 hpf (between 9:00–13:00) in 200 μL of media. For the thigmotaxis assay embryos were placed in 24 well plates (Corning® Primaria™) with one embryo per well in 1 mL exposure media. Embryos were exposed either to the POP mixtures in two concentrations, POP10 and POP70, or two concentrations of PFOS, PFOS10 and PFOS70. Each well plate also included a control with embryos immersed in sterile embryo media (DMSO 0.1%). For the thigmotaxis assay two controls were used, one for the POP mixture treatment and one for the PFOS treatment respectively, as the plate layout meant that each group could not be equally represented on each row and column without the addition of the extra controls. All well plates were pre-incubated with the respective exposure for 24 h. The media was removed before the embryos were placed in the well and replaced with a fresh working solution. All groups were spread equally on each row and column to avoid position-bias during behavioral testing. For the light-dark transition test, each well plate included 10 embryos per condition and was repeated in five independent experiments. For the thigmotaxis assay, each well plate contained three embryos per condition with three well plates per replicate and was repeated in three independent experiments.

2.5. Exposure for transcriptome analysis

Fertilized embryos were exposed to five different conditions (Control, POP10, POP70, PFOS10, PFOS70) in six well plates (Falcon® 6-well). Each well plate included only one condition with four pseudoreplicates. In each well 15 embryos were placed with 3 mL exposure media. All wells were pre-saturated for 24 h prior to the experiment and the media was replaced with fresh working solution at the start of the experiment. Embryos were exposed in static conditions from 6 hpf until 96 hpf. Replicates of 10 non-deformed embryos per experimental condition were collected, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis (RNA extraction for high-throughput sequencing analysis and RT-qPCR for gene validation purposes).

2.6. Exposures for behavioral tests with RyR agonist and antagonist

Based on the transcriptome analysis, we designed an experiment to uncover a potential mechanism of action of POPs on the ryanodine receptor (RyR) using the light-dark transition behavioral test. Caffeine, an established agonist, and dantrolene, an established antagonist of RyR were used (Brennan et al., 2005; Hernández-Fonseca and Massieu, 2005; Yuen et al., 2013). Each compound was used in two different exposure scenarios. In the chronic exposure the larvae were exposed from 6 hpf until the behavioral testing at 96–100 hpf (between 9:00–13:00). In the acute exposure the compound was added in the well 10 min prior to the behavioral testing. Each compound and exposure scenario (chronic and acute) were tested in a separate well plate. Six conditions were included in each well plate, Control (DMSO 0.1% for the caffeine study or DMSO 0.68% for the dantrolene study), Control with DAN or CAF, POP70, POP70 with DAN or CAF, PFOS70 and PFOS70 with DAN or CAF. We chose the highest concentration of the chemicals in question because only the high concentrations had an effect on the behavioral outcome of zebrafish. For each test, 96 well plates (Nunc™ MicroWell™) were used with one embryo per well in 200 μL exposure media. Three independent experiments with 16 individuals per condition were performed. All groups were spread equally over each row and column. All wells were pre-incubated prior to the start of each experiment.

2.7. Behavioral assays

Behavioral assays were performed in a ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France). Behavioral tests were conducted between 9:00–13:00 in 96–100 hpf zebrafish larvae. For the light-dark transition test and the thigmotaxis test, the cumulative distance travelled and the time spent active were measured simultaneously for all larvae in a well-plate. The light-dark cycle lasted 20 min and consisted of 10 min of light and 10 min of dark. Prior to the test the larvae were acclimatized in the behavioral chamber for 10 min with the lights on. For the light-dark transition test the mean swimming speed was calculated by dividing the cumulated distance travelled by the total time spent active. For the thigmotaxis test the percent of the total distance moved in the outer zone was calculated. For this purpose, each well in a 24 well plate was divided in two zones (total diameter of each well 16.2 mm). The width of the outer zone was set at 5 mm relative to the border of the well. The light level was set to 100% on the ViewPoint software (7.45 Klux, TES 1337 light meter). Infrared light (850 nm) tracks larval activity during the “dark” periods. The threshold for determining movement was set at 5 mm/s. Dead and deformed (coagulated, unhatched, notochord deformations, yolk sac or cardiac edema, swim bladder development, loss of equilibrium) larvae were excluded from the analysis (Supplementary data, Table S2).

2.8. RNA purification

RNA from samples for RNA-seq analysis and gene expression for validation were purified using NucleoSpin® RNA extraction kit (Macherey-Nagel, Germany). RA1 lysismix (Macherey-Nagel, Germany) was added to each sample (10 larvae per sample) and samples were passed through a 21-gauge needle (HSW HENKE-JECT®, Germany) until complete homogenization. Total RNA was extracted from samples following manufacturer's instructions. Each sample was eluted in 50 μL RNase-free water and stored at −80 °C until further analysis. RNA purity for all samples was assessed using a Nanodrop-1000 Spectrophotometer (NanoDrop Technologies, DE, USA). For samples sent for RNA-seq analysis, RNA integrity number (RIN) was determined with Agilent 2100 Bioanalyzer (Agilent Technologies, Ca, USA) using RNA Nano LabChip Kit (Agilent Technologies, Ca, USA). All samples were found to be of acceptable quality for sequencing (RIN $>$ 9.0).

2.9. RNA-seq and transcriptome analysis

Samples for sequencing were sent to Novogene (Hong Kong). A total of 2 μg was used for library preparation. A quality check (QC) of total RNA was performed with a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), Agilent 2100 (Agilent Technologies, CA, USA) and agarose gel electrophoresis prior to library construction. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations (more information about RNA-seq analysis can be found in Supplementary data).

For the transcriptome analysis raw fastq files were adapter trimmed using trim_galore (v0.4.5, Babraham institute, UK) under standard parameters. We used the STAR aligner (v2.5.4 b) (Dobin et al., 2012) to align and map sequences to the zebrafish genome (GRCz11, https://www.ensembl.org) with a recent release of the zebrafish transcriptome GTF (v92, www.ensembl.org). After alignment, the generated BAM files were loaded to SeqMonk sequence analysis tool (v1.41, Babraham institute, UK) and mRNAs were quantified using the built-in mRNA seq pipeline. Transcriptome analysis was performed as described in Hurem et al. (2018) with minor modifications (see Supplementary data). Differentially expressed genes (DEG) were chosen based on a false discovery rate, FDR $<$ 0.05 and an absolute fold change, FC $>$ 1.5.

A principal component analysis was performed in all expressed genes that had a log 2 $\geq$ 0 expression in all groups (Control, POP10,

POP70, PFOS10, PFOS70) using ClustVis, a web tool for visualizing clustering of multivariate data (Metsalu and Vilo, 2015). PCA scores were loaded to R version 3.6.1 (R Development Core Thayer et al., 2012, http://www.r-project.org) and biplots of principal components were designed with "ggplot2" library, while the stat_ellipse argument within the "ggplot2" library was used to compute 95% confidence ellipses. Venn diagrams of DEG were created using Venny (version 2.1, https://bioinfogp.cnb.csic.es/tools/venny/).

2.10. Pathway analysis

Ingenuity Pathway Analysis (IPA) (version 49,309,495, Qiagen) was used to uncover enriched pathways in each condition (PFOS10, PFOS70, POP10, POP70). Differentially expressed genes were imported and used with the user dataset as background. Around 60% of the genes were annotated as having a human orthologue, and these genes were used for pathways analysis. IPA uses human orthologues for pathway analysis; hence we used the IPA nomenclature inside the context of pathways (e.g. MYH4) and use the official zebrafish gene nomenclature when referred to outside IPA context (e.g. *myhz1.1*). IPA calculates over representation of genes and gene lists involved in known pathways, upstream regulators, and diseases, using Fisher's exact tests. Furthermore, it uses the direction of the differentially expressed genes to predict activation or inhibition of pathways and upstream regulators by means of Z-scores. P values < 0.01 were considered significant. Another tool was employed to explore affected pathways, differentially expressed genes were also imported in Webgestalt (www.webgestalt.org) for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis and gene ontology (GO) analysis.

2.11. RT-qPCR for gene validation

For gene validation analysis equal amounts of RNA were reverse transcribed with the high-capacity cDNA RT kit (Applied Biosystems, NY, USA), followed by a 10 times dilution of the cDNA reaction with nuclease free water. QPCR on the diluted cDNA were performed in 10 μL, containing 5 μL FastStart Essential DNA Green Master (Roche, Norway), 250 nM of forward and reverse primers, 2 μL of diluted cDNA, and nuclease free water, in technical duplicates. PCR was performed on a Roche Lightcycler 96 (Roche, Norway), with 10-min denaturation at 95 °C, followed by 45 cycles of 10 s at 95 °C and 30 s at 60 °C. After the run a melting curve was generated from 65 to 95 °C. Primers for validation genes were developed using the Primer-BLAST software from NCBI (https://www.ncbi.nlm.nih.gov) (Supplementary data Table S3). Primer sequences for reference genes (*beta-actin, ef1a*) were taken from a previous publication (Kamstra et al., 2017). All primers were validated for specificity by melting curve analysis and gel electrophoresis. Efficiency of primers was determined against a dilution curve of pooled zebrafish cDNA. Cq values were calculated using linreg (Ramakers et al., 2003). These values were used for calculating normalized gene expression using the geometric average of the 2 reference genes (*beta-actin, ef1a*). Correlation was calculated with non-parametric Spearman correlation in Graphpad (v8.3, CA, USA).

2.12. Chemical analysis

Embryos from 6 until 96 hpf were exposed statically to either POP70 or PFOS70 in a 96 well plate (1 plate per condition) with one embryo per well in 200 μL exposure media. All wells were pre-saturated with the respective exposure for 24 h. Larvae were then collected in 2 mL eppendorf tubes. In total, 10 tubes were collected with each tube having an ascending number of embryos (the 1st tube contained 1 embryo, the 2nd tube contained 2 embryos, and so on, up to the final tube that contained 10 embryos). The reason was to make certain that chemicals would be detected during chemical analysis. The exposure media was also collected and sent for chemical analysis. Pools of 6, 7, and 8 embryos were used as replicates to evaluate the chemical uptake in the tissue of larvae zebrafish.

The measurements were performed at the Norwegian University of Life Sciences (NMBU), Department of Food Safety and Infection Biology, Laboratory of Environmental Toxicology. 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 (Polder et al., 2014). 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) (Bytingsvik et al., 2012). Details from the extraction, clean-up, and instrument run for the samples and quality control parameters can be found in the supplementary data.

2.13. Statistical analysis for behavioral data

Behavioral data were transferred to R version 3.6.1 (R Development Core Thayer et al., 2012, http://www.r-project.org). Dead and deformed larvae were discounted for behavioral analyses. Here, no more than 8 larvae were removed (chronically exposed to dantrolene only, Supplementary data Table S2). For all test scenarios, only motility during the dark phase was analyzed as movement was minimal during the light periods as expected based on previous work (e.g. Fraser et al. (2017)). Linear mixed effect (LME) models within the "nlme" package were used to assess behavior. For the initial light dark transition tests on exposed larvae, the dependent variable was either the cumulative time spent active (seconds), the cumulative distance travelled (mm), or average swimming speed, with group (5 levels, control, POP10, POP70, PFOS10, PFOS70) as a categorical independent variable, and replicate as a random effect. For the thigmotaxis assay, the same models were used, with the addition of a fourth dependent variable, thigmotaxis (% of time spent in the outer zone) and the independent variable group had 6 levels (control-POP, POP10, POP70, control-PFOS, PFOS10, PFOS70). For the mechanistic work, the dependent variable was either cumulative distance moved, cumulative time active, or swimming speed, with dantrolene or caffeine, (2 levels, Y/N) and treatment (3 levels, control, POPs, PFOS) as categorical independent variables, and replicate as a random effect. Here, the variables dantrolene or caffeine, and treatment were initially allowed to interact. We then compared the interaction model to a model without the interaction using the Akaike Information Criterion with a correction for small sample size (AICc). The model with the lowest AICc score was then considered the true model (Aho et al., 2014). If the interaction was the true model, this provides evidence of a mechanistic action, whereas no interaction provides evidence against a mechanistic action. For model validation, all models were also compared to a null model to verify results were not based on the random effect, also using AICc. More information about the statistical analysis parameters can be found in Supplementary data. Significance was assigned at $p = < 0.05$. All graphs were plotted in Graphpad (v8.3, CA, USA).

3. Results

3.1. Behavioral outcome of larvae exposed to the POP mixture or PFOS

Statistical analysis of the light-dark transition test revealed that the cumulative distance moved and swimming speed were significantly affected by chemical exposure, but not the time spent active (Supplementary data Fig. S1). The cumulative distance moved was higher compared to controls for PFOS70 and POP70 and swimming speed increased when exposed to PFOS70, POP10, and POP70. The thigmotaxis test revealed that the percent of the total distance moved in

the outer zone (thigmotaxis) was affected by exposure to chemicals. Larvae exposed to PFOS10, PFOS70, and POP70 moved significantly further in the outer zone of the well compared to controls (Supplementary data Fig. S2).

3.2. Sequencing analysis

Analysis with FastQC revealed high quality sequences with phred scores generally above 35 over all reads except for the paired-end read towards the 3' end where reads were still above the acceptable phred score of 20 (data not shown). Average mapping efficiency was over 90% unique reads (Supplementary data Table S4).

Quality analysis, performed in SeqMonk on aligned reads, showed a high proportion of reads that fell into genes (90%). Additionally, a high proportion of those reads fell into exons (around 94%) which means that the library contained very few unspliced transcripts. A low percent of reads was present in ribosomal and mitochondrial RNA and 87% of the annotated zebrafish genes were mapped. The reads mapped equally on the sense and anti-sense strands which confirms that our library was non-strand specific (Supplementary data, Fig. S3). A cumulative distribution analysis of expressed genes over all samples revealed the same profile over all levels of expression, which indicated highly similar sequencing libraries, and that further normalization based on reads per million (RPM) was not biased (Supplementary data, Fig. S4).

3.3. RNA seq expression results in exposed larvae

Deseq2 analysis revealed 41 (1 downregulated, 2.4%) and 18 (10 downregulated, 55%) differentially expressed genes (DEG) for POP10 and POP70 exposures respectively. For the PFOS10 exposure, analysis revealed 879 DEG (842 downregulated 95.8%) and 164 (2 downregulated, 1.2%) for PFOS70. (Fig. 1A–D and Supplemental File 1). Principal component analysis was performed on all measured genes in the 5 conditions (Control, POP10, POP70, PFOS10, PFOS70) (Fig. 1E). The first principle component explained 30.3% of the variance and

Table 1
KEGG analysis of canonical pathways for the exposed groups, FDR false discovery rate.

ID	Name	#Gene	p-value	FDR
PFOS10				
dre01100	Metabolic pathways	1301	1.56E-11	2.51E-09
dre00980	Metabolism of xenobiotics by cytochrome P450	36	1.18E-07	9.49E-06
dre00982	Drug metabolism	33	5E-06	0.000268
dre03320	PPAR signaling pathway	62	3.83E-05	0.001544
dre01200	Carbon metabolism	129	7.34E-05	0.002365
dre00380	Tryptophan metabolism	48	0.000126	0.003386
dre04512	ECM-receptor interaction	76	0.000259	0.004328
dre00350	Tyrosine metabolism	32	0.000266	0.004328
dre00051	Fructose and mannose metabolism	42	0.000269	0.004328
dre00010	Glycolysis/Gluconeogenesis	76	0.000259	0.004328
PFOS70				
dre04512	ECM-receptor interaction	76	3.88E-05	0.006253
dre04260	Cardiac muscle contraction	91	0.001176	0.094696
dre00533	Glycosaminoglycan biosynthesis	14	0.00231	0.12398
dre04510	Focal adhesion	226	0.005724	0.23041
dre00052	Galactose metabolism	30	0.010486	0.33765
dre04261	Adrenergic signaling in cardiomyocytes	188	0.015635	0.41953
dre04020	Calcium signaling pathway	248	0.038453	0.88442
POP10				
dre04260	Cardiac muscle contraction	91	0.000334	0.053788
dre04261	Adrenergic signaling in cardiomyocytes	188	0.002767	0.22278
dre04020	Calcium signaling pathway	248	0.058234	1
POP70				
dre04020	Calcium signaling pathway	248	0.018203	1
dre05132	Salmonella infection	90	0.078562	1
dre04260	Cardiac muscle contraction	91	0.079406	1

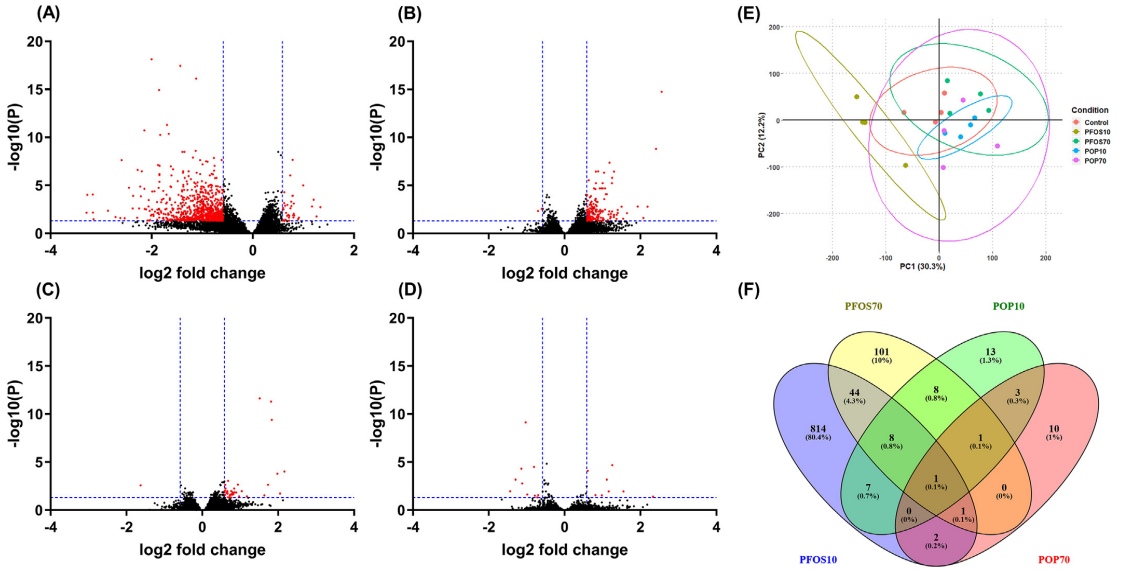

Fig. 1. Volcano plots of genes of exposed groups (A) PFOS10 (B) PFOS70 (C) POP10 (D) POP70. Red dots represent differentially expressed genes. P = p-value. (E) Principal components analysis of all expressed genes in all conditions. Circles around each condition represent the 95% confidence ellipses. Percent in parentheses shows the variation explained by each principal component. (F) Venn diagram showing the number of overlapping differentially expressed genes from each exposure scenario. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
GO analysis of canonical pathways involved in biological processes for the exposed groups, FDR false discovery rate.

ID	Name	#Gene	p-value	FDR
PFOS10				
GO:0006641	triglyceride metabolic process	19	3.87E-10	1.53E-06
GO:0044281	small molecule metabolic process	673	9.39E-10	1.86E-06
GO:0042632	cholesterol homeostasis	17	2.84E-09	2.81E-06
GO:0055092	sterol homeostasis	17	2.84E-09	2.81E-06
GO:0055088	lipid homeostasis	29	3.94E-09	3.10E-06
GO:0006869	lipid transport	110	4.71E-09	3.10E-06
GO:0006638	neutral lipid metabolic process	26	1.75E-08	0.0000076
GO:0006639	acylglycerol metabolic process	26	1.75E-08	0.0000076
GO:0006629	lipid metabolic process	400	1.78E-08	0.0000076
GO:0010876	lipid localization	119	1.92E-08	0.0000076
PFOS70				
GO:0006941	striated muscle contraction	28	9.85E-07	0.002904
GO:0006936	muscle contraction	59	2.38E-06	0.002904
GO:0048513	animal organ development	1447	2.63E-06	0.002904
GO:0003009	skeletal muscle contraction	16	3.09E-06	0.002904
GO:0003012	muscle system process	65	0.0000042	0.002904
GO:0050879	multicellular organismal movement	18	5.14E-06	0.002904
GO:0050881	musculoskeletal movement	18	5.14E-06	0.002904
GO:0060350	endochondral bone morphogenesis	19	6.47E-06	0.003089
GO:0060348	bone development	41	7.02E-06	0.003089
GO:0060048	cardiac muscle contraction	22	0.000012	0.0047629
POP10				
GO:0003009	skeletal muscle contraction	16	1.37E-08	0.0000304
GO:0050879	multicellular organismal movement	18	2.30E-08	0.0000304
GO:0050881	musculoskeletal movement	18	2.30E-08	0.0000304
GO:0006941	striated muscle contraction	28	1.52E-07	0.0001507
GO:0003008	system process	460	5.92E-07	0.0004689
GO:0006936	muscle contraction	59	3.27E-06	0.0021565
GO:0008015	blood circulation	145	0.0000046	0.0023215
GO:0003012	muscle system process	65	4.83E-06	0.0023215
GO:0003013	circulatory system process	149	5.28E-06	0.0023215
GO:0060048	cardiac muscle contraction	22	7.60E-06	0.0030091
POP70				
GO:0006357	regulation of transcription by RNA polymerase II	682	0.0012485	0.89514
GO:0006366	transcription by RNA polymerase II	716	0.0014999	0.89514
GO:0097755	positive regulation of blood vessel diameter	5	0.0040343	0.89514
GO:0032945	negative regulation of mononuclear cell proliferation	5	0.0040343	0.89514
GO:0042130	negative regulation of T cell proliferation	5	0.0040343	0.89514
GO:0050672	negative regulation of lymphocyte proliferation	5	0.0040343	0.89514
GO:0070664	negative regulation of leukocyte proliferation	5	0.0040343	0.89514
GO:0035176	social behavior	5	0.0040343	0.89514
GO:0051703	intraspecies interaction between organisms	5	0.0040343	0.89514
GO:0051705	multi-organism behavior	5	0.0040343	0.89514

separated the PFOS10 group from all the other groups whereas we did not observe any separation along the first and the second principal component, indicating marginal effects in the other exposure groups. Furthermore, the confidence ellipses containing each group revealed that there is higher variation in PFOS10 and POP70 than in the Control, POP10 and PFOS70 groups. Venn diagrams showed that PFOS10 and PFOS70 share the highest number of overlapping DEGs with 54 common genes (Fig. 1F). Interestingly these two groups show a clear separation in the PCA analysis, since all the common genes are downregulated in PFOS10 and upregulated in PFOS70. Between POP10 and POP70 the number of mutually DEGs was much lower, only 5 genes (Fig. 1F). Finally, *myhz1.1* was mutually differentially expressed in all groups (Supplementary File 2).

3.4. Pathway analysis with IPA and Webgestalt

DEGs were imported to Webgestalt using a custom background of all measured genes for gene enrichment analysis. Results of enriched pathways are summarized in Tables 1 and 2. The most enriched pathways were evident in the PFOS10 and PFOS70 groups. This result was expected due to the fact that these two groups had the highest number of DEGs. KEGG analysis revealed that the most enriched pathways in the PFOS10 group were involved in metabolism, peroxisome proliferator-activated receptors (PPAR) signaling (Supplementary data Fig. S5) and extracellular matrix-receptor (ECM) interaction. Gene ontology (GO) analysis for biological functions revealed multiple pathways involved in lipid metabolic and homeostatic processes. KEGG analysis for PFOS70 revealed that significantly enriched pathways ($p < 0.05$) were involved in ECM-receptor interaction, cardiac muscle contraction and calcium signaling. GO analysis revealed multiple pathways involved in muscle contraction. Interestingly, albeit not significant, pathways involved in muscle contraction (both cardiac and skeletal) and calcium signaling were also observed in the remaining 2 groups POP10 and POP70 (Tables 1 and 2 and Supplemental File 3). Using these results, we used IPA to further explore the calcium signaling pathway. Datasets of PFOS70, POP10 and POP70 were overlaid ignoring the DE analysis cutoff values, to include all genes involved in the pathway in question, and the molecule activity predictor (MAP) function revealed activation of muscle contraction via increased influx of calcium through ryanodine receptors (RyR) (Fig. 2).

3.5. Validation with RT-qPCR

We performed a validation of the sequencing results by measuring 10 genes that were differentially expressed in either or all conditions. Validation of these 10 genes showed a significant correlation ($p < 0.05$) between qPCR and sequencing data (Supplementary data Fig. S6, $r = 0.902$ and Table S5).

3.6. Behavioral outcome of larvae exposed to RyR agonist and antagonist

Based on the results of the transcriptomic analysis, we performed an experiment using the light-dark transition behavioral assay to uncover a potential mechanism of action of POPs on the calcium signaling pathway via ryanodine receptors (RyR). Caffeine, an established agonist, and dantrolene, an established antagonist of RyR were used (Hernández-Fonseca and Massieu, 2005). All details of the statistical results are summarized in Supplementary data Table S6.

In the acute (10 min before test) and chronic (6–96 h post fertilization, hpf) exposure scenarios, three groups were tested with or without the addition of 50 μM caffeine; Control, PFOS70, and POP70. For the controls, both the chronic and acute exposure to caffeine resulted in a significant decrease in the time spent active and an increase in swimming speed, but caffeine had no effect on the distance moved. Although the raw data and model selection suggested a possible interaction between PFOS and POPs with chronic caffeine on swimming speed and distance moved, the interaction was not significant for any endpoint (Supplementary data Fig. S7). However, all groups responded similarly to acute caffeine exposure with no tendencies towards any interaction effect (Supplementary data Fig. S7).

We followed the same exposure scenario as the caffeine experiments for the dantrolene chronic and acute exposure. Three groups were tested with or without the addition of 50 μM dantrolene, Control, PFOS70, and POP70. Acute and chronic dantrolene exposure resulted in a decrease in distance moved and time spent active. There was no

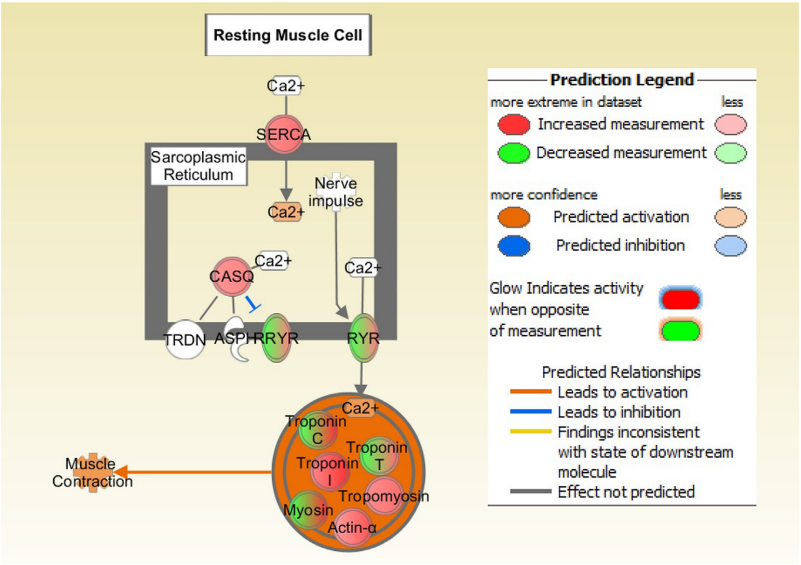

Fig. 2. IPA Calcium signaling pathway of muscle cell predict activation of ryanodine receptor (RYR) and activation of muscle contraction. SERCA: Sarcoplasmic/endoplasmic reticulum calcium ATPase, CASQ: Calsequestrin, TRDN: Triadin, ASPH: Aspartate beta-hydroxylase.

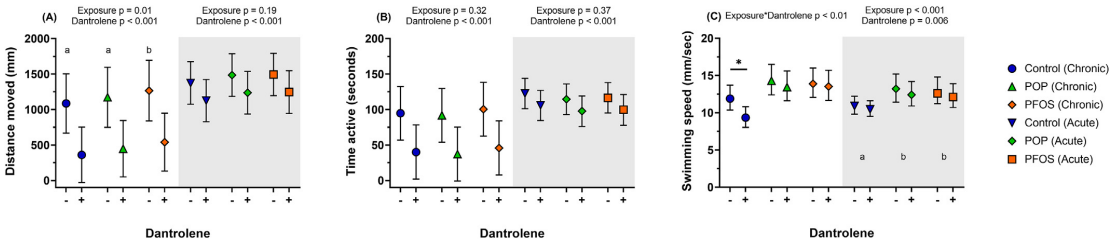

Fig. 3. Behavioral responses following the light-dark transition test in larval zebrafish exposed to PFOS or a POP mixture and co-exposed to RyR antagonist dantrolene chronically (90 h) or acutely (10 min prior to the behavioral test). (A) Distance moved. (B) Time spent active. (C) Swimming speed. Statistics are from linear mixed effect models. Data shown are least square means ± 95% CI. Different lowercase letters indicate general exposure effects (i.e. Control vs POPs vs PFOS). Asterisk indicates an effect of dantrolene within exposure group (for the significant interaction model only).

interaction on any endpoint following acute exposure to dantrolene (Fig. 3). On the contrary there was an interaction effect between PFOS and POPs following chronic dantrolene exposure (Fig. 3C). Here, whereas swimming speed was reduced in the control following dantrolene exposure (p-value < 0.01), there was no effect of dantrolene in either the PFOS or POPs exposed fish.

3.7. Chemical analysis

We used a variety of methods to determine chemical accumulation in zebrafish larvae tissue after 90 h of exposure (6–96 hpf). Results of the chemical analysis are summarized in Table 3. In general, the POP mixture analysis revealed that the chemicals with the highest accumulation in the zebrafish larvae were PFUnDA, PFDA, PFOS, HBCD, α-chlordane, α-HCH, β-HCH and γ-HCH. For the single PFOS exposure, results showed an accumulation of 31.5% of the nominal input concentration in the tissue of larvae zebrafish (Table 3). The accumulation of PFOS incorporated in the POP mixture, in the tissue of larvae, was lower than the one from the single PFOS exposure and reached a level of 12.7% of the nominal input concentration.

4. Discussion

In the present study, we observed behavioral parameters in zebrafish larvae after exposure to a POP mixture or PFOS for 90 h, followed by transcriptome analysis to try and identify potential toxic mechanisms. Subsequently, we identified the calcium signaling pathway via the activation of RyRs as a potential mechanism of POPs and PFOS exposure and found interactions between POPs and PFOS with the RYR antagonist dantrolene on our behavioral endpoint. As such, although POPs and PFOS resulted in some differences in transcriptome profiles, a common mechanism may be behind behavioral aberrations.

The mixture of POPs used in the present study was based on the average levels of chemicals found in human blood of the Scandinavian population and includes 29 chemicals (Berntsen et al., 2017). Following chemical analysis, and based on the recovered percentage in larvae and media we estimated that the low concentration of PFOS was equal to 106.5 μg/L and the high concentration was equal to 731.3 μg/L. In comparison, the concentration of PFOS incorporated in the POP mixture was equal to 55.3 μg/L and 380.1 μg/L for POP10 and POP70 exposures respectively. These values are very relevant for human toxicity. For example, in human blood, PFOS serum levels range from 1

Table 3
Chemical analysis of the uptake of all chemicals in the POPs mixture and single PFOS and their respective concentration in the exposure media.

Chemical	Nominal input concentration (ng/L)	Levels in larva (ng)	Concentration measured in media (ng/L)	% in larva	% in media
POPs mixture					
PFASs					
PFOA	316610	0.83	16351.3	1.32	5.16
PFOS	2,059,750	52.27	118378.4	12.69	5.75
PFDA	34650	1.14	1350	16.48	3.90
PFNA	56000	0.51	2905.4	4.52	5.19
PFHxS	241500	0.84	21081.1	1.75	8.73
PFUnDA	39200	1.65	471.6	21.05	1.20
BFRs					
BDE-47	630	0.00	0.00	2.85	0.00
BDE-99	280	0.00	0.00	0.18	0.00
BDE-100	210	0.00	0.00	1.89	0.00
BDE-153	70	0.00	0.00	0.00	0.00
BDE-154	140	0.00	0.00	0.00	0.00
BDE-209	770	0.01	40.4	4.45	5.25
HBCD	1750	0.04	0.00	11.86	0.00
PCBs					
PCB 28	910	0.00	0.00	0.00	0.00
PCB 52	700	0.00	0.00	0.00	0.00
PCB 101	560	0.00	0.00	0.00	0.00
PCB 118	4480	0.02	1.1	2.54	0.03
PCB 138	15540	0.15	19.1	4.77	0.12
PCB 153	25340	0.10	0.00	1.92	0.00
PCB 180	13580	0.05	2.2	1.99	0.02
Other organochlorines					
p,p-DDE	35140	0.25	140	3.49	0.40
HCB	8190	0.03	0.00	2.07	0.00
α-chlordane	770	0.02	0.00	10.85	0.00
Oxy-chlordane	1540	0.03	0.00	8.62	0.00
Trans-nonachlor	2870	0.05	3.4	7.91	0.12
α-HCH	420	0.01	57.3	10.91	13.64
β-HCH	3710	0.11	1314	14.74	35.42
γ-HCH (lindane)	420	0.01	114.6	16.05	27.28
Dieldrin	1680	0.02	0.00	6.91	0.00
Single PFOS	2,059,750	129.67	83471.2	31.47	4.05

to 10 μg/L in multiple surveys of general populations, and from 100 to 1000 μg/L for highly exposed populations (Kato et al., 2015; Olsen, 2015). In addition, our PFOS measurements within the larvae (52.3 ng) are similar to the levels found by Huang et al. (2010) in zebrafish exposed to 2000 μg/L between 0 and 96 hpf (≈38 ng). Results from the chemical analysis showed that the highest recovery based on tissue accumulation in larva for the POP mixture was 21% for PFUnDA, followed by PFDA at 16.5%, PFOS at 12.7%, HBCD at 11.8%, β-HCH at 14.7% and γ-HCH at 16% of the nominal input concentrations. For the single PFOS exposure tissue, we recovered 31.5% of the nominal exposure. It is unclear where the remaining quantities went, since the chemical analysis revealed that most of the remaining quantities of chemicals were only recovered to some extent (up to 35%) from the media. Xenobiotic metabolism by CYP450 enzymes is active by 72 hpf in zebrafish larvae but it is highly unlikely that zebrafish at this stage could metabolize the missing quantities of chemicals (Chen et al., 2012). Additionally, even though test vessels were pre-conditioned with exposure media some adsorption might have also occurred, for instance in the case for PFASs high affinity to polystyrene vessels has been already documented (Llorca et al., 2018). Finally, we cannot rule out interactions between chemicals in our mixture since the recovered level in larval tissue for the single PFOS exposure was more than double of what was recovered from the POP mixture. Nevertheless, the levels of POPs recovered from the chemical analysis are closer to human exposure levels than would be expected from the nominal input.

We tested the effect of exposure to the POP mixture or PFOS alone on the behavioral parameters of zebrafish larvae at 96 hpf using a light-dark transition test and a thigmotaxis assay. The increase in "wall-hugging" identified by the thigmotaxis assay, and the increase in locomotor activity identified in the light-dark transition test, are both perceived to indicate increased anxiety (MacPhail et al., 2009; Ali et al., 2011; Schnörr et al., 2012). Our results matched those from a previous study in our lab (Khezri et al., 2017), whereby the POP mixture and PFOS led to similar increases in swimming speed. Here, it is noted that Khezri et al. (2017) failed to identify a behavioral effect following exposures to separate sub-mixtures of the POP mixture that didn't contain PFOS. It was notable that although there was a twofold reduction in tissue concentrations, there was no difference in the behavioral response between the highest concentration of the POP mixture and PFOS. This could suggest that either the chemicals within the POP mixture had an additive or synergistic effect with PFOS on behavior, or that the behavioral assay is not sensitive enough to detect such relatively small differences in chemical burden. Nevertheless, our results are in agreement with previous studies where developmental PFOS exposure has been linked with hyperactivity in zebrafish (Huang et al., 2010; Spulber et al., 2014; Jantzen et al., 2016) and in mammalian models like mice (Johansson et al., 2008) where similar concentrations of PFOS were used.

Transcriptomics analysis revealed that groups exposed to PFOS10 (0.55 μM) and PFOS70 (3.83 μM) were most affected having 879 and 164 DEGs, respectively. The low number of DEGs in the POP10 and POP70 exposed groups (41 and 18 genes respectively) suggests that larvae exposed to the mixture do not show as strong of a response compared to our singular PFOS exposure. Whether this is through

unknown additive, synergistic, or antagonizing interactions between the chemicals present in our mixture or the fact that larvae exposed to PFOS had a higher accumulation of PFOS in their tissue than the level found after exposure to the mix cannot be determined at present. Nevertheless, we observed mainly a downregulation of genes in PFOS10 and an upregulation of genes in PFOS70, but also high numbers of unique DEGs in PFOS10 (814 genes) and PFOS70 (101 genes). This observation agrees with the notion of non-monotonic response, which states that a chemical can produce a decrease in response relative to controls at a low dose and a higher response at higher doses or *vice versa*, also known as a U-shape or inverted U-shape response (Conolly and Lutz, 2004). In a previous study with adult zebrafish exposed to bisphenol A an inverted U-shape relationship was observed between exposure and expression of cytochrome P450 aromatase (*cyp19a*) (Molina et al., 2018). Additionally, low-dose effect studies with chemicals representing environmental concentrations may produce a greater response as seen in this study where more DEGs were detected in the low PFOS exposure and can affect different systems as shown by the number of unique number of DEGs and enriched pathways in the two conditions (Vandenberg et al., 2012). This needs to be taken into consideration and further studies could focus on the testing of multiple concentrations of PFOS and the effects on the transcriptome level.

Focusing on the PFOS70, POP10 and POP70 groups, IPA analysis identified the calcium signaling pathway as one of the significantly affected pathways. Although our initial hypothesis was that this would be mostly linked to brain development and behavior, we surprisingly found limited pathways linked to behavior, but strong enrichments in genes related to muscle development. Genes involved in this pathway were significantly upregulated and included actin alpha cardiac muscle 1 (ACTC1), myosin heavy chain 4 (MYH4, zebrafish orthologue *myhz1.1*), myosin heavy chain 7 (MYH7, zebrafish orthologue *myh7l*), myosin light chain 4 (MYL4, zebrafish orthologue *myl4*), myosin light chain 7 (MYL7, zebrafish orthologue *myl7*), troponin C1 (TNNC1, zebrafish orthologue *tnnc1a*), troponin I2 (TNNI2, zebrafish orthologue *tnni2a.1*) and troponin T2 (TNNT2, zebrafish orthologue *tnnt2a*) with many of these genes, such as MYH4, MYH7, TNNC1, TNNI2, being commonly expressed in two or all three groups. In particular, MYH4 was expressed in all four groups but showed a downregulation in PFOS10. KEGG analysis confirmed the results of IPA that the calcium signaling pathway was one of the commonly affected pathways. Calcium is loaded in the endoplasmic reticulum (ER) through the activity of the sarcoendoplasmic reticulum Ca^{+2} ATPase (SERCA). Postsynaptic calcium signals in vertebrate skeletal and cardiac muscles are generated via mechanical coupling of specialized calcium channels, the ryanodine receptors (RyRs) (Brennan et al., 2005; Hernández-Fonseca and Massieu, 2005). Troponin and myosin genes provide instructions for making myosin and troponin proteins. The troponin and myosin complex are part of a structure called sarcomere, which is the basic unit of muscle contraction. The troponin complex, together with calcium, helps regulate contraction of the cardiac and skeletal muscle (Schiaffino and Reggiani, 1996). Activation of RyR from cerebral cortex microsomes and increased influx of calcium has been observed after exposure to chemicals, including PCBs, polybrominated diphenyl ethers, and PFOS (Dusza et al., 2018). Although our canonical pathway results point to the direction of calcium release by muscle specific RyR isoforms, the results of Dusza et al. (2018) seem to agree with our study. An upregulation of genes related to muscle function such as actins, myosins and tropomyosins has also been observed in another study where zebrafish were exposed to 1 mg/L of PFOS from 2 to 5 dpf (Martínez et al., 2019). The upregulation of these genes might also suggest a higher translation of muscle related proteins (actins, myosins and troponins) which in turn might relate to the longer distance moved and higher swimming speed of zebrafish larvae exposed to PFOS or POP mixture.

Based on the transcriptomic findings, we looked for treatment interactions with the RyR agonist caffeine and the RyR antagonist dantrolene. Results for the caffeine experiments failed to reveal an interaction effect between exposure to chemicals and caffeine, however, dantrolene exposure revealed an interaction with chemical exposure for swimming speed in chronically exposed larvae. Dantrolene can suppress intracellular calcium release from the sarcoplasmic reticulum by inactivating the RyR (Paul-Pletzer et al., 2002) and it has been shown to reduce the number of spontaneous muscular contractions in zebrafish embryos exposed to 10–1000 μM (Brennan et al., 2005). In controls, chronic dantrolene exposure led to a reduction in swimming speed that was not observed in the POPs or PFOS treated larvae. This could indicate that the chemicals used in this study have a higher affinity to RyR binding sites than dantrolene. Further work into endpoints within this pathway, such as mRNA transcription levels and protein, or Crispr knockouts that could pinpoint the exact mechanisms and genes that are responsible for these effects. would therefore be of interest.

Lipid metabolism, transport, and homeostasis were the main biological processes affected by the exposure to the lowest concentration of PFOS (0.55 μM), through the involvement of PPAR signaling pathway (Supplementary data Fig. S5). Transcriptomic changes in the genes involved in the PPAR signaling pathway may lead to inhibition of all lipid biological processes since all genes in our dataset were downregulated (Supplementary data Table S7). Previous studies have also proposed the mechanism of action for PFOS toxicity via an interaction with nuclear receptors associated with metabolic regulation like PPAR alpha (Lau et al., 2007; Shi et al., 2009; White et al., 2011) with dysregulation in lipid metabolism and homeostasis often observed after exposure to PFOS (Cui et al., 2017; Das et al., 2017; Martínez et al., 2019).

In this study we aimed to assess the effect of exposure to a complex POP mixture or single PFOS exposure to zebrafish larvae behavior and transcriptome response. Exposure to chemicals led to hyperactivity in zebrafish larvae and this might be correlated with the activation of muscle contraction observed during pathway analysis. Whilst further research is necessary, our results point to a possible involvement in muscle related toxicity via calcium signaling, rather than via neural related pathways. Lipid biological processes were also affected by PFOS exposure. Further studies should focus on the effect of PFOS on the lipid profile of zebrafish larvae to understand how dysregulation of lipid processes correlates with changes of lipid composition.

Author contributions

Maria Christou: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing, Thomas W. K. Fraser: Conceptualization, Formal analysis, Methodology, Supervision, Writing - review & editing. Vidar Berg: Writing - review & editing. Erik Ropstad: Funding acquisition, Supervision, Writing - review & editing. Jorke H. Kamstra: Conceptualization, Formal analysis, Methodology, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109702.

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Supplementary data

Fish maintenance

System water originated from the local water supply and was treated with reverse osmosis and filtered through particle and active charcoal. Additionally, the water was treated with UV irradiation for sterilization. System water was conditioned by the addition of 155 mg synthetic sea salt (Instant Ocean, Blacksburg, USA), 53 mg sodium carbonate and 15 mg calcium chloride (Sigma-Aldrich, Norway AS) per liter to reach a conductivity of 500 µS/cm, a general hardness of 4 – 5 and a pH of 7 - 7.5 (adjusted with 1M HCl). Adults were fed 3 times daily, twice with Gemma Micro (Skretting, Stavanger, Norway) and once with artemia (Sep-art Artemia, Ocean Nutrition, Belgium).

RNA-seq and transcriptome analysis

mRNA was purified from total RNA using poly-T oligo attached magnetic beads. Fragments were randomly created in fragmentation buffer. First strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase. After first-strand synthesis, a custom second-strand synthesis buffer (Illumina, CA, USA) was added with dNTPs, RNase H and *Escherichia coli* polymerase I to generate the second strand. The final cDNA library was ready after a round of purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection, and PCR enrichment. PCR products were purified (AMPure XP system, Beckman, US) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Libraries were analyzed with a Hiseq 4000 (Illumina, Ca, USA), using 150bp paired-end reads, with a depth of 20 million reads per sample.

Library quality was evaluated with the RNA-seq QC plot and cumulative distribution plots using SeqMonk. Filter was applied to analyze mRNAs that had at least a log2 = 0 expression in either of the samples, to assure that for statistical analysis only mRNAs with enough reads were

included. Deseq2 filter using R (v3.5.0) within SeqMonk was used on raw read counts to calculate differential expression. With this analysis mRNA isoforms were merged, since Deseq2 cannot assess differential expressed transcript isoforms. Deseq2 is a differential gene expression analysis based on the negative binomial distribution, with Benjamini Hochberg false discovery rate multiple comparisons adjustments (FDR) (Love *et al.* 2014). After Deseq2 analysis, data were normalized by reads per million (RPM) to calculate fold change (FC) per gene combining all replicates in each condition to calculate the average.

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 persistent organic pollutants (POPs) has been described by Polder *et al.* (2014). Briefly the samples were weighed, internal standards added (PCB 29, 112 and 207 (Ultra Scientific, RI, USA); BDE 77, 119 and 181 and 13C12-BDE 209 (Cambridge Isotope Laboratories, Inc., MA, USA)) and solvents (cyclohexane/acetone/water) were added, followed by homogenization using a T25 Ika Ultra-Turrax®. 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 organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), brominated diphenyl ethers (BDEs) and hexabromocyclododecane (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 Polder *et 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 (hexachlorocyclohexanes, HCHs), 284 (hexachlorobenzene, 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) (Polder *et 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 Bytingsvik *et 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).

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 Polder *et al.* (2014). Briefly, every analytical series included three procedural blanks (solvents), a reference sample with known amounts of added chemicals and clean animal tissue, a blind sample, 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)) in media and relative recoveries (%) were for HCB 0.0017 (73 %), HCHs 0,0034-0,0068 (89-92%), p,p'- DDE 0.0014 (95%), dieldrin 0.01 (75%), PCBs 0.0017–0.0034 (88-100%), chlordanes 0.0017-0,0034 (97-10093%), BDEs 0.0017-0.025 (80-103%), HBCD 0.022 (59%). For perfluorinated compounds 0.01-0.02 (104-126%), except for

perfluorooctanoic acid (PFOA) 0.0428 (117%) in media. The perfluorinated detection limits were approximately the same as in one embryo. Positive consistent blanks in media were found for dieldrin (0.046 ng/g), For PCBs 118, 153, 138 and 180, (0.006, 0.065, 0.068 and 0.008 ng/g respectively), and for BDEs 47, 99 and 100 (0.007 ng/g). The results were corrected for these blanks. The quality control parameters were within the accepted ranges for the methods applied. The analytical quality is regularly approved by routinely analyzing 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.

Statistical analysis for behavioral data

The “Anova” command within the “car” library was used to extract the results for the main effects whereas the “lsmeans” command within the “emmeans” library was used as a post-hoc test to compare groups against one another while adjusting for the means of other factors within the model (Lenth 2016). Type II sum of squares were used for models without interactions, whereas main effects were calculated using type III sum of squares when interactions were present within the final model. The R^2 of the model was determined using the command “r.squaredGLMM” that returns the marginal and conditional R^2 that represent the variance explained by the fixed actors alone excluding the random effect and the variance of the entire model including the random effect, respectively (Nakagawa and Schielzeth 2013). For all models, examination of the residual plots verified that no systematic patterns occurred in the errors (e.g. standardized residuals vs fitted values). The dantrolene and caffeine data were natural log transformed to improve model residuals against right skew. Comparisons of transformed and non-transformed models demonstrated minor differences in p-values, with each model resulting in the same conclusions. For clarity, only the transformed models are reported in the results.

Table S1. Composition and concentration of chemicals in the POP mixture

Chemicals	Nominal concentration of stock solution (μM)	Nominal concentration of stock solution (mg/ml)
PFASs		
PFOA	10923	4.523
PFOS	54801	29.425
PFDA	962	0.495
PFNA	1723	0.800
PFHxS	7873	3.450
PFUnDA	990	0.560
BFRs		
BDE-47	18	0.009
BDE-99	7	0.004
BDE-100	3	0.003
BDE-153	1	0.00
BDE-154	3	0.002
BDE-209	11	0.011
HBCD	38	0.025
PCBs		
PCB 28	50	0.013
PCB 52	34	0.010
PCB 101	24	0.008
PCB 118	196	0.064
PCB 138	615	0.222
PCB 153	1003	0.362
PCB 180	490	0.194
Other organochlorines		
p,p-DDE	1578	0.502
HCB	410	0.117
α-chlordane	26	0.011
Oxy-chlordane	51	0.022
Trans-nonachlor	92	0.041
α-HCH	20	0.006
β-HCH	182	0.053
γ-HCH (lindane)	20	0.006
Dieldrin	63	0.024

Table S2. Mortality and deformation numbers of larvae intended for behavioural assays

Experiment	Dead (replicate no)	Deformed (replicate no)
Light/dark transition test		
Control		
POP10	1(1)	
POP70	1(1), 1(3), 1(5)	1(1)
PFOS10	1(1), 1(3)	1(1), 1(4)
PFOS70		
Thigmotaxis test		
Control		
POP10	1(1)	
POP70	2(2)	
Control		
PFOS10		
PFOS70		
Dantrolene behavioral assay		
Acute		
Control	1(1)	1(2)
Control + Dantrolene		
POP70		1(1), 1(3)
POP70 + Dantrolene		1(1)
PFOS70		
PFOS70 + Dantrolene	1(2), 1(3)	1(3)
Chronic		
Control		1(1)
Control + Dantrolene	1(1)	2(1), 3(2), 2(3)
POP70	2(3)	1(1), 1(2)
POP70 + Dantrolene	1(1)	2(1), 3(3)
PFOS70	1(1), 2(3)	
PFOS70 + Dantrolene	1(2), 4(3)	1(1), 1(3)
Caffeine 250µM (no behavioral assay)		
Acute		
Control	1(3)	1(2)

Control + Caffeine	2(3)	
POP70	1(2)	
POP70 + Caffeine	2(3)	
PFOS70		
PFOS70 + Caffeine	1(3)	
Chronic		
Control	1(3)	
Control + Caffeine		3(1), 1(3)
POP70		
POP70 + Caffeine	1(3)	9(1), 8(2), 3(3)
PFOS70	1(3)	1(2), 1(3)
PFOS70 + Caffeine		9(1), 4(2), 7(3)
Caffeine 50µM behavioral assay		
Acute		
Control		2(2)
Control + Caffeine	1(2)	
POP70		1(1), 1(2)
POP70 + Caffeine		
PFOS70	1(2)	1(3)
PFOS70 + Caffeine	1(1), 1(3)	1(1), 1(3)
Chronic		
Control		1(3)
Control + Caffeine		
POP70		1(2), 1(3)
POP70 + Caffeine		1(2), 1(3)
PFOS70	1(1), 2(2)	1(2), 1(3)
PFOS70 + Caffeine	1(2)	1(1), 2(2)

Figure S1. Responses following the light-dark transition test in larval zebrafish exposed to PFOS or a POP mixture in two different concentrations. (**A**) Distance moved. (**B**) Time spent active. (**C**) Swimming speed. The model with the lowest AICc score (underlined) is presented along with the marginal (m) and conditional (c) R^2. Results are those of linear mixed effect models. Data shown are least square means ± 95% CI. * = $p < 0.05$ compared to control

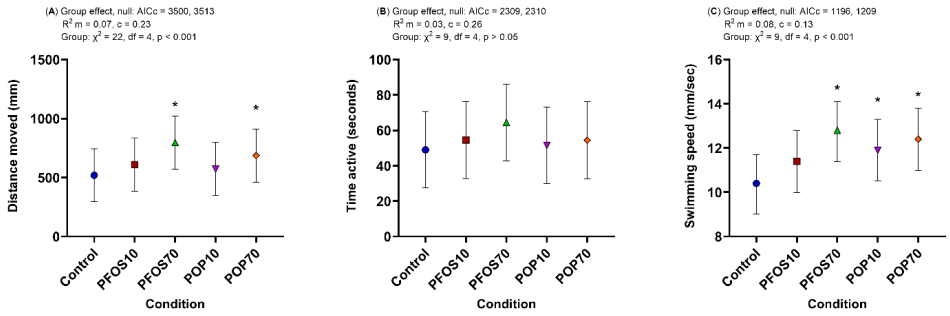

Figure S2. Percent of total distance moved in the outer zone following thigmotaxis test in larval zebrafish exposed to PFOS or a POP mixture in two different concentrations. The model with the lowest AICc score (underlined) is presented along with the marginal (m) and conditional (c) R^2. Results are those of linear mixed effect models. Data shown are least square means ± 95% CI. * = $p < 0.05$ compared to control

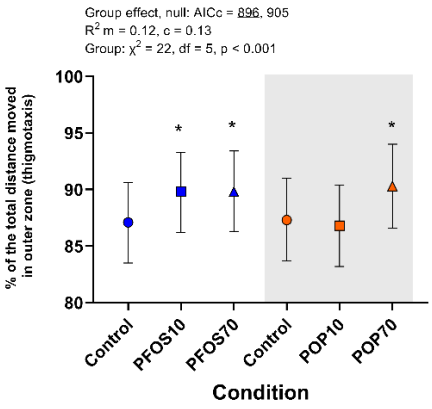

Table S3. Primer sequences for validation with RT-qPCR

Accession number	Gene	Forward primer	Reverse primer
XM_021469469	*myhz1.1*	GTGACCAGCCACAGAATCCAT	TCGTCTGCATCCACCACAAA
NM_131329	*myl7*	TTCGACCCTAATGCCACAGG	AAGCCTGGTCAACCTCTTCTG
NM_001077464	*myh7l*	GAACGCAGGGATGCCTTACT	CGTGCCTCAGACTTCGCATA
NM_152893	*tnnt2a*	TCAGTGACCATCAGAAAACGTCA	ACAGTGGTCAGCTCCTCTCT
NM_001083827	*col10a1a*	CCCAAGTATGCCGATTTGACC	GAGTAAGGCTGGTACTGCGG
NM_001037420	*ugt5a1*	TTGTCAGGTCGCGTGCAAA	GGAAGGTCTTACCACAGTCACA
NM_001002363	*cd36*	GGTCGGAATGAACCCCAACT	AGAACGGCCGTCTCATTCAA
NM_131128	*apoa1a*	GTGGCTCTTGCACTGACTCT	AGCTGGAGTTTGTACTGCTCA
NM_001145236	*scpp5*	CTCCGCGATTCCCTGCTAAT	GGTTGATCCTGAGCTCTACCG
NM_001329865	*tg*	CCTGCCAAAAGCCTCAGGTA	CAGCAAGGCAGCCTGTAGAT

Table S4. Mapping results of STAR alignment. Overview of total reads and percent of unique reads per sample

Sample	Total Reads (M)	% unique reads
Control 1	28.0	92.4
Control 2	28.2	92.5
Control 3	28.6	92.2
Control 4	27.3	92.3
PFOS10 1	25.1	91.9
PFOS10 2	27.1	92.3
PFOS10 3	26.2	92.1
PFOS10 4	24.0	92.4
PFOS70 1	27.0	92.3
PFOS70 2	22.0	92.7
PFOS70 3	26.7	92.6
PFOS70 4	29.2	92.4
POP10 1	25.7	92.3
POP10 2	23.1	92.2
POP10 3	25.1	92.4
POP10 4	27.0	92.4
POP70 1	23.5	92.4
POP70 2	23.6	92.1
POP70 3	22.6	92.1
POP70 4	24.1	92.2

Figure S3. RNA quality control plot. Mapping of reads to different features in all individual samples

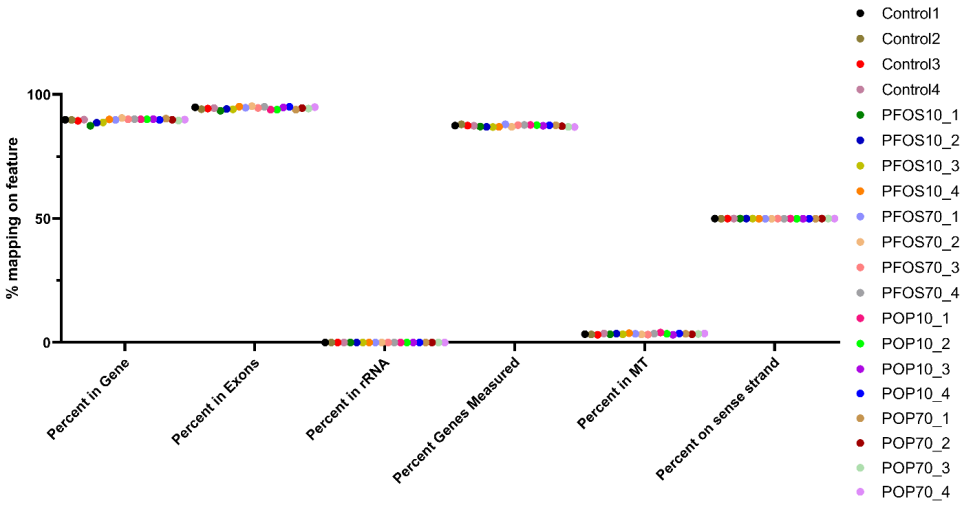

Figure S4. Cumulative distribution plot showing the cumulative distribution of log2 RPM values from low to highly expressed genes in all samples

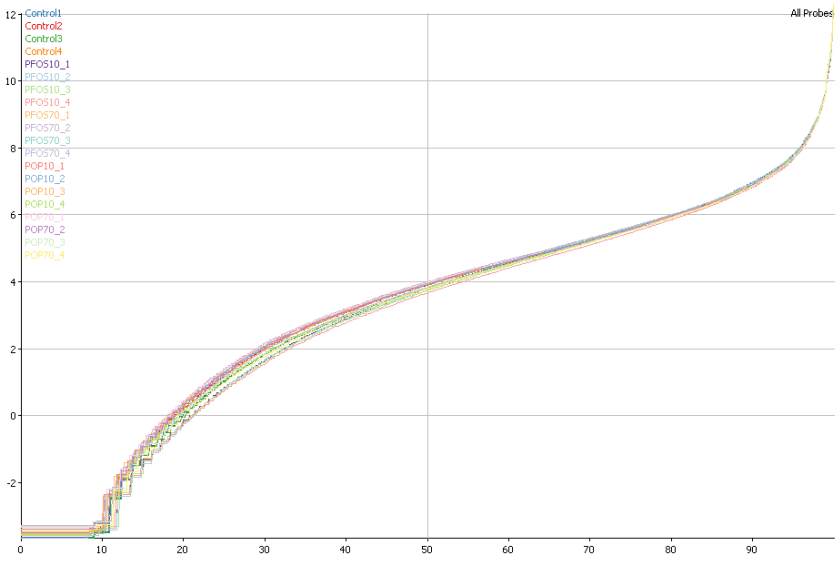

Figure S5. PPAR signalling pathway regulation after exposure to PFOS10. Components in red represent genes that are present in the DEG dataset of PFOS10 transcriptome analysis. Pathway was created in KEGG

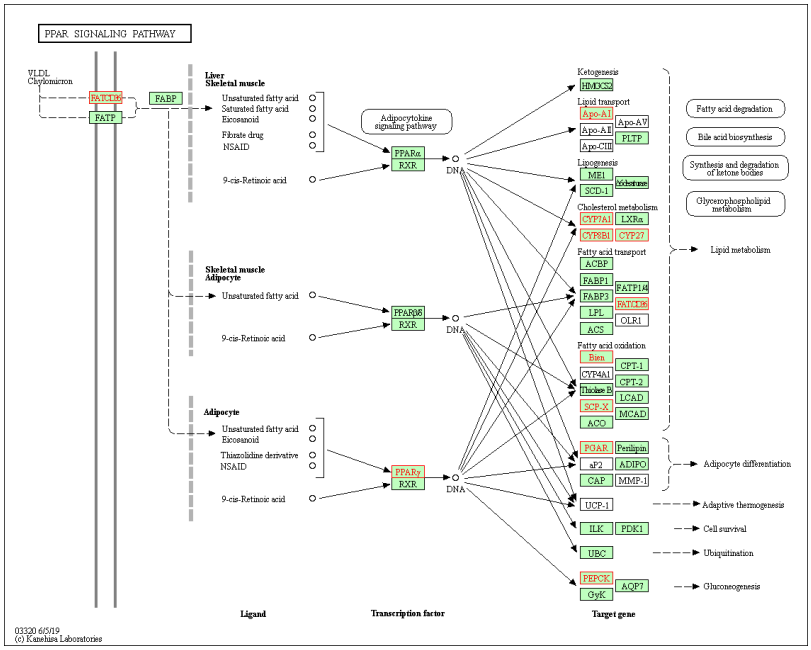

Table S5. qPCR results as shown in absolute fold changes relative to controls for gene validation of RNA-seq results

Gene	PFOS10	PFOS70	POP10	POP70
myhz1.1	-1.97	2.57	2.19	1.77
col10a1a	-2.65	1.59	1.19	1.28
myl7	-2.20	1.83	2.00	1.66
myh7l	-1.43	1.89	2.87	2.18
tnnt2a	-1.44	1.53	1.44	1.72
ugt5a1	-2.88	1.26	1.30	1.10
cd36	-1.97	1.18	-1.05	-1.25
apoa1a	-1.34	1.19	1.42	-1.04
scpp5	-4.68	2.36	1.45	1.43
tg	-1.19	1.19	2.03	1.58

Figure S6. RT-qPCR validation of sequencing results. Scatterplot showing the log2 fold changes from qPCR and sequencing data

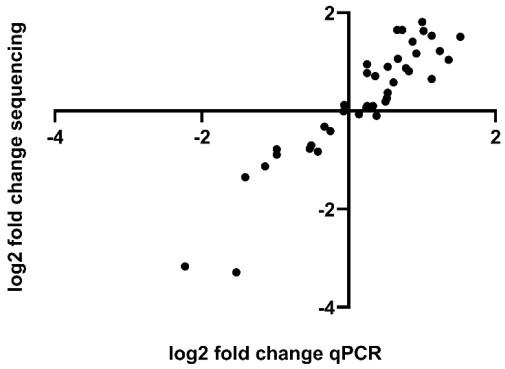

Table S6. Statistical results for larval zebrafish exposed to POP mixture and dantrolene or caffeine in a chronic or acute scenario. For model comparison the AICc score is reported. The model with the lowest AICc is underlined, and the statistics are from lme models, and include the marginal (m) and conditional (c) R^2 of the chosen model.

Parameter	Model comparison (AICc score)	R^2(m, c)	Model results (χ^2, df, p)		
	Exposure×Caffeine, Exposure+Caffeine, null		Exposure×Caffeine	Exposure	Caffeine
Chronic caffeine					
Distance moved (mm)	4157.6, 4157.7, 4161.4	0.05, 0.13	4.3, 2, 0.11	6.5, 2, 0.04	1.2, 1, 0.26
Time active (seconds)	2811, 2810, 2818	0.04, 0.16		0.9, 2, 0.64	13.2, 1, <0.001
Swimming speed (mm/s)	-277, -276, -191	0.29, 0.32	4.6, 2, 0.09	19.2, 2, <0.001	7.8, 1, 0.005
Acute caffeine					
Distance moved (mm)	4171, 4172, 4182	0.07, 0.15	4.8, 2, 0.09	14.2, 2, <0.001	0.4, 1, 0.5
Time active (seconds)	2805.3, 2805.1, 2815.2	0.05, 0.14		3.6, 2, 0.16	13.4, 1, <0.001
Swimming speed (mm/s)	-374, -377, -275	0.31, 0.35		76, 2, <0.001	57, 1, <0.001
	Exposure×Dantrolene, Exposure+Dantrolene, null		Exposure×Dantrolene	Exposure	Dantrolene
Chronic dantrolene					
Distance moved (mm)	3893, 3892, 4042	0.88, 0.99		8.7, 2, 0.01	180, 1, <0.001
Time active (seconds)	2647, 2643, 2745	0.32, 0.38		2.3, 2, 0.32	130.6, 1, <0.001
Swimming speed (mm/s)	-37, -29, 44	0.30, 0.30	12.1, 2, 0.002	18.9, 2, <0.001	26.7, 1, <0.001
Acute dantrolene					
Distance moved (mm)	4298, 4294, 4308	0.07, 0.08		3.3, 2, 0.19	16.9, 1, <0.001
Time active (seconds)	2900, 2896, 2903	0.04, 0.04		1.9, 2, 0.37	11.3, 1, <0.001
Swimming speed (mm/s)	-1837, -1841, -1768	0.23, 0.28		83.3, 2, <0.001	7.6, 1, 0.006

Figure S7. Behavioral responses following the light-dark transition test in larval zebrafish exposed to PFOS or a POP mixture and co-exposed to RyR agonist caffeine chronically (90 hours) or acutely (10 minutes prior to the behavioral test). (**A**) Distance moved. (**B**) Time spent active. (**C**) Swimming speed. Statistics are from linear mixed effect models. Data shown are least square means ± 95% CI. Different lowercase letters indicate general exposure effect (i.e. Control vs POPs vs PFOS).

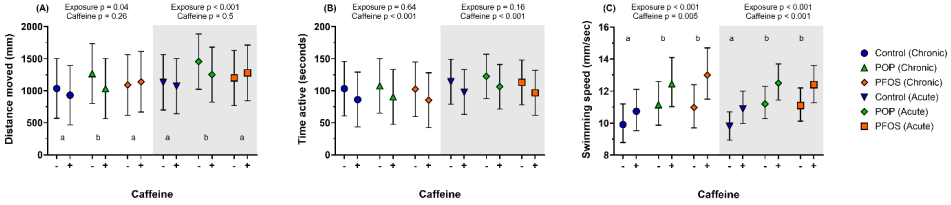

Table S7. Genes in the DEG dataset of PFOS10 transcriptome analysis involved in the regulation of PPAR signalling pathway. absFC: absolute fold change

Ensembl ID	Gene Symbol	Gene Name	absFC
ENSDARG00000012076	apoa1a	apolipoprotein A-Ia	-1.8
ENSDARG00000012194	scp2a	sterol carrier protein 2a	-1.6
ENSDARG00000013522	pck1	phosphoenolpyruvate carboxykinase 1 (soluble)	-9.6
ENSDARG00000020956	pck2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	-2.4
ENSDARG00000031848	pparg	peroxisome proliferator-activated receptor gamma	-2.5
ENSDARG00000032639	cd36	CD36 molecule (thrombospondin receptor)	-1.8
ENSDARG00000035859	angptl4	angiopoietin-like 4	-1.5
ENSDARG00000053068	cyp8b1	cytochrome P450, family 8, subfamily B, polypeptide 1	-2.0
ENSDARG00000057262	si:dkey-91i10.3	si:dkey-91i10.3	-2.2
ENSDARG00000069018	cyp7a1	cytochrome P450, family 7, subfamily A, polypeptide 1	-2.6
ENSDARG00000070029	ehhadh	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	-1.8

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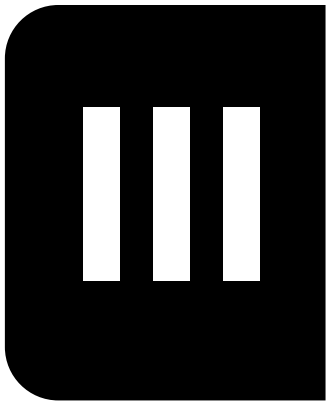


Manuscript

Developmental exposure to a POPs mixture or PFOS increased body weight and reduced swimming ability but had no effect on reproduction or behavior in zebrafish adults.

Maria Christou[a,*], Erik Ropstad[a], Stephen Brown[a], Jorke H. Kamstra[b], Thomas W. K. Fraser[a]

[a] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, P.O Box 369 Sentrum, 0102 Oslo, Norway
Erik Ropstad *email:* erik.ropstad@nmbu.no
Stephen Brown *email:* brownstephen323@gmail.com
Thomas Fraser *email:* thomas.fraser@nmbu.no

[b] Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences, Utrecht University, 3584 CM Utrecht, the Netherlands
Jorke H. Kamstra *email:* j.h.kamstra@uu.nl

[*] Corresponding author. *E-mail address:* maria.christou@nmbu.no (M. Christou)

Abstract

Complex mixtures of persistent organic pollutants (POPs) are regularly detected in the environment and animal tissues. Often these chemicals are associated with latent effects following early-life exposures, following the developmental origin of health and disease paradigm. We investigated the long-term effects of a human relevant mixture of 29 POPs on adult zebrafish following a developmental exposure, in addition to a single PFOS exposure for comparison, as it was the compound with the highest concentration within the mixture. Zebrafish embryos were exposed from 6 to 96 hours post fertilization to x10 and x70 the level of POP mixture or PFOS found in human blood before being transferred to clean water. We measured growth, swimming performance, and reproductive output at different life stages. In addition, we assessed anxiety behavior of the adults and their offspring, as well as performing a transcriptomic analysis on the adult zebrafish brain, as the POP mixture and PFOS concentrations used are known to affect larval behavior. Exposure to POP mixture and PFOS reduced swimming performance and increased length and weight, compared to controls. No effect of developmental exposure was observed on reproductive output or anxiety behavior. Additionally, RNA-seq did not reveal pathways related to anxiety although pathways related to synapse biology were affected at the x10 PFOS level. Furthermore, pathway analysis of the brain transcriptome of adults exposed as larvae to the low concentration of PFOS revealed enrichment in pathways such as calcium, MAPK, and GABA signaling, all of which are important for learning and memory. Based on our results we can conclude that some mild effects on the endpoints measured were apparent, but if these effects lead to adversities at population levels remains elusive.

Keywords: U_{crit}, RNA-seq, behavior, growth, reproduction, pathway analysis

1. Introduction

Persistent organic pollutants (POPs) are omnipresent in the environment leading to humans and wildlife experiencing a near continuous exposure to these chemicals (WHO/UNEP 2012). POPs include many chemicals with anthropologic origins such as polychlorinated biphenyls (PCBs), pesticides such as dichlorodiphenyltrichloroethane (DDT), brominated flame retardants (BFRs) including polybrominated diphenyl ethers (PBDEs), dioxins, and *per*- and poly-fluoroalkylated substances (PFASs) (UNEP 2005). POPs are found in numerous products that were, or are still used, such as plasticizers, pharmaceuticals, pesticides, and industrial chemicals (Ritter *et al.* 1998, Birnbaum and Fenton 2003). Levels of persistent organic pollutants (POPs) are increasing in the environment due to the consequences of human activity and lipophilicity and persistency makes them very potent for bioaccumulation and biomagnification (Ritter *et al.* 1998).

Many POPs are endocrine disrupting chemicals (EDCs) defined as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” (EC 2019). Since hormone balance is of particular importance during early development, early-life exposure to EDCs is expected to increase the susceptibility to disease in later-life, following the developmental origins of health and disease (DOHaD) hypothesis (Hanson and Gluckman 2014). Indeed, early-life exposure to EDCs are associated to later-life effects on cardiovascular, metabolic and reproductive function, as well as being associated with the development of obesity, diabetes and cancer in humans and experimental models (Dolinoy and Jirtle 2008, Zhang and Ho 2011, Barouki *et al.* 2012). Furthermore, the DOHaD proposes that exposure to environmental stressors early in life can produce changes to the genome or epigenome leading to adverse effects in the offspring of individuals during their life leading to transgenerational effects (Anway *et al.* 2006, Guerrero-Bosagna *et al.* 2010). For example, exposure of pregnant female rats to a PCB mixture led to increased body weight and lineage-specific effects of exposure were found for serum progesterone and estradiol in the F2 and F3 generations that were not observed in the F1 offspring that had been directly exposed (Mennigen *et al.* 2018).

Vertebrate models have been used for many years in research studies concerning the effects of chemical exposure (Parasuraman 2011, Gad 2014). These studies aimed to uncover mechanisms of toxicity at the molecular or physiological level thus providing evidence that associates effects of chemicals to human health, but also how exposure to chemicals can affect wildlife populations (Hodson 1985, Suda *et al.* 1999, Brown *et al.* 2015, Marty *et al.* 2017, White *et al.* 2017). In such studies, the zebrafish is a widely used vertebrate model that is ideal for studying chemical toxicity due to its many advantages such as small size, external fertilization, and embryonic transparency that facilitates early chemical exposure and visualization of exposure effects. Additionally, large clutch sizes and short generation time of 3 – 4 months allows for the evaluation of chemical effects over multiple generations. Lastly, the availability of genomic and bioinformatic resources enables the investigation of mechanisms of action (Hill *et al.* 2005).

POPs are part of complex mixtures in the environment, yet many toxicological studies are based on single compound exposures. Such studies fail to detect complex additive, synergistic or antagonistic interactions as is the case in environmental mixtures. For example, zebrafish exposed to a mixture of EDCs had lower egg production even if each compound was present in the mixture at a concentration which on its own would not produce an observable effect (Thrupp *et al.* 2018). This is not well studied in POPs although we know that POP mixture or single compounds can have effects at multiple endpoints. These include survival, swimming performance, body growth and weight, skewed sex ratio, reproductive defects including fertility and fecundity and adult anxiety-like behaviors accompanied by reduced survival and altered behavior of offspring over multiple generations (McCarthy *et al.* 2003, Nourizadeh-Lillabadi *et al.* 2009, Daouk *et al.* 2011, Lyche *et al.* 2011, Lyche *et al.* 2013, Pean *et al.* 2013, Xia *et al.* 2014, Vignet *et al.* 2015, Horri *et al.* 2018, Alfonso *et al.* 2019). In addition, we find no information on developmental exposures and transgenerational effects with mixtures and the single compounds incorporated in the mixture.

In our study we employ zebrafish as an experimental model to explore the long-term impacts of early developmental exposure (6 – 96 hpf) to an environmentally relevant mixture of 29 POPs, or single PFOS the most abundant constituent of the mixture and

111 solely responsible for larval behavioral effects (Khezri *et al.* 2017). The POPs mixture is
112 based on the average levels of chemicals found in human blood of the Scandinavian
113 population (Berntsen *et al.* 2017), but we previously found the levels within larval
114 zebrafish tissues following developmental exposure are also similar to the concentrations
115 of chemicals detected in fish from Norwegian lakes (Nourizadeh-Lillabadi *et al.* 2009).
116 Here, we hypothesize that early life exposure of zebrafish larvae and related effects on
117 behavior, will also produce adverse long-term health effects in adults and their progeny.
118 We investigated the effect of chemical exposure on different key life-traits such as
119 survival, growth, swimming ability, reproduction, sex ratio, and adult anxiety like behavior.
120 In addition, because of the previously reported results on anxiety-like behavior in exposed
121 larvae (Christou *et al.* 2020), we also performed transcriptomic analysis on adult zebrafish
122 brains to reveal long-term effects of early exposure. Finally, we assessed F1 offspring
123 behavior to reveal multigenerational effects of exposure.

124

125 **2. Materials and methods**

126 The study was approved by the Institutional Animal Care and Use Committee at
127 the Norwegian University of Life Sciences (NMBU) and the Norwegian Food Safety
128 Authority (application ID: FOTS 13094). It was conducted in strict accordance with The
129 Norwegian Regulation on Animal Experimentation at the Section for Experimental
130 Biomedicine, NMBU-Faculty of Veterinary Medicine, in Oslo, Norway.

131

132 **2.1 Fish maintenance and breeding**

133 AB wild-type (AB) were kept at 28 ± 1 °C under a 14:10 light/dark photoperiod.
134 Animal care was performed in accordance with lab protocols. System water originated
135 from the local water supply and was treated with reverse osmosis and filtered through
136 particles and active charcoal. Furthermore, the water was treated with UV radiation for
137 sterilization. System water was conditioned by the addition of 155 mg synthetic sea salt
138 (Instant Ocean, Blacksburg, USA), 53 mg sodium carbonate and 15 mg calcium chloride
139 (Sigma-Aldrich, Norway AS) per liter to reach a conductivity of 500 µS/cm, a general

hardness of 4 – 5, and a pH of 7 - 7.5 (adjusted with 1M HCL). Adults were fed 3 times daily, twice with Gemma Micro (Skretting, Stavanger, Norway) and once with artemia (Sep-art Artemia, Ocean Nutrition, Belgium). For embryo production, adults were placed in breeding tanks in the afternoon. The next morning the separator was removed as soon as the lights turned on (08:00) and embryos were collected an hour later. Embryos were maintained in sterile embryo media (60 µg/mL Instant Ocean® sea salts) until the time of exposure.

2.2 POPs mixture and chemicals

Dimethyl sulfoxide (DMSO, >99.7%, CAS number 67-68-5), PFOS (≥ 98%, CAS number 2795-29-3) and MS-222 (98%, CAS number 886-86-2), were purchased from Sigma-Aldrich. The composition of the POPs mixture is described in Table 1 and further details of its preparation can be found in Berntsen *et al.* (2017). Stock solutions of POPs and PFOS were prepared in DMSO and were stored at -20 °C until use.

Table 1. Composition and concentration of chemicals in the POP mixture.

Chemicals	Nominal concentration of stock solution (μM)	Nominal concentration of stock solution (mg/ml)
PFASs		
PFOA	10923	4.523
PFOS	54801	29.425
PFDA	962	0.495
PFNA	1723	0.800
PFHxS	7873	3.450
PFUnDA	990	0.560
BFRs		
BDE-47	18	0.009
BDE-99	7	0.004
BDE-100	3	0.003
BDE-153	1	0.00
BDE-154	3	0.002
BDE-209	11	0.011
HBCD	38	0.025
PCBs		
PCB 28	50	0.013
PCB 52	34	0.010
PCB 101	24	0.008
PCB 118	196	0.064
PCB 138	615	0.222
PCB 153	1003	0.362
PCB 180	490	0.194
Other organochlorines		
p,p-DDE	1578	0.502
HCB	410	0.117
α-chlordane	26	0.011
Oxy-chlordane	51	0.022
Trans-nonachlor	92	0.041
α-HCH	20	0.006
β-HCH	182	0.053
γ-HCH (lindane)	20	0.006
Dieldrin	63	0.024

2.3 Solutions preparation

For exposure experiments, two concentrations of the POP mixture were used. The low concentration was equal to the levels of chemicals that are 10 times higher than what is found in average Scandinavian human blood levels and the high concentration corresponds to levels 70 times higher (exposures will be referred to as POP10 and

POP70 from here on). Working solutions of the POP mixture were prepared at the day of the experiment by diluting the stock solution (1,000,000x) in sterile embryo media and adjusting the concentration of DMSO to 0.1%. Concentrations of the POPs mixture were based on previous work done in our group (Khezri *et al.* 2017). The concentrations of PFOS were based on the nominal concentration found in the POP mixture exposures and corresponded to 0.3 mg/L (0.55 µM, will be referred from now as PFOS10) for the low concentration and 2.06 mg/L (3.83 µM, will be referred to as PFOS70) for the high concentration. PFOS working solutions were prepared on the day of the experiments by diluting the stock solution (54.8 mM) in sterile embryo media and adjusting the DMSO concentration to 0.1%. A stock solution of MS-222 (500 mg/L) was prepared in phosphate buffer solution and the pH was adjusted to 7 – 7.5. MS-222 was used accordingly for either anesthesia or euthanasia.

2.4 Larval exposures and maintenance of experimental populations

For the establishment of the F0 generation, five populations of larvae were produced per replicate with four independent biological replicates produced in total. Each population consisted of 300 fertilized eggs. The control population consisted of eggs exposed only to the solvent (0.1% DMSO) and treated larvae were exposed to either POP10, POP70, PFOS10 or PFOS70. Eggs and larvae were kept in exposure media from 6 – 96 hours post fertilization (hpf) in petri dishes with 60 mL exposure media (size 150 mm x 15 mm, Sigma-Aldrich, Norway AS). After 96 hpf the larvae were transferred to 1 L beakers (VWR®) with clean system water with a stocking density of 150 larvae/L and 90% daily renewal of water. At 15 days post fertilization (dpf) the larvae were transferred to a ZebTEC Stand Alone system (Tecniplast S.p.A, Italy) until the termination of the experiments when fish were 15 months old. Larvae and adult fish were kept in 8 L tanks. The initial stocking density of larvae was 50 individuals/L and at 50 dpf the populations were divided to reach a final stocking density of 8 individuals/L. For the duration of the experiments the photoperiod was kept at 14:10 hr light/dark, cycle, pH at 7 – 7.5, conductivity at 500 – 550 µS/cm and temperature at 28 – 28.5 °C. Oxygen saturation levels were > 95% and there was 100% water recirculation rate per hour with a 15% daily

renewal rate. Larvae were fed 3 times daily with artemia (Sep-art Artemia, Ocean Nutrition, Belgium) and were gradually introduced to dry feed of different sizes according to the manufacturer's instructions (ZebraFeed, Sparos, Portugal). Adult fish from each condition and replicate were used for all performed subsequent tests.

2.5 Survival and growth

Survival of experimental populations was monitored daily until 150 dpf. After this age mortality was below 10%. Random samples of 15 – 20 individuals/population were taken at 5, 15, 30, 60, 90 and 120 dpf for the evaluation of growth rate. At 5 and 15 dpf, larvae were placed under a stereomicroscope and from 30 dpf onwards fish were placed under a camera mounted on a tripod. Fish were anesthetized and photographed for the measurement of standard length in mm (SL, tpsDig v2.30, Rohlf (2005)). Fish taken for growth rate estimation were returned to their respective tanks. One-way analysis of variance (ANOVA) followed by Dunnett's test with the Control population as a reference group was performed to test the effect of chemical exposure on fish growth in each sampling day and G-test was applied with a significance level of 0.05 to test whether chemical exposure had an effect on survival rates. All graphs were plotted in Graphpad (v8.3, CA, USA).

2.6 Behavioral test adults

At 7 months old, adult zebrafish were submitted to a novel tank diving test and recorded using the Ethovision XT13 software (Noldus Information Technology, The Netherlands). Two females and two males per condition and replicate were subjected to the behavioral test with a total of 40 fish per sex and condition (Control, POP10, POP70, PFOS10, PFOS70). Fish were immediately introduced to a 1.5 L tank (trapezoid tank Aquatic Habitats, Apopka, Florida, USA, size in cm: height 15.2 × width 7.1 × length 27.9 at the top and 22.5 at bottom) and their swimming pattern was recorded for 5 minutes. Recording started immediately after the transfer. The camera used was able to capture two tanks in one frame. Tanks were divided by a separator to ensure individuals could

not see one another. Between trials, the tanks were rinsed, and water renewed to remove waterborne pheromones. Cumulative duration (seconds), distance moved (cm), and mean velocity (cm/s) were calculated for each fish in two predefined zones, the bottom zone (the bottom half of the tank) and the top zone (top half of the tank). Additionally, the number of zone crossings was calculated (Cachat *et al.* 2010).

Behavioral data were imported to R (version 3.6.1). Linear mixed effect (LME) models within the "nlme" package were used to assess behavioral parameters. The dependent variables tested were either cumulative time in bottom zone (seconds), cumulative distance moved in bottom zone (cm), mean velocity (cm/s), and number of crossings between zones, with group (5 levels, control, POP10, POP70, PFOS10, PFOS70) and sex (2 levels, Males or Females) as categorical independent variables, and replicate as a random effect. Here, the variables Group and Sex were initially allowed to interact. We then compared the interaction model to a model without interaction between the two variables and another model where Group was the only independent variable using the Akaike Information Criterion with a correction for small sample size (AICc). The model with the lowest AICc score was then considered the true model (Aho *et al.* 2014). For model variation, all models were also compared to a null model, where the variation seen in our data is not explained by our independent variables, also using AICc. The "Anova" command within the "car" library was used to extract the results for the main effect whereas the "lsmeans" command with "emmeans" library was used as a post-hoc test to compare groups against one another while adjusting for the means of other factors within the model (Lenth 2016). Type II sum of squares were used for models without interactions. The R^2 of the model was determined using the command "r.squaredGLMM" that returns the marginal and conditional R^2 that represent the variance explained by the fixed actors alone excluding the random effect and the variance of the entire model including the random effect, respectively (Nakagawa and Schielzeth 2013). For all models, examination of the residual plots verified that no systematic patterns occurred in the errors (e.g. standardized residuals vs fitted values). Significance was assigned at $a = < 0.05$. All graphs were plotted in Graphpad (v8.3, CA, USA).

2.7 Reproductive tests adults

Reproductive tests were performed when zebrafish were 6 months old. Reproductive tests were performed on 3 of the 4 replicates. The experimental protocol was based on the paper published by Uusi-Heikkila *et al.* (2010). Briefly, female and male fish from control and exposed populations were kept in separate tanks for one week prior to the start of the reproductive tests. All fish were anesthetized and measured for standard length (SL), to make sure there were no statistical differences in length between the tested populations, as there is a positive correlation between size and reproductive output (Uusi-Heikkila *et al.* 2010). After one week fish were placed in breeding tanks for 5 days with a ratio of 1 female to 2 males per tank. Seven breeding pairs were set for each condition. Each morning eggs were collected from each tank and pooled together for each condition. The number of fertilized and unfertilized eggs was counted using ImageJ (v1.51k, https://imagej.nih.gov/ij/) and the fertilization rate was calculated. Afterwards, 100 fertilized eggs from each condition were placed in a petri dish. These eggs were monitored daily for mortality at 24 hpf and hatching rates at 72 hpf. The procedure was repeated for each of the five days, and the number of breeding pairs that laid eggs was also recorded. At the end of each reproductive trial, fish were euthanized and weighted both for total and gonadal weight. The gonadosomatic index (GSI = [gonad weight / total tissue weight] × 100) was calculated for each sex.

One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test relative to control was performed to test the effect of early chemical exposure on the gonadosomatic index of female and male zebrafish. Reproductive data were imported to R (version 3.6.1). Fertilization rate and average number of fertilized and unfertilized eggs per day were normalized based on the number of breeding pairs on each day. The depended variables tested were the fertilization rate, the mean number of fertilized and unfertilized eggs, mortality at 24 hpf, and hatching at 72 hpf. All variables were treated as continuous. Two models were tested, model A was a generalized least square fit model without a random effect whereas model B was a linear mixed-effect model with a random effect (Condition + Replicate). Independent variables for both models were the exposure condition (Control, POP10, POP70, PFOS10 and PFOS70) the day of reproductive trial

(1 to 5) and their interaction. The model with the lower AICc score was selected for further statistical comparisons. The “lsmeans” command was used with a post-hoc tukey adjustment to test for statistical differences at a significance level of $a = < 0.05$. All graphs were plotted in Graphpad (v8.3, CA, USA).

2.8 Swimming tests adults

Swimming performance was tested by estimating the sustained critical swimming speed (U_{crit}) in three males from each replicate (n = 12 males/condition) beginning at 10 months of age (tests started in Jan 2019). Adult males were measured for length and then placed in a modified Blazka-type swim tunnel (Loligo systems, Denmark) 18 h prior to testing and maintained on a flow rate of 1cm/s whilst unfed. The system consists of a 170 ml swim tunnel submerged in a 20 L tank supplied with system water (described above) and maintained at 28°C using a submerged heating element. For the U_{crit} experiment, individual males were subjected to a stepwise increment in swimming velocity of 2.7 body lengths/s every 39 minutes until exhaustion (when the fish was unable to swim). Critical swimming speed was calculated using the equation described in Brett (1964). In total, 60 males were assessed over a 25-week period. To prevent issues with age, one round of all groups within a replicate were done within 8 days of one another and one male from each group per replicate was done prior to assessing the second male from each group per replicate and so on.

Swimming data were imported to R (version 3.6.1). Linear mixed effect (LME) models within the “nlme” package were used to assess swimming activity. The dependent variable tested was Ucrit (BL/s), with group (5 levels, control, POP10, POP70, PFOS10, PFOS70) and length and weight as categorical independent variables and replicate as a random effect. Here the variables Group, Length and Weight were initially allowed to interact (Group + Length, Group + Length * Mass). We then compared the interaction model to a model without interaction between the two variables and another model where Group was the only independent variable using the Akaike Information Criterion with a correction for small size (AICc). Details concerning model selection and generation of

results is described in section *2.6 Behavioral tests adults*. Significance was assigned at $a = < 0.05$. All graphs were plotted in Graphpad (v8.3, CA, USA).

2.9 Brain sampling and transcriptome analysis

Whole brain tissue from 2 females and 2 males per condition and replicate (total 8 females and 8 males per condition) were collected after euthanasia of adult fish in MS-222. Brain tissue was collected individually in eppendorf tubes, snap frozen in liquid nitrogen, and stored at -80 °C until RNA extraction for high-throughput sequencing analysis.

RNA from samples for RNA-seq analysis were purified using NucleoSpin® RNA extraction kit (Macherey-Nagel, Germany). RA1 lysismix (Macherey-Nagel, Germany) was added to each sample (10 larvae per sample) and samples were passed through a 21-gauge needle (HSW HENKE-JECT®, Germany) until complete homogenization. Total RNA was extracted from samples following manufacturer's instructions. Each sample was eluted in 50 µL RNase-free water and stored at -80 °C until further analysis. RNA quantity and purity for all samples was assessed using a Nanodrop-1000 Spectrophotometer (NanoDrop Technologies, DE, USA). RNA integrity number (RIN) was determined with Agilent 2100 Bioanalyzer (Agilent Technologies, Ca, USA) using RNA Nano LabChip Kit (Agilent Technologies, Ca, USA). All samples were found to be of acceptable quality for sequencing (RIN > 7.0).

Samples for sequencing were sent to Novogene (Hong Kong). A total of 2 µg was used for library preparation. A quality check (QC) of total RNA was performed with a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), Agilent 2100 (Agilent Technologies, CA, USA) and agarose gel electrophoresis prior to library construction. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo attached magnetic beads. Fragments were randomly created in fragmentation buffer. First strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase. After first-strand

synthesis, a custom second-strand synthesis buffer (Illumina, CA, USA) was added with dNTPs, RNase H and *Escherichia coli* polymerase I to generate the second strand. The final cDNA library was ready after a round of purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection, and PCR enrichment. PCR products were purified (AMPure XP system, Beckman, US) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Libraries were analyzed with a Hiseq 4000 (Illumina, Ca, USA), using 150bp paired-end reads, with a depth of 20 million reads per sample.

For the transcriptome analysis we used the recently developed pipeline SnakePipes for high throughput omics analysis (Bhardwaj *et al.* 2019). Within SnakePipes, raw fastq files were adapter trimmed using trim_galore (v0.4.5, Babraham institute, UK) under standard parameters, followed by STAR alignment (v2.5.4b) (Dobin *et al.* 2012) to map sequences to the zebrafish genome (GRCz11, https://www.ensembl.org) with a recent release of the zebrafish transcriptome GTF (v92, www.ensembl.org) . After alignment the generated filtered BAM files were loaded to SeqMonk sequence analysis tool (v1.45, Babraham institute, UK) and mRNAs were quantified using the built-in mRNA seq pipeline. Library quality was evaluated with the RNA-seq QC plot and cumulative distribution plots using SeqMonk. Filter was applied to analyze mRNAs that had at least a log2 expression > 0 in either of the replicate sets (Control, POP10, POP70, PFOS10, PFOS70) to assure that for statistical analysis only mRNAs with enough reads were included. A principal component analysis was performed in all expressed genes that had a log 2 ≥ 0 expression in all groups and sex separately (Control F, Control M, POP10 F, POP10 M, POP70 F, POP70 M, PFOS10 F, PFOS10 M, PFOS70 F, PFOS M) using ClustVis, a web tool for visualizing clustering of multivariate data (Metsalu and Vilo 2015). PCA scores were loaded to R (version 3.6.1) and biplots of principal components were designed with “ggplot2” library, while the stat_ellipse argument within the “ggplot2” library was used to compute 95% confidence ellipses to test whether there was a clear separation of different groups and sex. Deseq2 filter using R (v3.6.1) within SeqMonk was used on raw read counts to calculate differential expression. With this analysis mRNA isoforms were merged, sinceDeseq2 cannot assess differential expressed transcript isoforms. Deseq2 is a differential gene expression analysis based on the negative binomial distribution, with Benjamini Hochberg false

discovery rate multiple comparisons adjustments (FDR) (Love *et al.* 2014). After Deseq2 analysis, data were normalized by reads per million (RPM) to calculate fold change (FC) per gene combining all samples in each condition to calculate the average. Differentially expressed genes (DEG) were chosen based on an FDR < 0.05 and an absolute FC > 1.5. Venn diagrams were created using Venny (version 2.1, https://bioinfogp.cnb.csic.es/tools/venny/).

Differentially expressed genes were imported in Webgestalt (Liao *et al.* 2019a) for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis and gene ontology (GO) analysis to explore affected pathways. Only pathways that had a p-value < 0.05 were considered significantly enriched.

2.10 Behavioral tests of F1 larvae

No re-exposures

To test whether early life exposure of F0 generation had a multigenerational effect on the behavioral outcome of F1 generation, larvae were submitted to a light/dark transition test and a thigmotaxis assay according to Christou *et al.* (2020). Fertilized embryos derived from adult zebrafish of the F0 generation (belonging to Control, POP10, POP70, PFOS10 and PFOS70 populations) were transferred into clear polystyrene 96 well plates (Nunc™ MicroWell™) with one embryo per well from 6 hpf until the time of testing at 96 – 100 hpf (between 9:00 – 13:00) in 200 µL of sterile embryo media. For the thigmotaxis assay, embryos were placed in 24 well plates (Corning® Primaria™) with one embryo per well in 1 mL sterile embryo media. For the thigmotaxis assay two controls were used, one for the POPs mixture F1 larvae and one for the PFOS treatment respectively, as the plate layout meant that each group could not be equally represented on each row and column without the addition of extra controls. All groups were spread equally on each row and column to avoid bias based on position during behavioral testing. For the light-dark transition test, each well plate included 10 embryos per condition and was repeated 4 times, one for each replicate of the F0 generation. For the thigmotaxis

assay, each well plate contained 3 embryos per condition with 3 well plates per replicate. The experiment was also repeated 4 times.

Re-exposures

To test whether F1 larvae had the same response to chemical exposure as their F0 counterparts, embryos derived from F0 adults of Control, POP70 and PFOS70 conditions were re-exposed to either a control medium (0.1% DMSO), a POP10, or a POP70 exposure medium for embryo originating from Control and POP70 adults or control medium, PFOS10 and PFOS70 exposure medium for embryos originating from Control and PFOS70 adults. The behavioral outcome was evaluated with the light-dark transition test. Fertilized embryos were placed in a 96 well plate with 200 µL exposure media from 6 hpf until the time of testing at 96 – 100 hpf. Two well plates were included in each replicate, one containing embryos originating from F0 Control and F0 POP70 adults and another with embryos from F0 Control and F0 PFOS70 adults. Each well plate included 16 embryos per condition and the experiment was performed 4 times.

Behavioral assays were performed in a ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France). Behavioral tests were conducted between 9:00 – 13:00 in 96 – 100 hpf zebrafish larvae. For the light/dark transition test and the thigmotaxis test, the cumulative distance travelled, and the time spent active were measured simultaneously for all larvae in a well-plate. The light-dark cycle lasted 20 minutes and consisted of 10 minutes of light and 10 minutes of dark. Prior to the test the larvae were acclimatized in the behavioral chamber for 10 minutes with the lights on. For the light-dark transition test the mean swimming speed was calculated by dividing the cumulative distance travelled by the total time spent active. For the thigmotaxis test the percent of the total distance moved in the outer zone was calculated. For this purpose, each well in a 24 well plate was divided into two zones (total diameter of each well 16.2 mm). The width of the outer zone was set at 5mm relative to the border of the well. The light level was set to 100% on the ViewPoint software (7.45 Klux, TES 1337 light meter). Infrared light (850 nm) tracks larval activity during the “dark” periods. The threshold for determining movement was set at 5mm/sec. Dead, deformed, and non-moving

(coagulated, unhatched, notochord deformations, yolk sac or cardiac edema, swim bladder development, loss of equilibrium) larvae were excluded from the analysis (data not shown).

Behavioral data were imported to R (version 3.6.1). The procedure of statistical analysis was the same as the one described for the behavioral data of adult zebrafish. The dependent variable for the light-dark transition test was either the cumulative time spent active (seconds), the cumulative distance travelled (mm), or average swimming speed (calculated as the cumulated distance travelled/cumulated time spent active). For the thigmotaxis assay, the variable used was the percent of the total distance moved in the outer zone. For the re-exposure experiments, models also tested the interaction between exposure and history. Exposure was either Control, POP10 and POP70 for the POP experiment or Control, PFOS10 and PFOS70 for the PFOS experiment. History described the origin of F1 larvae and was either Control, POP70 or PFOS70. Interaction meant that F1 larvae derived from POP70 or PFOS70 adults responded to the chemical exposure in a different manner than their F1 Control counterparts.

2.11 Final sampling

All remaining fish (N = 54 – 71/condition and replicate) were euthanized with an excess of MS-222 at the end of the experiment at 15 months. All fish were individually measured for length (mm) and weight (mg) and internally inspected for sex determination. Condition factor indices (*K*) were calculated for each fish using their weight and length measurements (K = [weight × 100]/length^3) (Jones *et al.* 1999).

A chi square test was performed (JMP PRO v15.0, SAS Institute Inc.) to test whether there was a difference in sex ratio between conditions. G-test was applied with a significance level of 0.05 to test differences in final survival rates between conditions. Length, weight and condition factor data were imported R version as dependent variables (R Development Core Team 2018, http://www.r-project.org). Statistical analysis was performed with group (5 levels, control, POP10, POP70, PFOS10, PFOS70) and sex (2 levels, Males or Females) as categorical independent variables, and replicate as a

random effect. The process of statistical analysis is mentioned in the section of adult behavioral analysis. All graphs were plotted in Graphpad (v8.3, CA, USA).

3. Results

3.1 Survival and growth

No significant differences were observed in length between control and exposed populations on any of the sampling days up to 120 dpf (Supplementary material Figure S1). Furthermore, no differences were observed on the survival rates at 150 dpf (Supplementary material Figure S2).

3.2 Behavioral test adults

All details of the statistical results are summarized in Supplementary material Table S1. No significant differences were observed between Control and exposed groups for the time spent in bottom zone, mean velocity (cm/sec), or number of crossings between the two zones. Here, the null model was selected for all three tested variables using the AICc meaning that the variation in our results could not be explained by any of our independent variables i.e. group and sex. A significant effect of group was observed in cumulative distance moved in the bottom zone ($p = 0.03$). Here POP10, PFOS10, and PFOS70 tended to move less in the bottom zone compared to controls, but post-hoc analysis with Control as a reference group failed to return significant pairwise differences (Figure 1).

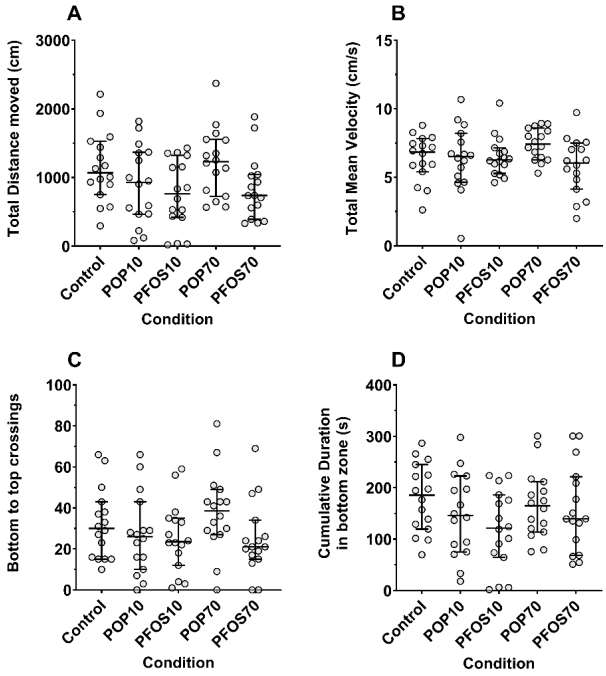


Figure 1. Behavioral responses of adult zebrafish following the novel tank diving test, **A)** Total distance moved, **B)** Total mean velocity, **C)** Bottom to top crossings and **D)** Cumulative duration in bottom zone. Data shown are median values ± 95% CI

3.3 Reproductive tests

Fertilization rate, mean number of fertilized and unfertilized eggs, mortality at 24 hpf, and hatching at 72 hpf did not present significant differences between control and treated populations (Figure 2A). The number of fertilized and unfertilized eggs reduced gradually from day 1 to day 5 of the reproductive test (Figure 2B, C). The fertilization rate however was relatively consistent during the 5 days of the reproductive test. Additionally, some conditions e.g. Control's group fertilization rate (Figure 2A) were characterized by large variation as shown with the confidence intervals hence the lack of statistical differences between groups. No statistical differences where observed on the GSI of males and females of different conditions (Figure 2F).

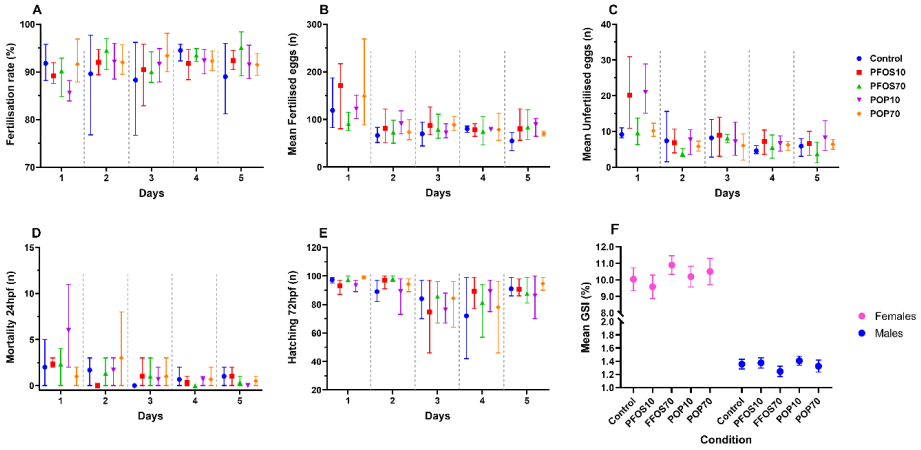
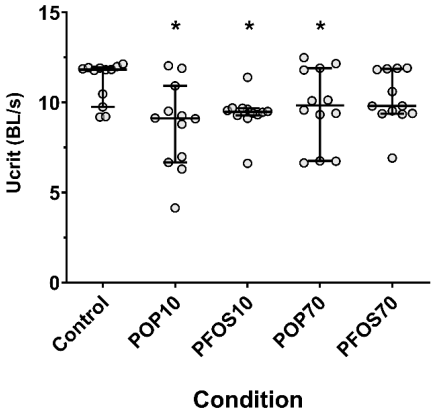


Figure 2. Reproductive variables obtained from the reproductive tests performed on adult zebrafish. **A)** Fertilization rate **B)** Mean number of fertilized eggs **C)** Mean number of unfertilized eggs **D)** Mortality of embryos at 24hpf **E)** Hatching at 72hpf and **F)** gonadosomatic index of females and males. Data shown are **A-E** Least square means ± 95% CI, **F** Mean ± SE

3.4 Swimming tests

All details of the statistical results are summarized in Supplementary material Table S1.

Critical swimming speed, the speed when the fish cannot keep their position in the swim tunnel and fatigue sets in (Brett 1964) (Body length/second, BL/s) was significantly affected by early life exposure ($p < 0.001$). When compared to Control, the critical swimming speed of POP10, PFOS10, and POP70 individuals was significantly lower (Figure 3).



519

520 **Figure 3.** Reduced swimming speed in adult fish exposed as larvae to POP10, POP70, PFOS10
521 and PFOS70. Values represent median ± 95% CI relative to body length (BL/s). Asterisks indicate
522 statistical differences relative to control ($p < 0.05$).

523

524 ### 3.5 Sequencing analysis

525 Analysis with FastQC revealed high quality sequences with phred scores above
526 30 over all reads (data not shown). Average mapping efficiency was over 87% unique
527 reads (Supplementary material Table S2). Quality control analysis performed with
528 SeqMonk on aligned reads, revealed a high proportion of reads falling into genes (77%)
529 with a high proportion of these reads falling into exons (89%) meaning our library was
530 mature, containing a low amount of unspliced transcripts. Additionally, a low amount of
531 reads fell into ribosomal or mitochondrial RNA and 84% of the annotated zebrafish genes
532 were mapped. Reads were mapped equally on the sense and anti-sense strand
533 confirming that our library was non-strand specific (Supplementary material Figure S3).
534 A cumulative distribution analysis of expressed genes over all samples revealed the same
535 expression profile, which indicated highly similar sequencing libraries, and further
536 normalization using reads per million (RPM) was unbiased (Supplementary material
537 Figure S4).

538

3.6 RNA sequencing expression results on adult brains

Principal component analysis (PCA) revealed no clustering of samples coming from different condition and sex so all samples were pooled together irrespective of their sex (data not shown). Additionally, PCA analysis revealed a consistent outlier in Control male group therefore this sample was excluded from further analyses (data not shown).

Deseq2 analysis revealed that PFOS10 was the group with the highest number of differentially expressed genes (DEG) with 466 genes (463 upregulated). The rest of the conditions had a very low number of DEG, 2 genes (1 upregulated), 5 genes (5 upregulated), 13 genes (12 upregulated) for PFOS70, POP10, and POP70 respectively (Supplementary material Table S3). Principal component analysis was subsequently performed on all measured genes in the 5 conditions (Control, POP10, POP70, PFOS10, PFOS70) (Figure 4). The first principle component explained 24.6% and the second 10.6% of the variance. We did not observe any separation along either of the principle components indicating no effects in the global transcriptome of the exposure groups.

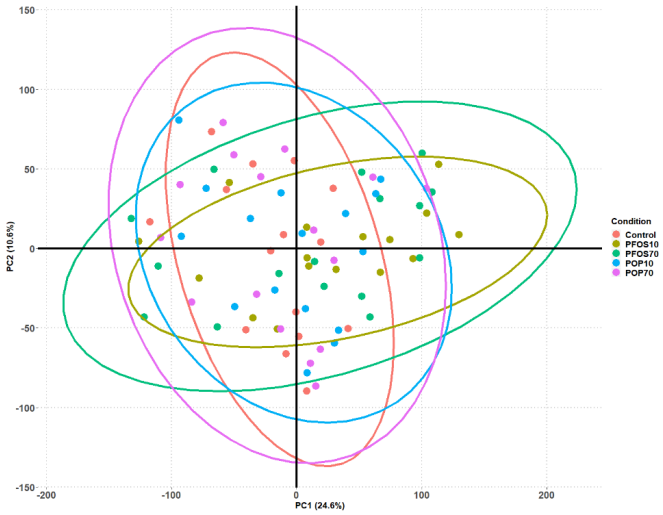


Figure 4. Principal component analysis of all adult groups of all genes expressed in each group. Circles around each condition represent 95% confidence ellipses. Percent explained by each principal component is presented in parenthesis of x and y axes.

According to the venn diagram, 1 gene was common between PFOS10 and POP10 (*ier2a*), 2 genes were common between PFOS10, POP10, and PFOS70 (*egr2b, npas4a*), 1 gene was common between POP10 and POP70 (*egr1*) and 1 gene was common between PFOS70 and POP70 (*anxa1a*). PFOS10 and POP70 share the highest number of overlapping DEGs with 5 common genes (*fosl1a, rtn4rl2a, egr2a, nr4a1, zgc:122979*) (Figure 5 and Supplementary material Table S4).

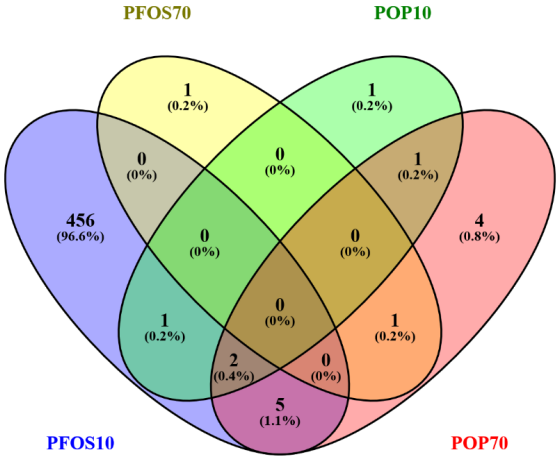


Figure 5. Venn diagram showing the number of overlapping differentially expressed genes from each exposure scenario

3.7 Webgestalt pathway analysis

DEGs were imported to Webgestalt (Liao *et al.* 2019b) using a custom reference list of all measured genes for gene enrichment analysis. An overview of all pathways from GO and KEGG analysis are presented in Tables 2 – 3. Only PFOS10 and POP70 had an adequate number of DEGs for pathway analysis. PFOS10 had the most enriched pathways since it presented with the highest number of DEGs (463 genes). GO analysis of biological functions revealed multiple pathways in synaptic or post-synaptic transmission and signaling pathways along with transmembrane transport pathways (Table 2). KEGG analysis revealed enrichment in pathways such as mitogen-activated

protein kinases (MAPK), apelin, calcium, ErbB (epidermal growth factor receptors), Wnt and adipocytokine signaling (Table 3). For the POP70 group, GO analysis revealed pathways involved in transcription, metabolic and biosynthetic processes, and immune system response (Table 2). KEGG pathway analysis failed to return significantly enriched pathways (Table 3).

Table 2. GO analysis of canonical pathways involved in biological processes for the adults exposed as larvae in PFOS10 or POP70, FDR = false discovery rate

ID	Name	#Gene	p-value	FDR
PFOS10				
GO:0007268	chemical synaptic transmission	166	3.12E-07	0.000337
GO:0098916	anterograde trans-synaptic signaling	166	3.12E-07	0.000337
GO:0099537	trans-synaptic signaling	167	3.41E-07	0.000337
GO:0099536	synaptic signaling	168	3.71E-07	0.000337
GO:0051932	synaptic transmission, GABAergic	6	4.29E-06	0.003113
GO:0034220	ion transmembrane transport	401	6.93E-06	0.004193
GO:0030001	metal ion transport	219	1.44E-05	0.007266
GO:0007214	gamma-aminobutyric acid signaling pathway	8	1.93E-05	0.007266
GO:0055085	transmembrane transport	566	1.99E-05	0.007266
GO:0098660	inorganic ion transmembrane transport	272	2E-05	0.007266
POP70				
GO:0006357	regulation of transcription by RNA polymerase II	566	0.000165	0.23612
GO:0006366	transcription by RNA polymerase II	596	0.000211	0.23612
GO:0080090	regulation of primary metabolic process	1585	0.000274	0.23612
GO:0031323	regulation of cellular metabolic process	1611	0.000305	0.23612
GO:0031326	regulation of cellular biosynthetic process	1106	0.000377	0.23612
GO:0009889	regulation of biosynthetic process	1115	0.000395	0.23612
GO:0019222	regulation of metabolic process	1712	0.000455	0.23612
GO:0050778	positive regulation of immune response	38	0.00076	0.3162
GO:0002252	immune effector process	42	0.000928	0.3162
GO:0050776	regulation of immune response	50	0.001315	0.3162

589 **Table 3.** KEGG analysis of canonical pathways for the adults exposed as larvae in PFOS10 or
590 POP70, FDR = false discovery rate

ID	Name	#Gene	p-value	FDR
PFOS10				
dre04010	MAPK signaling pathway	308	2.6E-06	0.000416
dre04371	Apelin signaling pathway	145	7.75E-05	0.006199
dre04020	Calcium signaling pathway	173	0.000457	0.024363
dre04012	ErbB signaling pathway	96	0.000745	0.0298
dre04080	Neuroactive ligand-receptor interaction	206	0.002314	0.074049
dre04144	Endocytosis	264	0.003009	0.08025
dre04310	Wnt signaling pathway	145	0.003971	0.090761
dre04912	GnRH signaling pathway	103	0.00482	0.096395
dre04920	Adipocytokine signaling pathway	69	0.008347	0.14839
dre04914	Progesterone-mediated oocyte maturation	98	0.01281	0.20495
POP70				
dre03018	RNA degradation	81	0.082893	1
dre04933	AGE-RAGE signaling pathway in diabetic complications	102	0.10346	1
dre04912	GnRH signaling pathway	103	0.10443	1
dre04625	C-type lectin receptor signaling pathway	105	0.10637	1
dre04310	Wnt signaling pathway	145	0.14442	1
dre04371	Apelin signaling pathway	145	0.14442	1
dre04010	MAPK signaling pathway	308	0.28633	1

591

592 ### 3.8 Behavioral tests of F1 larvae

593 All details of the statistical results are summarized in Supplementary material
594 Table S1.

595

596 *No re-exposures*

597 No behavioral effects were observed in F1 larvae submitted to the light/dark
598 transition test or the thigmotaxis assay. The null model explained all the variation

observed for cumulative distance moved, total time spent active, swimming speed, and percent of total distance moved in the outer zone (Figure S5, Supplementary material).

Re-exposures

Re-exposure to PFOS10 and PFOS70 had a significant effect on all variables tested in the light/dark transition test for larvae originating from F0 Control and PFOS70 adults but with no tendency of interaction with history. All groups reacted with a dose-dependent increase of their responses to PFOS re-exposure (Figure 6A-C).

Exposure to the POP mixture revealed an interaction between exposure and history for cumulative distance moved and total time spent active. For larvae originating from Control F0 adults, exposure to the POP mixture caused a significant increase in time spent active whereas for larvae originating from POP70 F0 adults showed no increase in time active. In contrast, for distance moved although the model suggested an interaction, this interaction was not significant. No interaction was suggested for swimming speed, but exposure had a significant effect, causing an increase in average swimming speed in larvae originating both from Control and POP70 F0 adults (Figure 6D-F).

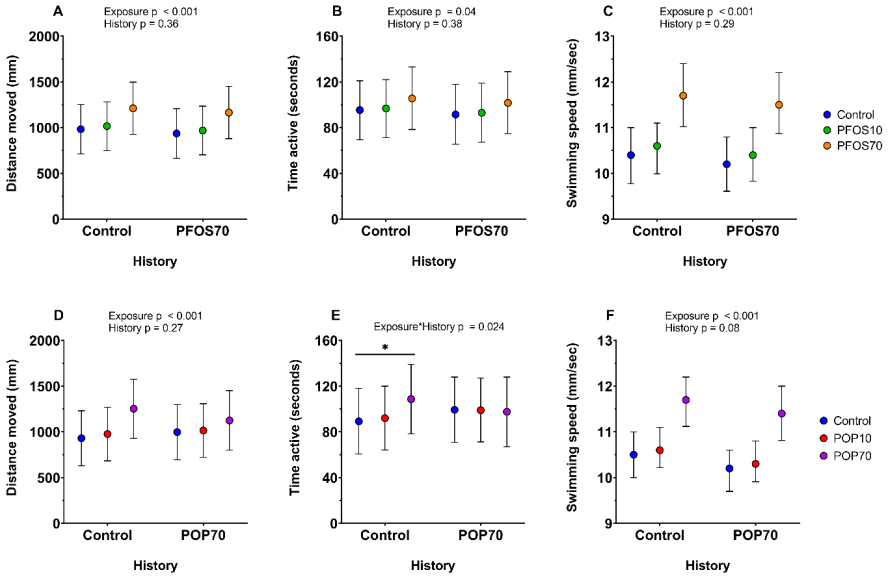


Figure 6. Behavioral responses of F1 larvae originating from Control and PFOS70 F0 adults (A-C), and from Control and POP70 F0 adults (D-F). **(A, D)** Distance moved. **(B, E)** Time spent active. **(C, F)** Swimming speed. Statistics are from linear mixed effect models. Data shown are least square means ± 95% CI. Asterisk indicates an effect of re-exposure within different history groups (for the significant interaction model only). For panels A, B, C, D and F the exposure effect is 0 < x10 < x70.

3.9 Final sampling

All details of the statistical results are summarized in Supplementary material Table S1.

No significant differences were observed in the sex ratio and survival rate at the end of the experiments (Figures 7A and B).

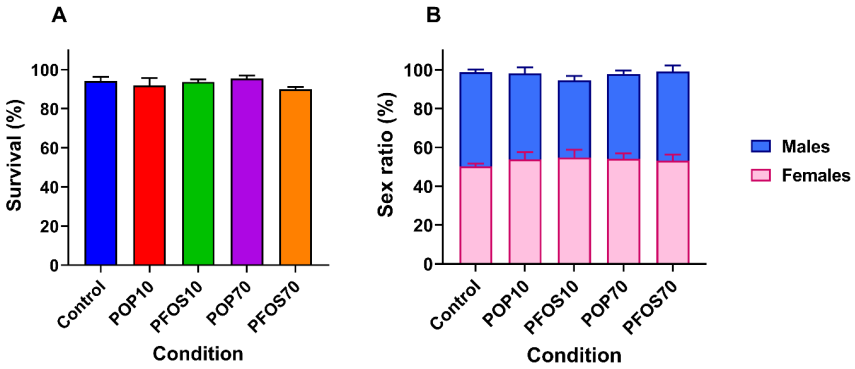


Figure 7. Final survival and sex ratio of all conditions. Values presented are mean ± SE

Model testing revealed no interaction between group and sex for all dependent variables. Sex had a significant effect on weight, length and *K* with females always having greater values than males. We also observed differences between groups. Specifically, the POP10 and PFOS70 population had greater weight compared to control, in both females and males. The PFOS70 population was also larger in terms of length. Condition

factor was determined to be significantly greater in the POP10 population compared to control (Figure 8).

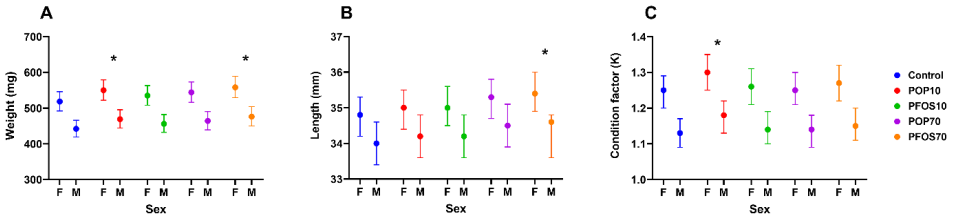


Figure 8. Final weight, length and condition factor (K) of each condition for females (F) and males (M). Statistics are from linear mixed effect models. Data shown are least square means ± 95% CI. Asterisk indicate significant differences compared to control with $p < 0.05$.

4. Discussion

In the present study we addressed a current knowledge gap by investigating the developmental origins of adult health and disease in zebrafish. We observed effects of developmental exposure to POPs or PFOS on adult swimming performance and body size parameters whereas effects on the brain transcriptome were only found following developmental exposure to PFOS. However, developmental exposure had no effect on reproduction or anxiety-like behavior, or larval behavior in the F1 generation. These results suggest developmental exposure can have long-lasting effects on key life-traits, but subtle differences exist between single compound exposures and related mixtures.

We observed latent effects on body size parameters, with developmental exposure to the POP mixture and PFOS increasing body size at 15 months of age. Interestingly, both POP10 and PFOS70 had significantly higher body weights and lengths (exclusively for PFOS70) than controls. Increased body mass was recorded in 5-month-old zebrafish exposed to a mixture of POPs through feeding accompanied with changes in pathways involved in endocrine signaling and weight homeostasis (Nourizadeh-Lillabadi *et al.* 2009, Lyche *et al.* 2011). This increased weight gain may be due to many of the POPs having

been characterized as having obesogenic effects via disruption of the PPAR signaling pathway which is involved in lipid biological processes such as metabolism, transport and homeostasis (White *et al.* 2011, Darbre 2017, Tian *et al.* 2019). Condition factor was only significantly increased in POP10 individuals due to the relationship between weight and length, since only weight was elevated, this led to an increase in condition factor, whereas in PFOS70 adults, weight was affected by exposure only based on its allometric relationship with length.

We found both the POP mixture and PFOS significantly lowered U_{crit} values. This agrees with a study by Xia *et al.* (2014) where exposure of adult topmouth gudgeon (*Pseudorasbora parva*) to PFOS (8 and 32 mg/L) for 96 hours led to a decrease in U_{crit}. Previous work following developmental exposure to crude oil also found a significant reduction in the swimming speed of adult zebrafish. This was associated with subtle changes in heart shape (Hicken et al 2011) that is important for heart function and swimming performance (Farrell 2002). Acute exposure to PFOS (4 and 16 mg/L) additionally was found to affect the development and function of heart in the marine medaka (4 -10 dpf) (Huang *et al.* 2011). We did not investigate whether there were changes in heart shape, however, we previously found developmental exposure to the POP mixture or PFOS resulted in significant changes in gene expression in larvae related to cardiovascular disease such as atherosclerotic lesions, cardiomyopathy, hypertrophy, effect on diastolic function, and cardiac contraction (Christou *et al.* 2020). Therefore, future work should investigate heart function following developmental exposure to POPs.

We found no effect of developmental exposure on anxiety-like behavior in adults. This was unexpected, as we previously found increased levels of thigmotaxis in larvae exposed to POP70 and PFOS70 (Christou *et al.* 2020), which is a measure of anxiety (Schnörr *et al.* 2012). The lack of an effect later in life might suggest that persistent organic pollutants in our study only affect early developmental stages, which have been shown to be more sensitive in chemical exposures (Makri *et al.* 2004, Lau *et al.* 2006), whereas detoxification during the growing phase might account for the lack of effects. One other study using the novel tank test have concluded that chemical exposure leads to higher anxiety levels as shown by less time that zebrafish occupy the top area of the

691 tank. This study however exposed zebrafish chronically to a chemical mixture of
692 polycyclic aromatic hydrocarbons through diet for 6 months (Vignet *et al.* 2014)

693 Pathway analysis of transcriptomic results agrees with the lack of effects on
694 anxiety-like behavior of adult zebrafish since we did not observe enriched pathways that
695 might induce anxiety such as the corticotropin-releasing hormone pathway (Timpl *et al.*
696 1998). Multiple pathways relating to synaptic transmission and signaling were observed
697 however in the brains of PFOS10 adults that might be related to other behavioral
698 endpoints. KEGG analysis of DEGs revealed enrichment of the calcium signaling pathway
699 (Supplementary material Figure S6). All genes in this pathway showed upregulation
700 (Table 4). Alteration of calcium signaling pathway can further affect downstream signaling
701 pathways. One of the pathways directly affected by changes in the calcium signaling
702 pathways was the MAPK signaling pathway (Supplementary material Figure S7) which is
703 involved in many of the cellular processes such as proliferation, differentiation and
704 apoptosis. Changes in both signaling pathways have also been observed in the brains of
705 6 mo zebrafish after developmental exposure (4 – 24 hpf) to non-lethal doses of PCB126
706 (0.3 and 1.2nM) (Aluru *et al.* 2017). MAPK signaling pathway is involved in brain
707 development and has been suggested to play a role in synaptic plasticity, learning and
708 memory, and depression-like behaviors (Thomas and Huganir 2004, Jeanneteau and
709 Deinhardt 2011, Wefers *et al.* 2012).

710

711 **Table 4.** Lists of DEGs involved in each significant pathway in PFOS10 adult brains

Pathway	Fold change	Description
Calcium signaling pathway		
adcy1a	3.2	adenylate cyclase 1a
camk2b	2.3	calcium/calmodulin dependent protein kinase II beta
chrm2a	4.9	cholinergic receptor, muscarinic 2a
erbb4b	2.4	erb-b2 receptor tyrosine kinase 4b
gna11a	2.5	guanine nucleotide binding protein (G protein), alpha 11a (Gq class)
grm5b	3.7	glutamate receptor, metabotropic 5b
ppp3r1a	2.5	protein phosphatase 3, regulatory subunit B, alpha a
prkacaa	2.3	protein kinase, cAMP-dependent, catalytic, alpha, genome duplicate a

Table 4 *(continued)*

si:ch73-374l24.1	2.6	si:ch73-374l24.1
slc8a1b	2.1	solute carrier family 8 (sodium/calcium exchanger), member 1b
slc8a3	2.2	solute carrier family 8 (sodium/calcium exchanger), member 3
slc8a4a	2.8	solute carrier family 8 (sodium/calcium exchanger), member 4a
MAPK signaling pathway		
akt3a	1.7	v-akt murine thymoma viral oncogene homolog 3a
arrb1	2.3	arrestin, beta 1
cacnb3a	2.9	calcium channel, voltage-dependent, beta 3a
cacng4b	3.8	calcium channel, voltage-dependent, gamma subunit 4b
dusp4	1.7	dual specificity phosphatase 4
erbb4b	2.4	erb-b2 receptor tyrosine kinase 4b
igf1ra	2.3	insulin-like growth factor 1a receptor
map2k4a	2.5	mitogen-activated protein kinase kinase 4a
mapk10	2.1	mitogen-activated protein kinase 10
mapk8b	2.7	mitogen-activated protein kinase 8b
mapk8ip2	3	mitogen-activated protein kinase 8 interacting protein 2
nf1b	2.7	neurofibromin 1b
nr4a1	3.5	nuclear receptor subfamily 4, group A, member 1
ntrk2a	2	neurotrophic tyrosine kinase, receptor, type 2a
ppm1aa	2.3	protein phosphatase, Mg2+/Mn2+ dependent, 1Aa
ppp3r1a	2.5	protein phosphatase 3, regulatory subunit B, alpha a
prkacaa	2.3	protein kinase, cAMP-dependent, catalytic, alpha, genome duplicate a
prkcg	2.8	protein kinase C, gamma
rasgrf2b	4	Ras protein-specific guanine nucleotide-releasing factor 2b
si:ch73-374l24.1	2.6	si:ch73-374l24.1
taok2b	2.5	TAO kinase 2b
tgfbr1b	2.6	transforming growth factor, beta receptor 1 b
traf2b	1.6	Tnf receptor-associated factor 2b
synaptic transmission, GABAergic		
gabra1	2.6	gamma-aminobutyric acid (GABA) A receptor, alpha 1
gabra2a	3.7	gamma-aminobutyric acid type A receptor alpha2 subunit a
gabra4	2	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4
npas4a	3.3	neuronal PAS domain protein 4a

712

Another pathway that is suggested to be involved in learning and memory, but also depression-like and anxiolytic effects in pathological conditions is the gamma aminobutyric acid (GABA) signaling pathway. The GABA signaling pathway was enriched in the brains of PFOS10 adults and an upregulation of genes encoding GABA receptors was observed (Table 4) (Collinson *et al.* 2002, Liu *et al.* 2007). A study has suggested an association between GABAA receptor and cognitive and spatial memory of rats exposed to pesticides (Godinho *et al.* 2016). We did not observe any behavioral alterations suggesting elevated anxiety in zebrafish subjected to the novel tank diving test but additional behavioral tests could be more informative, such as the T-maze test, to evaluate the effects of early life exposure on learning and memory of adult zebrafish (Bailey *et al.* 2015).

We found no transgenerational effect in larvae, either on basal behavior or in response to a second developmental exposure. Transgenerational effects have previously been seen in F1 zebrafish larvae in terms of higher swimming speed in a light/dark assay derived from parents that were exposed chronically to PFOS at three time periods 1 – 20, 21 - 120 or 1 – 120 dpf or 5 months continuously. Behavioral changes were highly correlated with residues of PFOS in F1 embryos.(Wang *et al.* 2011, Chen *et al.* 2013). Lack of effect in F1 larvae in our study may be due to shorter exposure periods that might facilitated clearance of chemical burden from the body of adults thus no maternal transfer of chemicals in the developing eggs.

No effects of early life exposure on survival rates were evident at 5 and 15 mo zebrafish in this study. Reduced survival was observed in zebrafish only between 10 and 20 dpf when fed with an environmentally relevant mixture of POPs containing PCBs, PBDEs and organochlorine pesticides (Nourizadeh-Lillabadi *et al.* 2009). In contrast, no effect on acute or late mortality was observed in zebrafish fed with an environmentally relevant mixture containing 22 PCB congeners and 7 PBDE congeners (Horri *et al.* 2018) suggesting that zebrafish may be particularly sensitive to chemical stress during early life stages and that different routes, composition of exposures and duration might affect the outcome.

We found no effect of developmental exposure to the POP mixture or PFOS on the sex ratio or reproduction. Zebrafish exposure to PFOS for 5 months led towards a female dominant sex ratio whereas exposure to a POP mixture led to a male dominance in the exposed groups compared to control (Nourizadeh-Lillabadi *et al.* 2009, Wang *et al.* 2011). Since zebrafish do not have highly differentiated sex chromosomes the mechanisms involved in sex determination and how this is affected by chemical exposures are still unclear. Studies investigating effects of PCBs, PBDEs and organochlorine pesticides on the reproductive output have shown effects on fertilization rate but also number of eggs produced, hatching success, survival and gonadosomatic index of females and males (Johnson *et al.* (2013) and references therein). Results from previous studies were mostly observed when fish were either chronically exposed to chemicals or exposed as adults prior to the reproductive tests (Johnson *et al.* (2013) and references therein).

Concentration differences were observed in most of the variables that were affected by early chemical exposure. Weight was only affected at the highest concentration of PFOS whereas it was affected at the low concentration of the POP mixture. This might suggest a possible synergistic effect of PFOS with other compounds in the mixture. The lack of effect at the higher concentration of the POP mixture might imply a shift to an antagonistic relationship due to possible oversaturation of cellular binding sites (Vandenberg *et al.* 2012). Transcriptomic analysis of adult brains also responded in a non-monotonic manner where the PFOS10 group had the highest number of DEGs with 466 genes. Interestingly the PFOS10 group had the most DEGs following transcriptomic analysis in larvae from our previous study (Christou *et al.* 2020). In contrast to the gene profile of PFOS10 adult brains which was mainly characterized by upregulation of genes (463 genes), there was a downregulation of 96% of the total number of DEGs in PFOS10 larvae. The higher number of DEGs in the lower concentrations than in the high concentrations suggest that the mechanisms of action (MoA) might be different and can be attributed to the non-monotonic effects of toxicants. Acute non-monotonic effects of toxicants have been previously demonstrated (Birnbaum 2012), but this is one of the few studies that underline the non-monotonic effects of toxicants in a DOHaD scenario (Aluru *et al.* 2017). Additionally, these observations point

to the necessity of sampling at different time points and different tissues for a more thorough evaluation of MoA of chemical exposure.

The mixture used in the present study is based on the average concentration of chemicals detected in human blood and results from our study seem to agree with epidemiological studies that associate exposure to POPs and obesity (Guo *et al.* 2019). Additionally, overweight and obesity are significantly associated with an increased risk of metabolic disorders such as diabetes (Mokdad *et al.* 2003).Thus exposure to chemicals during early life might lead to metabolic syndrome both in fish and humans.

Although the POP mixture is designed for humans, the sum of PCBs, PBDEs and OCPs in the low and high exposure used here are comparable to the concentrations found accumulated in fish from Norwegian lakes (Nourizadeh-Lillabadi et al. 2009). Therefore, our results also have relevance for wildlife. Examination of swimming abilities is emerging as an effective method to evaluate the effects of chemical exposure in fish. During this study we observed that the critical swimming speed of adult fish was significantly reduced. A decrease in swimming performance might hinder the survivability of individuals making them prone to predation or unable to acquire food which in consequence might affect the population size (Hammer 1995).Furthermore it is not clear, whether an increased condition factor or weight can be considered an unfavorable outcome for a wildlife population. However, potential changes of behavior for larger fish may include unsuitable timing for migration, season inappropriate behavior, as an increased appetite during winter, and higher activity leading to higher metabolic demands (Meador 2011).

In conclusion early developmental exposure to an environmentally relevant POP mixture or single PFOS led to some effects on adult zebrafish physiology but absence of effects in their offspring. This might mean that adults have the ability to detoxify once they are removed from the chemical exposure and that the effects are reversible. Effects on weight of adult fish exposed as larvae to POPs and PFOS might indicate an obesogenic effect of persistent organic pollutants as these have been reported before (Yang *et al.* 2017). Differences in DEGs and affected pathways between the larval and adult stage pinpoint the need of multiple sampling across time points and tissues for a more precise evaluation of the chemical's effects during each life stage of an organism.

Conflict of interest

The authors declare that there are no conflicts of interest

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Supplementary material

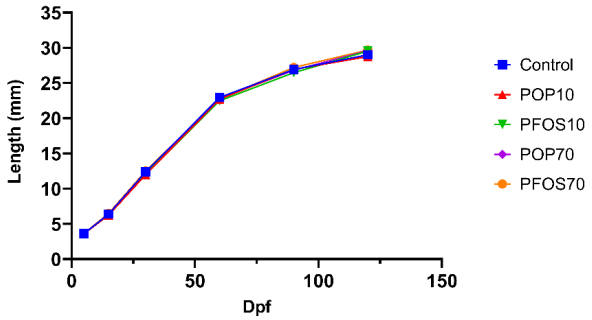

Figure S1. Growth curves of all conditions from 5 until 120 days post fertilization (dpf)

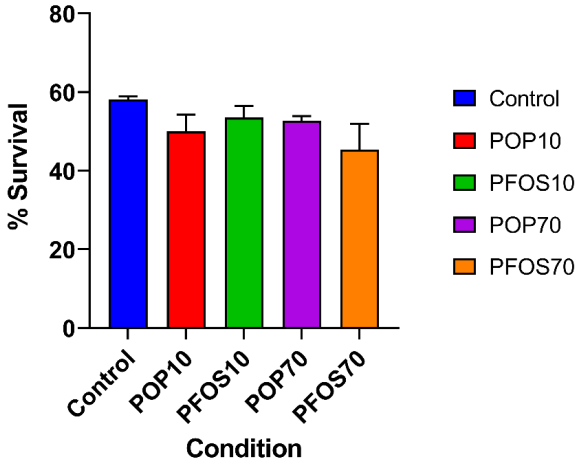

Figure S2. Percent survival of experimental populations at 150 dpf

Table S1. Statistical results for adult behavioral tests, swimming test, final sampling parameters and F1 behavioral test. For model comparison the AICc score is reported. The model with the lowest AICc is underlined, and the statistics are from lme models, and include the marginal (m) and conditional (c) R2 of the chosen model.

Parameter	Model comparison (AICc score)	R2(m, c)	Model results (χ2, df, p)		
	Group×Sex, Group+Sex, Group, Null		Group		
Behavior test adults					
Time in bottom zone (s)	936, 929, 926, 923	0.07, 0.11			
Velocity (cm/s)	342, 333, 331, 328	0.08, 0.08			
Distance moved (cm)	1243, 1236, 1234, 1235	0.12, 0.14	10.96, 4, 0.027		
Number of crossings	708, 703, 701, 698	0.07, 0.07			
	Group, Group+Length, Group+Length*Mass, null		Group		
Swimming test adults					
Ucrit (BL/s)	241, 249, 254, 250	2,36e-08, 3.05e-08	25.87, 4, <0.001		
	Group×Sex, Group+Sex, null		Group	Sex	
Final sampling					
Weight	-39.13, -39.18, 90.69	0.11, 0.11	14.3, 4, 0.006	132.2, 1, <0.001	
Length	5676, 5672, 5700	0.03, 0.03	9.9, 4, 0.41	28.1, 1, <0.001	
Condition factor	-1913, -1914, -1687	0.18, 0.20	22.9, 4, <0.001	238.6, 1, <0.001	
Behavior of F1 larvae					
	Group, null		Group		
No re-exposures					
Distance moved (mm)	2894, 2892	0.03, 0.05			
Time active (s)	1966, 1964	0.03, 0.07			
Swimming speed (mm/s)	622, 620	0.03, 0.09			
Thigmotaxis	1156, 1150	0.02, 0.02			
	Exposure×History, Exposure+History, null		Exposure×History	Exposure	History
Re-exposures					
PFOS - Distance moved (mm)	4185, 4184, 4196	0.05, 0.15		16, 1, <0.001	0.85, 1, 0.36
PFOS - Time active (s)	2827, 2824.6, 2825.3	0.15, 0.15		4, 1, 0.04	0.77, 1, 0.38
PFOS - Swimming speed (mm/s)	-2449, -2451, -2405	0.16, 0.18		53.7, 1, <0.001	1.11, 1, 0.29
POP - Distance moved (mm)	4077, 4079, 4092	0.06, 0.21	3.4, 1, 0.06	19, 1, <0.001	1.2, 1, 0.27
POP - Time active (s)	2747, 2750, 2750	0.03, 0.20	5.1, 1, 0.024	8.7, 1, 0.003	3.4, 1, 0.065
POP - Swimming speed (mm/s)	994.7, 994.5, 1027.8	0.13, 0.13		36.9, 1, <0.001	2.9, 1, 0.083

Table S2. Mapping results of STAR alignment. Overview of total reads and percent of unique reads per sample

Sample	Total reads (M)	% unique reads
CF3_1	22.1	89.5
CF3_2	20.9	89.5
CF4_1	22.2	90.7
CF4_2	25.8	90.0
CF5_1	25.0	89.6
CF5_2	26.3	90.0
CF6_1	22.9	87.9
CF6_2	21.1	88.2
CM3_1	20.0	89.9
CM3_2	27.2	87.3
CM4_1	21.8	90.5
CM4_2	28.8	90.0
CM5_1	26.1	90.1
CM5_2	31.2	90.9
CM6_1	24.4	89.8
CM6_2	21.6	89.3
PF10F3_1	20.6	88.0
PF10F3_2	21.5	89.5
PF10F4_1	21.4	90.9
PF10F4_2	29.7	90.2
PF10F5_1	24.7	90.6
PF10F5_2	28.7	90.2
PF10F6_1	22.9	88.3
PF10F6_2	22.1	88.8
PF10M3_1	24.4	89.3
PF10M3_2	23.3	90.0
PF10M4_1	26.8	90.9
PF10M4_2	21.6	89.9
PF10M5_1	25.4	90.7
PF10M5_2	21.3	87.4
PF10M6_1	23.4	90.8
PF10M6_2	23.7	88.8
PF70F3_1	28.7	89.6
PF70F3_2	21.3	88.5
PF70F4_1	29.9	88.7
PF70F4_2	28.0	90.4
PF70F5_1	31.5	87.9
PF70F5_2	24.4	88.8
PF70F6_1	31.6	89.2
PF70F6_2	21.9	89.9
PF70M3_1	26.8	90.4

Table S2 *(continued)*

PF70M3_2	33.3	90.1
PF70M4_1	22.5	88.9
PF70M4_2	33.0	90.2
PF70M5_1	20.4	90.7
PF70M5_2	30.5	90.6
PF70M6_1	20.0	90.4
PF70M6_2	25.8	88.6
PO10F3_1	20.6	89.9
PO10F3_2	20.4	90.3
PO10F4_1	31.4	90.4
PO10F4_2	30.6	88.0
PO10F5_1	24.0	90.2
PO10F5_2	21.6	90.0
PO10F6_1	28.1	88.5
PO10F6_2	21.5	90.5
PO10M3_1	20.4	85.5
PO10M3_2	26.5	89.6
PO10M4_1	28.9	90.3
PO10M4_2	21.2	90.5
PO10M5_1	27.5	89.9
PO10M5_2	22.1	90.5
PO10M6_1	27.9	90.2
PO10M6_2	22.9	90.1
PO70F3_1	22.6	89.5
PO70F3_2	20.8	87.6
PO70F4_1	27.4	91.0
PO70F4_2	22.5	90.6
PO70F5_1	27.6	90.5
PO70F5_2	21.1	87.9
PO70F6_1	21.0	89.7
PO70F6_2	23.4	90.4
PO70M3_1	38.5	88.9
PO70M3_2	25.8	90.4
PO70M4_1	22.6	89.2
PO70M4_2	25.2	90.7
PO70M5_1	26.8	90.7
PO70M5_2	22.5	87.4
PO70M6_1	22.3	90.6
PO70M6_2	21.4	87.8

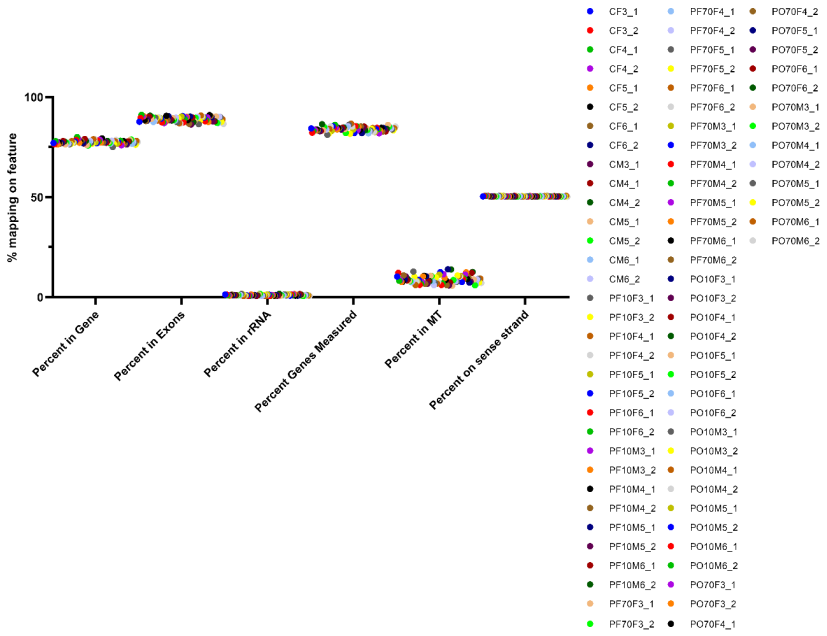

Figure S3. RNA quality control plot. Mapping of reads to different features in all individual samples

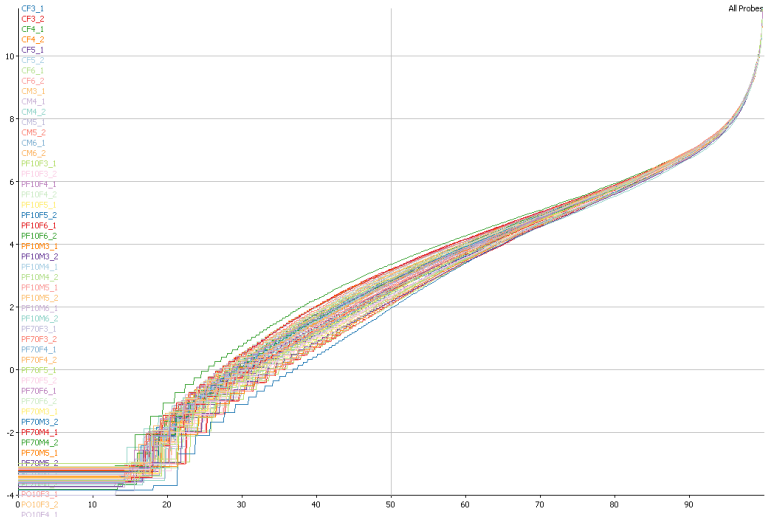

Figure S4. Cumulative distribution plot showing the distribution of log2 RPM values from low to highly expressed genes in all samples

Table S3. List of differentially expressed genes (DEG) in all conditions. FDR, false discovery rate, absFC, absolute fold change

Probe	Chromosome	ID	Description	FDR	absFC
			PFOS10		
march2	22	ENSDARG00000061738	membrane-associated ring finger (C3HC4) 2 [Source:ZFIN;Acc:ZDB-GENE-050208-777]	0.048468	2.390073
march5	13	ENSDARG00000032552	membrane-associated ring finger (C3HC4) 5 [Source:ZFIN;Acc:ZDB-GENE-070424-47]	0.035384	2.118165
aacs	5	ENSDARG00000012468	acetoacetyl-CoA synthetase [Source:ZFIN;Acc:ZDB-GENE-040426-903]	0.037536	2.468313
aak1a	8	ENSDARG00000011855	AP2 associated kinase 1a [Source:ZFIN;Acc:ZDB-GENE-081105-101]	0.047559	2.636593
aak1b	10	ENSDARG00000077686	AP2 associated kinase 1b [Source:ZFIN;Acc:ZDB-GENE-091118-25]	0.021669	2.389097
aatka	3	ENSDARG00000078222	apoptosis-associated tyrosine kinase a [Source:ZFIN;Acc:ZDB-GENE-100812-4]	0.033306	1.847537
abhd8a	2	ENSDARG00000103518	abhydrolase domain containing 8a [Source:ZFIN;Acc:ZDB-GENE-080204-70]	0.021669	3.488552
abi1b	2	ENSDARG00000062991	abl-interactor 1b [Source:ZFIN;Acc:ZDB-GENE- 060929-1182]	0.048918	2.325716
adam11	12	ENSDARG00000079204	ADAM metallopeptidase domain 11 [Source:ZFIN;Acc:ZDB-GENE-070808-3]	0.042374	2.279676
adam17b	20	ENSDARG00000093093	ADAM metallopeptidase domain 17b [Source:ZFIN;Acc:ZDB-GENE-041001-199]	0.016296	4.205786
adarb1b	9	ENSDARG00000003544	adenosine deaminase, RNA-specific, B1b [Source:ZFIN;Acc:ZDB-GENE-030219-15]	0.049903	2.230782
adcy1a	20	ENSDARG00000069026	adenylate cyclase 1a [Source:ZFIN;Acc:ZDB- GENE-070705-302]	0.042916	3.179953
add3b	17	ENSDARG00000056250	adducin 3 (gamma) b [Source:ZFIN;Acc:ZDB- GENE-080718-6]	0.043006	2.262113
aes	22	ENSDARG00000021805	amino-terminal enhancer of split [Source:ZFIN;Acc:ZDB-GENE-040426-1409]	0.041383	2.648737
aftphb	17	ENSDARG00000071347	aftiphilin b [Source:ZFIN;Acc:ZDB-GENE- 060503-388]	0.043006	2.102693
agap3	24	ENSDARG00000087822	ArfGAP with GTPase domain, ankyrin repeat and PH domain 3 [Source:ZFIN;Acc:ZDB-GENE-110927-1]	0.030054	2.300765
ago1	16	ENSDARG00000092644	argonaute RISC catalytic component 1 [Source:ZFIN;Acc:ZDB-GENE-110606-3]	0.040298	2.333229
ajap1	8	ENSDARG00000038655	adherens junctions associated protein 1 [Source:ZFIN;Acc:ZDB-GENE-041210-353]	0.039864	2.112101
akt3a	13	ENSDARG00000104810	v-akt murine thymoma viral oncogene homolog 3a [Source:ZFIN;Acc:ZDB-GENE-050419-180]	0.043006	1.721937
aldh3a2b	21	ENSDARG00000029381	aldehyde dehydrogenase 3 family, member A2b [Source:ZFIN;Acc:ZDB-GENE-040912-103]	0.025806	2.063036
ANKFN1	12	ENSDARG00000060941	si:ch211-277e21.2 [Source:ZFIN;Acc:ZDB-GENE- 130603-21]	0.037216	3.190741
ankib1a	19	ENSDARG00000060768	ankyrin repeat and IBR domain containing 1a [Source:ZFIN;Acc:ZDB-GENE-060503-156]	0.035032	1.566914
ankrd34ba	10	ENSDARG00000077086	ankyrin repeat domain 34Ba [Source:ZFIN;Acc:ZDB-GENE-091118-17]	0.039987	2.147942
ankrd52a	23	ENSDARG00000036826	ankyrin repeat domain 52a [Source:ZFIN;Acc:ZDB-GENE-050522-247]	0.016857	3.474452
ano3	25	ENSDARG00000058015	anoctamin 3 [Source:ZFIN;Acc:ZDB-GENE-111031- 3]	0.029884	2.610069
ano8b	11	ENSDARG00000077229	anoctamin 8b [Source:ZFIN;Acc:ZDB-GENE- 121024-1]	0.02293	2.269922

apbb2b	1	ENSDARG00000099995	amyloid beta (A4) precursor protein-binding, family B, member 2b [Source:ZFIN;Acc:ZDB-GENE-090313-73]	0.023224	2.78208
ar	5	ENSDARG00000067976	androgen receptor [Source:ZFIN;Acc:ZDB-GENE- 060131-1]	0.023224	3.055265
arf3b	6	ENSDARG00000036998	ADP-ribosylation factor 3b [Source:ZFIN;Acc:ZDB-GENE-030616-356]	0.023695	3.519107
arhgef11	7	ENSDARG00000052482	Rho guanine nucleotide exchange factor (GEF) 11 [Source:ZFIN;Acc:ZDB-GENE-070604-1]	0.038307	2.226122
ARMC6	2	ENSDARG00000103770	armadillo repeat containing 6 [Source:HGNC Symbol;Acc:HGNC:25049]	0.046367	2.526899
arrb1	15	ENSDARG00000043241	arrestin, beta 1 [Source:ZFIN;Acc:ZDB-GENE- 060824-1]	0.033899	2.352253
atf3	20	ENSDARG00000007823	activating transcription factor 3 [Source:ZFIN;Acc:ZDB-GENE-040426-728]	0.02563	1.691139
atg2b	17	ENSDARG00000097650	autophagy related 2B [Source:ZFIN;Acc:ZDB- GENE-131121-626]	0.046957	2.433373
atp6ap1la	11	ENSDARG00000091509	ATPase H+ transporting accessory protein 1 like a [Source:ZFIN;Acc:ZDB-GENE-110318-1]	0.038307	2.768707
atxn1a	19	ENSDARG00000061687	ataxin 1a [Source:ZFIN;Acc:ZDB-GENE-060503- 759]	0.045206	2.231781
atxn7l1	4	ENSDARG00000089860	ataxin 7-like 1 [Source:ZFIN;Acc:ZDB-GENE- 131127-383]	0.023364	1.956363
bahcc1a	3	ENSDARG00000103739	BAH domain and coiled-coil containing 1a [Source:ZFIN;Acc:ZDB-GENE-170301-1]	0.025927	2.363967
birc7	23	ENSDARG00000058082	baculoviral IAP repeat containing 7 [Source:ZFIN;Acc:ZDB-GENE-070615-35]	0.035384	2.429828
bnip3la	8	ENSDARG00000025468	BCL2 interacting protein 3 like a [Source:ZFIN;Acc:ZDB-GENE-030131-2283]	0.039864	2.444801
ca4a	5	ENSDARG00000043589	carbonic anhydrase IV a [Source:ZFIN;Acc:ZDB- GENE-080204-85]	0.021669	3.150616
cabin1	8	ENSDARG00000079326	calcineurin binding protein 1 [Source:ZFIN;Acc:ZDB-GENE-030131-1735]	0.033899	1.894907
cabp1b	10	ENSDARG00000033411	calcium binding protein 1b [Source:ZFIN;Acc:ZDB-GENE-040718-111]	0.024839	2.797894
CABZ01071171.1	7	ENSDARG00000116353	Danio rerio low density lipoprotein receptor- related protein 4 (lrp4), mRNA. [Source:RefSeq mRNA;Acc:NM_001351714]	0.038307	2.829086
cacna1eb	2	ENSDARG00000095614	calcium channel, voltage-dependent, R type, alpha 1E subunit b [Source:ZFIN;Acc:ZDB-GENE-070705-133]	0.029884	2.454817
cacna2d2a	6	ENSDARG00000103390	calcium channel, voltage-dependent, alpha 2/delta subunit 2a [Source:ZFIN;Acc:ZDB-GENE-050320-71]	0.029075	1.981818
cacnb3a	23	ENSDARG00000001881	calcium channel, voltage-dependent, beta 3a [Source:ZFIN;Acc:ZDB-GENE-060221-2]	0.032687	2.864907
cacnb3b	23	ENSDARG00000076030	calcium channel, voltage-dependent, beta 3b [Source:ZFIN;Acc:ZDB-GENE-090127-1]	0.032078	3.407017
cacng4b	3	ENSDARG00000074669	calcium channel, voltage-dependent, gamma subunit 4b [Source:ZFIN;Acc:ZDB-GENE-120104-6]	0.039864	3.821827
cadm2a	10	ENSDARG00000009930	cell adhesion molecule 2a [Source:ZFIN;Acc:ZDB-GENE-040426-1614]	0.038307	2.733213
cadm3	2	ENSDARG00000057013	cell adhesion molecule 3 [Source:ZFIN;Acc:ZDB-GENE-060512-196]	0.042916	2.225604
cadpsa	11	ENSDARG00000043661	Ca2+-dependent activator protein for secretion a [Source:ZFIN;Acc:ZDB-GENE-030616-525]	0.033899	1.836895
caln1	15	ENSDARG00000088898	calneuron 1 [Source:ZFIN;Acc:ZDB-GENE-130313- 1]	0.032687	2.554928
camk2b2	10	ENSDARG00000100089	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta 2 [Source:ZFIN;Acc:ZDB-GENE-090312- 34]	0.039987	2.263916

camsap2b	2	ENSDARG00000059965	calmodulin regulated spectrin-associated protein family, member 2b [Source:ZFIN;Acc:ZDB-GENE-070912- 80]	0.045766	2.044298
cax2	25	ENSDARG00000015732	cation/H+ exchanger protein 2 [Source:ZFIN;Acc:ZDB-GENE-100825-2]	0.039987	1.593262
ccdc85a	13	ENSDARG00000039497	coiled-coil domain containing 85A [Source:ZFIN;Acc:ZDB-GENE-080220-55]	0.046367	2.215634
ccser1	8	ENSDARG00000075919	coiled-coil serine-rich protein 1 [Source:ZFIN;Acc:ZDB-GENE-081104-422]	0.036572	2.614894
cd302	9	ENSDARG00000086100	CD302 molecule [Source:ZFIN;Acc:ZDB-GENE- 111118-1]	0.048918	2.322896
cdc42bpaa	17	ENSDARG00000104283	CDC42 binding protein kinase alpha (DMPK- like) a [Source:ZFIN;Acc:ZDB-GENE-120727-12]	0.043006	2.315322
cdh12a	2	ENSDARG00000078226	cadherin 12, type 2a (N-cadherin 2) [Source:ZFIN;Acc:ZDB-GENE-070912-673]	0.033669	3.316393
CDH22	6	ENSDARG00000098824	cadherin 22 [Source:HGNC Symbol;Acc:HGNC:13251]	0.021669	2.972451
cdh23	13	ENSDARG00000094454	cadherin-related 23 [Source:ZFIN;Acc:ZDB- GENE-040513-7]	0.025806	3.063285
cdk14	16	ENSDARG00000074665	cyclin-dependent kinase 14 [Source:ZFIN;Acc:ZDB-GENE-080220-44]	0.033669	2.737163
cdk16	8	ENSDARG00000032072	cyclin-dependent kinase 16 [Source:ZFIN;Acc:ZDB-GENE-030131-2939]	0.035384	2.284181
CDK18	11	ENSDARG00000012204	si:dkey-166c18.1 [Source:ZFIN;Acc:ZDB-GENE- 141215-51]	0.040771	2.364659
cdk19	20	ENSDARG00000043858	cyclin-dependent kinase 19 [Source:ZFIN;Acc:ZDB-GENE-040724-19]	0.044981	2.412832
celf2	4	ENSDARG00000002131	cugbp, Elav-like family member 2 [Source:ZFIN;Acc:ZDB-GENE-030826-35]	0.021669	2.70098
celf5b	2	ENSDARG00000090727	cugbp, Elav-like family member 5b [Source:ZFIN;Acc:ZDB-GENE-070912-150]	0.021669	3.113326
cep104	8	ENSDARG00000060361	centrosomal protein 104 [Source:ZFIN;Acc:ZDB- GENE-060503-195]	0.028641	2.491811
CEP170B (1 of many)	20	ENSDARG00000078327	si:ch73-212j7.1 [Source:ZFIN;Acc:ZDB-GENE- 081104-258]	0.02293	2.521138
chico	2	ENSDARG00000019588	chico [Source:ZFIN;Acc:ZDB-GENE-000210-28]	0.022485	3.28957
chrm2a	4	ENSDARG00000098612	cholinergic receptor, muscarinic 2a [Source:ZFIN;Acc:ZDB-GENE-030314-1]	0.021669	4.883126
clocka	20	ENSDARG00000011703	clock circadian regulator a [Source:ZFIN;Acc:ZDB-GENE-990630-14]	0.044042	2.147779
clvs2	20	ENSDARG00000053122	clavesin 2 [Source:ZFIN;Acc:ZDB-GENE-041014- 313]	0.02518	2.703785
cnksr2a	24	ENSDARG00000074480	connector enhancer of kinase suppressor of Ras 2a [Source:ZFIN;Acc:ZDB-GENE-060228-5]	0.021669	3.056543
cnnm4b	5	ENSDARG00000074309	cyclin and CBS domain divalent metal cation transport mediator 4b [Source:ZFIN;Acc:ZDB-GENE-070705- 181]	0.033899	2.282414
cnr1	20	ENSDARG00000009020	cannabinoid receptor 1 [Source:ZFIN;Acc:ZDB- GENE-040312-3]	0.04738	3.171806
cntn2	11	ENSDARG00000000472	contactin 2 [Source:ZFIN;Acc:ZDB-GENE-990630- 12]	0.047701	2.121425
cntn3b	6	ENSDARG00000053454	contactin 3b [Source:ZFIN;Acc:ZDB-GENE- 130530-645]	0.043006	2.483708
col4a1	9	ENSDARG00000055009	collagen, type IV, alpha 1 [Source:ZFIN;Acc:ZDB-GENE-081105-114]	0.043006	2.524556
cpeb2	23	ENSDARG00000052604	cytoplasmic polyadenylation element binding protein 2 [Source:ZFIN;Acc:ZDB-GENE-130530-639]	0.031289	2.739684
crebrf	21	ENSDARG00000105114	creb3 regulatory factor [Source:ZFIN;Acc:ZDB- GENE-120203-3]	0.016296	3.045096
crim1	17	ENSDARG00000029668	cysteine rich transmembrane BMP regulator 1 (chordin-like) [Source:ZFIN;Acc:ZDB-GENE-040312-2]	0.033899	2.376732

crtc1a	2	ENSDARG00000076068	CREB regulated transcription coactivator 1a [Source:ZFIN;Acc:ZDB-GENE-061027-225]	0.021669	3.78262
csmd2	19	ENSDARG00000001559	CUB and Sushi multiple domains 2 [Source:ZFIN;Acc:ZDB-GENE-030616-39]	0.024013	2.342655
CSMD3 (1 of many)	19	ENSDARG00000077920	si:ch211-233f16.1 [Source:ZFIN;Acc:ZDB-GENE- 120214-1]	0.025583	2.146847
CSMD3 (1 of many)	16	ENSDARG00000077905	si:ch211-236p22.1 [Source:ZFIN;Acc:ZDB-GENE- 050419-118]	0.023224	2.626227
csrnp1b	24	ENSDARG00000038429	cysteine-serine-rich nuclear protein 1b [Source:ZFIN;Acc:ZDB-GENE-030131-1515]	0.021669	2.879656
csrnp2	23	ENSDARG00000075972	cysteine-serine-rich nuclear protein 2 [Source:ZFIN;Acc:ZDB-GENE-090312-137]	0.02814	2.905714
CSRNP3	9	ENSDARG00000079741	cysteine and serine rich nuclear protein 3 [Source:HGNC Symbol;Acc:HGNC:30729]	0.03349	3.345865
CYTH2	16	ENSDARG00000028800	si:dkey-283b15.4 [Source:ZFIN;Acc:ZDB-GENE- 100921-55]	0.023364	3.054907
dab1a	20	ENSDARG00000059939	Dab, reelin signal transducer, homolog 1a (Drosophila) [Source:ZFIN;Acc:ZDB-GENE-060528-1]	0.033899	2.498208
dab2ipa	8	ENSDARG00000069484	DAB2 interacting protein a [Source:ZFIN;Acc:ZDB-GENE-081104-516]	0.021669	3.554203
dcbld2	9	ENSDARG00000062177	discoidin, CUB and LCCL domain containing 2 [Source:ZFIN;Acc:ZDB-GENE-070112-1822]	0.040888	1.943251
dgkg	9	ENSDARG00000062696	diacylglycerol kinase, gamma [Source:ZFIN;Acc:ZDB-GENE-081105-39]	0.031109	2.576108
dlg2	10	ENSDARG00000099323	discs, large homolog 2 (Drosophila) [Source:ZFIN;Acc:ZDB-GENE-050221-3]	0.034007	2.192902
dlg4a	5	ENSDARG00000041926	discs, large homolog 4a (Drosophila) [Source:ZFIN;Acc:ZDB-GENE-130530-613]	0.042916	2.138601
dlgap1a	2	ENSDARG00000014280	discs, large (Drosophila) homolog-associated protein 1a [Source:ZFIN;Acc:ZDB-GENE-030616-580]	0.037536	2.739567
dlgap1b	24	ENSDARG00000037415	discs, large (Drosophila) homolog-associated protein 1b [Source:ZFIN;Acc:ZDB-GENE-091116-98]	0.043006	2.22555
dnajb2	9	ENSDARG00000058644	DnaJ (Hsp40) homolog, subfamily B, member 2 [Source:ZFIN;Acc:ZDB-GENE-061013-537]	0.039987	1.826775
dnajb5	5	ENSDARG00000052881	DnaJ (Hsp40) homolog, subfamily B, member 5 [Source:ZFIN;Acc:ZDB-GENE-070705-308]	0.042912	2.697911
dnajc5aa	8	ENSDARG00000042948	DnaJ (Hsp40) homolog, subfamily C, member 5aa [Source:ZFIN;Acc:ZDB-GENE-031113-20]	0.033864	2.281757
dnal4a	3	ENSDARG00000031116	dynein, axonemal, light chain 4a [Source:ZFIN;Acc:ZDB-GENE-040801-122]	0.024013	2.21364
dner	18	ENSDARG00000061031	delta/notch-like EGF repeat containing [Source:ZFIN;Acc:ZDB-GENE-130502-1]	0.019757	3.542843
dnm1b	5	ENSDARG00000009281	dynamin 1b [Source:ZFIN;Acc:ZDB-GENE-100920- 3]	0.043006	2.363595
dnmt3aa	20	ENSDARG00000005394	DNA (cytosine-5-)-methyltransferase 3 alpha a [Source:ZFIN;Acc:ZDB-GENE-050314-5]	0.023237	2.376302
dock3	22	ENSDARG00000063180	dedicator of cytokinesis 3 [Source:ZFIN;Acc:ZDB-GENE-100624-1]	0.025297	2.644145
DOCK4 (1 of many)	25	ENSDARG00000076213	si:dkey-81e3.1 [Source:ZFIN;Acc:ZDB-GENE- 120703-34]	0.023224	2.437226
dot1l	22	ENSDARG00000061992	DOT1-like histone H3K79 methyltransferase [Source:ZFIN;Acc:ZDB-GENE-060503-341]	0.02293	2.644214
dscaml1	15	ENSDARG00000098057	Down syndrome cell adhesion molecule like 1 [Source:ZFIN;Acc:ZDB-GENE-110601-1]	0.025349	2.444644
dtna	24	ENSDARG00000031015	dystrobrevin, alpha [Source:ZFIN;Acc:ZDB- GENE-070117-2]	0.036465	2.136343

dtnbb	17	ENSDARG00000013020	dystrobrevin, beta b [Source:ZFIN;Acc:ZDB- GENE-070117-3]	0.031766	2.464709
dtx4	1	ENSDARG00000078016	deltex 4, E3 ubiquitin ligase [Source:ZFIN;Acc:ZDB-GENE-070410-129]	0.046367	2.358685
dusp4	21	ENSDARG00000044688	dual specificity phosphatase 4 [Source:ZFIN;Acc:ZDB-GENE-040426-709]	0.041383	1.758543
ECE2	15	ENSDARG00000102142	si:ch211-117a13.2 [Source:ZFIN;Acc:ZDB-GENE- 160113-64]	0.016296	3.878509
eea1	18	ENSDARG00000062868	early endosome antigen 1 [Source:ZFIN;Acc:ZDB-GENE-041111-270]	0.033899	2.125323
efnb3b	7	ENSDARG00000042277	ephrin-B3b [Source:ZFIN;Acc:ZDB-GENE-010618- 3]	0.043006	2.6168
egr2a	17	ENSDARG00000044098	early growth response 2a [Source:ZFIN;Acc:ZDB-GENE-030723-6]	0.033899	1.881464
egr2b	12	ENSDARG00000042826	early growth response 2b [Source:ZFIN;Acc:ZDB-GENE-980526-283]	0.023224	1.934403
egr3	8	ENSDARG00000089156	early growth response 3 [Source:ZFIN;Acc:ZDB- GENE-040718-394]	0.025806	1.609599
eif2ak2	13	ENSDARG00000068729	eukaryotic translation initiation factor 2- alpha kinase 2 [Source:ZFIN;Acc:ZDB-GENE-080422-1]	0.042916	2.684515
elac2	3	ENSDARG00000034060	elaC ribonuclease Z 2 [Source:ZFIN;Acc:ZDB- GENE-041111-227]	0.039738	-1.73336
epha4b	2	ENSDARG00000011600	eph receptor A4b [Source:ZFIN;Acc:ZDB-GENE- 030826-6]	0.029884	3.222029
erbb4b	9	ENSDARG00000089536	erb-b2 receptor tyrosine kinase 4b [Source:ZFIN;Acc:ZDB-GENE-030918-5]	0.038104	2.403314
erc2	11	ENSDARG00000105178	ELKS/RAB6-interacting/CAST family member 2 [Source:ZFIN;Acc:ZDB-GENE-130530-700]	0.041383	2.338745
faim2a	2	ENSDARG00000038784	Fas apoptotic inhibitory molecule 2a [Source:ZFIN;Acc:ZDB-GENE-050320-88]	0.038522	2.67265
fam102ab	5	ENSDARG00000016866	family with sequence similarity 102, member Ab [Source:ZFIN;Acc:ZDB-GENE-060929-412]	0.046367	2.220753
fam118b	18	ENSDARG00000078983	family with sequence similarity 118, member B [Source:ZFIN;Acc:ZDB-GENE-060810-6]	0.033899	3.07331
fam131a	2	ENSDARG00000077022	family with sequence similarity 131, member A [Source:ZFIN;Acc:ZDB-GENE-080204-112]	0.033899	1.925981
fam131bb	16	ENSDARG00000070575	family with sequence similarity 131, member Bb [Source:ZFIN;Acc:ZDB-GENE-100922-12]	0.049587	2.031972
fam155a	1	ENSDARG00000075858	family with sequence similarity 155, member A [Source:ZFIN;Acc:ZDB-GENE-090313-34]	0.033899	2.265623
fam163b	10	ENSDARG00000073914	family with sequence similarity 163, member B [Source:ZFIN;Acc:ZDB-GENE-091204-96]	0.026049	3.425725
fam168a	18	ENSDARG00000098832	family with sequence similarity 168, member A [Source:ZFIN;Acc:ZDB-GENE-060825-128]	0.029884	2.732562
fam171a2b	24	ENSDARG00000079656	family with sequence similarity 171, member A2b [Source:ZFIN;Acc:ZDB-GENE-161227-1]	0.045766	2.24892
fam219aa	21	ENSDARG00000016396	family with sequence similarity 219, member Aa [Source:ZFIN;Acc:ZDB-GENE-040801-110]	0.042916	2.029128
fam46d	14	ENSDARG00000062830	family with sequence similarity 46, member D [Source:ZFIN;Acc:ZDB-GENE-091118-44]	0.024013	3.750024
fam57bb	12	ENSDARG00000074564	family with sequence similarity 57, member Bb [Source:ZFIN;Acc:ZDB-GENE-110603-2]	0.038307	2.369031
fat3a	15	ENSDARG00000033840	FAT atypical cadherin 3a [Source:ZFIN;Acc:ZDB-GENE-060929-1254]	0.025806	2.310358
fbxo41	1	ENSDARG00000078908	F-box protein 41 [Source:ZFIN;Acc:ZDB-GENE- 090313-350]	0.029075	2.759635

fgd1	8	ENSDARG00000100402	FYVE, RhoGEF and PH domain containing 1 [Source:ZFIN;Acc:ZDB-GENE-081104-33]	0.025665	2.896535
fibcd1	5	ENSDARG00000060393	fibrinogen C domain containing 1 [Source:ZFIN;Acc:ZDB-GENE-070424-245]	0.033899	4.103113
fibpb	21	ENSDARG00000087666	fibroblast growth factor (acidic) intracellular binding protein b [Source:ZFIN;Acc:ZDB-GENE- 040630-2]	0.033899	2.012586
fkbp1ab	23	ENSDARG00000033567	FK506 binding protein 1Ab [Source:ZFIN;Acc:ZDB-GENE-040927-9]	0.038957	2.424225
fnbp1a	8	ENSDARG00000036156	formin binding protein 1a [Source:ZFIN;Acc:ZDB-GENE-081105-105]	0.022485	3.050935
fosb	18	ENSDARG00000055751	FBJ murine osteosarcoma viral oncogene homolog B [Source:ZFIN;Acc:ZDB-GENE-041114-181]	0.00943	12.35342
fosl1a	14	ENSDARG00000015355	FOS-like antigen 1a [Source:ZFIN;Acc:ZDB- GENE-061207-7]	0.038743	1.804413
FRMD4A	18	ENSDARG00000042249	si:ch211-220f12.1 [Source:ZFIN;Acc:ZDB-GENE- 050419-154]	0.025806	2.484215
fut8b	20	ENSDARG00000088117	fucosyltransferase 8b (alpha (1,6) fucosyltransferase) [Source:ZFIN;Acc:ZDB-GENE-130530-923]	0.046367	2.44043
fzd5	9	ENSDARG00000025420	frizzled class receptor 5 [Source:ZFIN;Acc:ZDB-GENE-990415-218]	0.043294	2.456655
gabbr1b	19	ENSDARG00000016667	gamma-aminobutyric acid (GABA) B receptor, 1b [Source:ZFIN;Acc:ZDB-GENE-060503-5]	0.033355	2.444207
gabra1	21	ENSDARG00000068989	gamma-aminobutyric acid (GABA) A receptor, alpha 1 [Source:ZFIN;Acc:ZDB-GENE-061013-194]	0.025927	2.585457
gabra2a	13	ENSDARG00000091459	gamma-aminobutyric acid type A receptor alpha2 subunit a [Source:ZFIN;Acc:ZDB-GENE-141216-16]	0.024013	3.716792
gabra4	14	ENSDARG00000013389	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4 [Source:ZFIN;Acc:ZDB-GENE-050417-360]	0.043006	1.99212
GABRB2 (1 of many)	21	ENSDARG00000100032	gamma-aminobutyric acid type A receptor beta2 subunit [Source:HGNC Symbol;Acc:HGNC:4082]	0.043006	2.762437
gabrg1	13	ENSDARG00000101995	gamma-aminobutyric acid type A receptor gamma1 subunit [Source:ZFIN;Acc:ZDB-GENE-141215-66]	0.021669	2.678598
gabrg2	21	ENSDARG00000053665	gamma-aminobutyric acid (GABA) A receptor, gamma 2 [Source:ZFIN;Acc:ZDB-GENE-091118-65]	0.021669	2.950838
GGA3 (1 of many)	3	ENSDARG00000098871	si:ch73-157i16.3 [Source:ZFIN;Acc:ZDB-GENE- 131126-18]	0.026044	2.406259
gipc1	3	ENSDARG00000104483	GIPC PDZ domain containing family, member 1 [Source:ZFIN;Acc:ZDB-GENE-060726-1]	0.032687	3.562859
gipr	15	ENSDARG00000025478	gastric inhibitory polypeptide receptor [Source:ZFIN;Acc:ZDB-GENE-050516-4]	0.039987	1.806442
glg1b	18	ENSDARG00000101210	golgi glycoprotein 1b [Source:ZFIN;Acc:ZDB- GENE-060825-255]	0.02518	2.050953
glis2b	3	ENSDARG00000100232	GLIS family zinc finger 2b [Source:ZFIN;Acc:ZDB-GENE-091204-120]	0.02814	2.485875
gna11a	22	ENSDARG00000010002	guanine nucleotide binding protein (G protein), alpha 11a (Gq class) [Source:ZFIN;Acc:ZDB-GENE- 050208-597]	0.032687	2.486415
GNG4	13	ENSDARG00000111259	G protein subunit gamma 4 [Source:HGNC Symbol;Acc:HGNC:4407]	0.023364	3.898309
gpd2	6	ENSDARG00000062430	glycerol-3-phosphate dehydrogenase 2 (mitochondrial) [Source:ZFIN;Acc:ZDB-GENE-030131-4869]	0.03886	2.119321
gpr12	24	ENSDARG00000062934	G protein-coupled receptor 12 [Source:ZFIN;Acc:ZDB-GENE-100209-3]	0.033967	2.486483
gpr173	8	ENSDARG00000042922	G protein-coupled receptor 173 [Source:ZFIN;Acc:ZDB-GENE-000710-1]	0.033899	2.197226
gpr186	19	ENSDARG00000094860	G protein-coupled receptor 186 [Source:ZFIN;Acc:ZDB-GENE-060503-489]	0.033899	1.877161

gpr37l1a	22	ENSDARG00000006079	G protein-coupled receptor 37 like 1a [Source:ZFIN;Acc:ZDB-GENE-110411-20]	0.039864	2.864447
gpr37l1b	8	ENSDARG00000056774	G protein-coupled receptor 37 like 1b [Source:ZFIN;Acc:ZDB-GENE-081104-324]	0.021669	3.489606
gpsm1b	5	ENSDARG00000054874	G protein signaling modulator 1b [Source:ZFIN;Acc:ZDB-GENE-110203-5]	0.049587	1.977653
gpsm2l	23	ENSDARG00000102026	G protein signaling modulator 2, like [Source:ZFIN;Acc:ZDB-GENE-041010-223]	0.031766	2.167896
gramd1ba	10	ENSDARG00000075383	GRAM domain containing 1Ba [Source:ZFIN;Acc:ZDB-GENE-091118-87]	0.029875	2.409963
gripap1	8	ENSDARG00000063069	GRIP1 associated protein 1 [Source:ZFIN;Acc:ZDB-GENE-070112-922]	0.039987	1.840856
grk3	10	ENSDARG00000104094	G protein-coupled receptor kinase 3 [Source:ZFIN;Acc:ZDB-GENE-030616-382]	0.043006	2.08301
grm3	18	ENSDARG00000031712	glutamate receptor, metabotropic 3 [Source:ZFIN;Acc:ZDB-GENE-061009-13]	0.021669	2.913914
grm5b	15	ENSDARG00000102067	glutamate receptor, metabotropic 5b [Source:ZFIN;Acc:ZDB-GENE-090821-6]	0.016296	3.758003
grm6a	8	ENSDARG00000017742	glutamate receptor, metabotropic 6a [Source:ZFIN;Acc:ZDB-GENE-060208-1]	0.033899	3.560265
hcn4l	25	ENSDARG00000074419	hyperpolarization activated cyclic nucleotide-gated potassium channel 4l [Source:ZFIN;Acc:ZDB- GENE-110411-3]	0.043006	2.307556
hdac5	3	ENSDARG00000075139	histone deacetylase 5 [Source:ZFIN;Acc:ZDB- GENE-121219-3]	0.042916	2.277728
HECTD4	7	ENSDARG00000101079	HECT domain E3 ubiquitin protein ligase 4 [Source:HGNC Symbol;Acc:HGNC:26611]	0.042916	2.081303
henmt1	20	ENSDARG00000018871	HEN methyltransferase 1 [Source:ZFIN;Acc:ZDB- GENE-050417-387]	0.039987	-1.92066
hic2	10	ENSDARG00000100497	hypermethylated in cancer 2 [Source:ZFIN;Acc:ZDB-GENE-030619-1]	0.025806	2.77012
hipk3b	18	ENSDARG00000062082	homeodomain interacting protein kinase 3b [Source:ZFIN;Acc:ZDB-GENE-030131-82]	0.025806	2.617963
hivep2b	17	ENSDARG00000018773	human immunodeficiency virus type I enhancer binding protein 2b [Source:ZFIN;Acc:ZDB-GENE-130530-562]	0.021669	3.218145
hlfa	3	ENSDARG00000074752	hepatic leukemia factor a [Source:ZFIN;Acc:ZDB-GENE-061013-159]	0.024617	1.767214
hlfb	12	ENSDARG00000061011	hepatic leukemia factor b [Source:ZFIN;Acc:ZDB-GENE-110420-3]	0.021669	3.578229
ier2a	3	ENSDARG00000099195	immediate early response 2a [Source:ZFIN;Acc:ZDB-GENE-030131-9126]	0.00943	2.101972
igf1ra	18	ENSDARG00000027423	insulin-like growth factor 1a receptor [Source:ZFIN;Acc:ZDB-GENE-020503-1]	0.023224	2.299126
igsf9bb	21	ENSDARG00000069467	immunoglobulin superfamily, member 9Bb [Source:ZFIN;Acc:ZDB-GENE-091112-15]	0.021669	3.345162
inpp5jb	5	ENSDARG00000093359	inositol polyphosphate-5-phosphatase Jb [Source:ZFIN;Acc:ZDB-GENE-081104-46]	0.03349	3.124116
iqsec2a	8	ENSDARG00000102125	IQ motif and Sec7 domain 2a [Source:ZFIN;Acc:ZDB-GENE-170302-3]	0.025806	2.152418
iqsec3b	18	ENSDARG00000093091	IQ motif and Sec7 domain 3b [Source:ZFIN;Acc:ZDB-GENE-050420-210]	0.027555	2.625581
jph1a	24	ENSDARG00000058603	junctophilin 1a [Source:ZFIN;Acc:ZDB-GENE- 040724-233]	0.043006	2.092961
jupb	19	ENSDARG00000059067	junction plakoglobin b [Source:ZFIN;Acc:ZDB- GENE-110407-10]	0.039767	2.417211
kcna1b	4	ENSDARG00000017108	potassium voltage-gated channel, shaker- related subfamily, member 1b [Source:ZFIN;Acc:ZDB-GENE- 140515-1]	0.033669	2.721795
kcna2b	23	ENSDARG00000102064	potassium voltage-gated channel, shaker- related subfamily, member 2b [Source:ZFIN;Acc:ZDB-GENE- 080204-89]	0.032255	2.274515
kcna4	7	ENSDARG00000078650	potassium voltage-gated channel, shaker- related subfamily, member 4 [Source:ZFIN;Acc:ZDB-GENE- 121127-2]	0.025927	2.505552

kcnc3b	24	ENSDARG00000098816	potassium voltage-gated channel, Shaw- related subfamily, member 3b [Source:ZFIN;Acc:ZDB-GENE- 100901-2]	0.025806	2.711286
kcnc4	8	ENSDARG00000061288	potassium voltage-gated channel, Shaw- related subfamily, member 4 [Source:ZFIN;Acc:ZDB-GENE- 060503-773]	0.028922	3.126769
kcnh3	9	ENSDARG00000069560	potassium voltage-gated channel, subfamily H (eag-related), member 3 [Source:ZFIN;Acc:ZDB-GENE-070912- 23]	0.016296	3.291888
kcnk1b	13	ENSDARG00000017254	potassium channel, subfamily K, member 1b [Source:ZFIN;Acc:ZDB-GENE-090312-78]	0.042916	2.804736
kcnq2b	6	ENSDARG00000091130	potassium voltage-gated channel, KQT-like subfamily, member 2b [Source:ZFIN;Acc:ZDB-GENE-120130-1]	0.025806	2.611187
kcnq3	2	ENSDARG00000060085	potassium voltage-gated channel, KQT-like subfamily, member 3 [Source:ZFIN;Acc:ZDB-GENE-070912-301]	0.032687	2.799858
kcnq3	2	ENSDARG00000060085	potassium voltage-gated channel, KQT-like subfamily, member 3 [Source:ZFIN;Acc:ZDB-GENE-070912-301]	0.028922	3.00683
kdm4aa	6	ENSDARG00000018782	lysine (K)-specific demethylase 4A, genome duplicate a [Source:ZFIN;Acc:ZDB-GENE-110609-4]	0.047559	1.792279
kif1ab	2	ENSDARG00000062024	kinesin family member 1Ab [Source:ZFIN;Acc:ZDB-GENE-070912-480]	0.030646	2.420209
kif21b	11	ENSDARG00000009733	kinesin family member 21B [Source:ZFIN;Acc:ZDB-GENE-130530-537]	0.046367	2.480112
kif5bb	12	ENSDARG00000103394	kinesin family member 5B, b [Source:ZFIN;Acc:ZDB-GENE-070629-4]	0.025806	2.726072
klf13	25	ENSDARG00000061368	Kruppel-like factor 13 [Source:ZFIN;Acc:ZDB- GENE-060929-1274]	0.03306	2.131947
klhdc8a	11	ENSDARG00000061000	kelch domain containing 8A [Source:ZFIN;Acc:ZDB-GENE-120810-3]	0.03718	2.84718
klhl12	8	ENSDARG00000100631	kelch-like family member 12 [Source:ZFIN;Acc:ZDB-GENE-041114-205]	0.04738	1.678261
klhl4	14	ENSDARG00000016531	kelch-like family member 4 [Source:ZFIN;Acc:ZDB-GENE-070410-63]	0.033899	2.319378
klhl5	1	ENSDARG00000062122	kelch-like family member 5 [Source:ZFIN;Acc:ZDB-GENE-080424-7]	0.039987	3.783713
l1camb	23	ENSDARG00000015025	L1 cell adhesion molecule, paralog b [Source:ZFIN;Acc:ZDB-GENE-980526-512]	0.048429	2.398347
large1	4	ENSDARG00000005126	LARGE xylosyl- and glucuronyltransferase 1 [Source:ZFIN;Acc:ZDB-GENE-061204-1]	0.036465	2.451505
lcor	22	ENSDARG00000100908	ligand dependent nuclear receptor corepressor [Source:ZFIN;Acc:ZDB-GENE-061201-6]	0.033899	3.33357
lgi3	8	ENSDARG00000041358	leucine-rich repeat LGI family, member 3 [Source:ZFIN;Acc:ZDB-GENE-060217-4]	0.037994	2.317809
lifra	5	ENSDARG00000098857	leukemia inhibitory factor receptor alpha a [Source:ZFIN;Acc:ZDB-GENE-050327-16]	0.030314	3.121661
lipeb	16	ENSDARG00000101145	lipase, hormone-sensitive b [Source:ZFIN;Acc:ZDB-GENE-100921-71]	0.025746	2.152665
lrfn5a	17	ENSDARG00000071230	leucine rich repeat and fibronectin type III domain containing 5a [Source:ZFIN;Acc:ZDB-GENE-080327-17]	0.029884	3.066816
lrrc3b	19	ENSDARG00000063215	leucine rich repeat containing 3B [Source:ZFIN;Acc:ZDB-GENE-070410-55]	0.02815	3.805995
LSAMP	15	ENSDARG00000103069	limbic system-associated membrane protein [Source:HGNC Symbol;Acc:HGNC:6705]	0.043006	2.620455
lzts3a	5	ENSDARG00000098751	leucine zipper, putative tumor suppressor family member 3a [Source:ZFIN;Acc:ZDB-GENE-060313-2]	0.026556	2.809752
magixa	8	ENSDARG00000025108	MAGI family member, X-linked a [Source:ZFIN;Acc:ZDB-GENE-060503-115]	0.040771	2.179979
MAN1C1	16	ENSDARG00000067912	si:ch73-373m9.1 [Source:ZFIN;Acc:ZDB-GENE- 110411-115]	0.046367	2.529239

map2k4a	3	ENSDARG00000063583	mitogen-activated protein kinase kinase 4a [Source:ZFIN;Acc:ZDB-GENE-060512-98]	0.024013	2.489291
map3k9	13	ENSDARG00000013491	mitogen-activated protein kinase kinase kinase 9 [Source:ZFIN;Acc:ZDB-GENE-081113-5]	0.035498	2.312513
map7a	17	ENSDARG00000078241	microtubule-associated protein 7a [Source:ZFIN;Acc:ZDB-GENE-131121-151]	0.037211	2.328485
mapk10	21	ENSDARG00000102730	mitogen-activated protein kinase 10 [Source:ZFIN;Acc:ZDB-GENE-051120-117]	0.042412	2.101348
mapk8b	12	ENSDARG00000009870	mitogen-activated protein kinase 8b [Source:ZFIN;Acc:ZDB-GENE-010202-1]	0.02518	2.704476
mapk8ip2	18	ENSDARG00000063157	mitogen-activated protein kinase 8 interacting protein 2 [Source:ZFIN;Acc:ZDB-GENE-070705- 326]	0.027932	3.011159
mark4a	5	ENSDARG00000023914	MAP/microtubule affinity-regulating kinase 4a [Source:ZFIN;Acc:ZDB-GENE-060531-156]	0.025264	2.711868
mbnl1	2	ENSDARG00000052978	muscleblind-like splicing regulator 1 [Source:ZFIN;Acc:ZDB-GENE-060929-704]	0.026551	2.331536
mcf2l2	11	ENSDARG00000079742	MCF.2 cell line derived transforming sequence-like 2 [Source:ZFIN;Acc:ZDB-GENE-100812-1]	0.016296	2.835508
mgat3b	6	ENSDARG00000036619	mannosyl (beta-1,4-)-glycoprotein beta-1,4-N- acetylglucosaminyltransferase b [Source:ZFIN;Acc:ZDB-GENE- 130820-2]	0.021669	3.381619
MIB2	8	ENSDARG00000103609	mindbomb E3 ubiquitin protein ligase 2 [Source:HGNC Symbol;Acc:HGNC:30577]	0.041341	2.038711
micall1a	6	ENSDARG00000079811	MICAL-like 1a [Source:ZFIN;Acc:ZDB-GENE- 110119-1]	0.031766	2.251959
mkl2a	12	ENSDARG00000088307	MKL/myocardin-like 2a [Source:ZFIN;Acc:ZDB- GENE-130531-36]	0.033899	2.698361
mlec	8	ENSDARG00000059630	malectin [Source:ZFIN;Acc:ZDB-GENE-081105- 187]	0.049903	2.41478
mmd	3	ENSDARG00000040387	monocyte to macrophage differentiation- associated [Source:ZFIN;Acc:ZDB-GENE-051120-39]	0.043294	2.516548
mmp24	6	ENSDARG00000018896	matrix metallopeptidase 24 [Source:ZFIN;Acc:ZDB-GENE-040724-262]	0.021669	3.04339
mpi	25	ENSDARG00000030786	mannose phosphate isomerase [Source:ZFIN;Acc:ZDB-GENE-050904-6]	0.036465	2.308759
mri1	1	ENSDARG00000075754	methylthioribose-1-phosphate isomerase 1 [Source:ZFIN;Acc:ZDB-GENE-080204-109]	0.038307	2.833444
msantd4	15	ENSDARG00000018040	Myb/SANT-like DNA-binding domain containing 4 with coiled-coils [Source:ZFIN;Acc:ZDB-GENE-121023-2]	0.037211	2.402476
myo10l1	6	ENSDARG00000074723	myosin X-like 1 [Source:ZFIN;Acc:ZDB-GENE- 080425-1]	0.023364	2.563987
myo16	9	ENSDARG00000078582	myosin XVI [Source:ZFIN;Acc:ZDB-GENE-070912- 470]	0.022829	2.147824
myo5aa	18	ENSDARG00000061635	myosin VAa [Source:ZFIN;Acc:ZDB-GENE-041027- 2]	0.033899	2.44503
nav1b	6	ENSDARG00000058771	neuron navigator 1b [Source:ZFIN;Acc:ZDB- GENE-120215-84]	0.010757	3.735929
nbeal1	6	ENSDARG00000099547	neurobeachin-like 1 [Source:ZFIN;Acc:ZDB- GENE-130530-544]	0.043196	2.223275
ncanb	22	ENSDARG00000005783	neurocan b [Source:ZFIN;Acc:ZDB-GENE-050208- 586]	0.048236	2.918642
ncoa1	20	ENSDARG00000018257	nuclear receptor coactivator 1 [Source:ZFIN;Acc:ZDB-GENE-041001-175]	0.033864	2.11049
nf1b	10	ENSDARG00000004184	neurofibromin 1b [Source:ZFIN;Acc:ZDB-GENE- 091111-4]	0.025414	2.696157
nlgn1	11	ENSDARG00000077710	neuroligin 1 [Source:ZFIN;Acc:ZDB-GENE- 090918-1]	0.016296	3.216914
nos1apb	2	ENSDARG00000058701	nitric oxide synthase 1 (neuronal) adaptor protein b [Source:ZFIN;Acc:ZDB-GENE-100824-1]	0.026556	3.535041

nova1	5	ENSDARG00000020178	neuro-oncological ventral antigen 1 [Source:ZFIN;Acc:ZDB-GENE-060526-113]	0.028922	2.123869
npas4a	14	ENSDARG00000055752	neuronal PAS domain protein 4a [Source:ZFIN;Acc:ZDB-GENE-060616-396]	5.23E-05	3.266818
nr1i2	9	ENSDARG00000029766	nuclear receptor subfamily 1, group I, member 2 [Source:ZFIN;Acc:ZDB-GENE-030903-3]	0.049786	1.940686
nr4a1	23	ENSDARG00000000796	nuclear receptor subfamily 4, group A, member 1 [Source:ZFIN;Acc:ZDB-GENE-040704-11]	0.004326	3.476529
ntrk2a	8	ENSDARG00000059897	neurotrophic tyrosine kinase, receptor, type 2a [Source:ZFIN;Acc:ZDB-GENE-010126-1]	0.025927	2.044431
nuak1a	4	ENSDARG00000020086	NUAK family, SNF1-like kinase, 1a [Source:ZFIN;Acc:ZDB-GENE-041210-122]	0.048488	2.007335
nudt14	20	ENSDARG00000062335	nudix (nucleoside diphosphate linked moiety X)-type motif 14 [Source:ZFIN;Acc:ZDB-GENE-041014-254]	0.043006	2.081784
numbl	15	ENSDARG00000101949	numb homolog (Drosophila)-like [Source:ZFIN;Acc:ZDB-GENE-051113-340]	0.025806	2.311578
osbp	1	ENSDARG00000004634	oxysterol binding protein [Source:ZFIN;Acc:ZDB-GENE-090312-18]	0.037994	2.225761
osbpl2a	23	ENSDARG00000053804	oxysterol binding protein-like 2a [Source:ZFIN;Acc:ZDB-GENE-091116-46]	0.032292	2.979684
otud7b	16	ENSDARG00000017220	OTU deubiquitinase 7B [Source:ZFIN;Acc:ZDB- GENE-060616-1]	0.023324	2.217343
pacs2	13	ENSDARG00000078185	phosphofurin acidic cluster sorting protein 2 [Source:ZFIN;Acc:ZDB-GENE-081218-1]	0.042916	2.081153
pak6a	17	ENSDARG00000027564	p21 protein (Cdc42/Rac)-activated kinase 6a [Source:ZFIN;Acc:ZDB-GENE-130410-1]	0.040745	2.606841
pbx1b	6	ENSDARG00000101131	pre-B-cell leukemia homeobox 1b [Source:ZFIN;Acc:ZDB-GENE-070424-11]	0.035652	2.400608
pcdh1g13	10	ENSDARG00000098963	protocadherin 1 gamma 13 [Source:ZFIN;Acc:ZDB-GENE-050609-8]	0.040745	2.581603
pcsk1	21	ENSDARG00000002600	proprotein convertase subtilisin/kexin type 1 [Source:ZFIN;Acc:ZDB-GENE-071009-1]	0.021669	3.523713
pctp	3	ENSDARG00000073985	phosphatidylcholine transfer protein [Source:ZFIN;Acc:ZDB-GENE-081022-182]	0.027655	3.294296
pde11a	9	ENSDARG00000063732	phosphodiesterase 11a [Source:ZFIN;Acc:ZDB- GENE-070912-207]	0.033899	3.197451
pde4d	8	ENSDARG00000032761	phosphodiesterase 4D, cAMP-specific [Source:ZFIN;Acc:ZDB-GENE-081105-16]	0.039987	2.499271
pdxkb	1	ENSDARG00000036546	pyridoxal (pyridoxine, vitamin B6) kinase b [Source:ZFIN;Acc:ZDB-GENE-030616-521]	0.033669	3.286603
pgap1	9	ENSDARG00000062465	post-GPI attachment to proteins 1 [Source:ZFIN;Acc:ZDB-GENE-090313-216]	0.029875	2.421878
pgap3	12	ENSDARG00000057531	post-GPI attachment to proteins 3 [Source:ZFIN;Acc:ZDB-GENE-080204-27]	0.025927	3.24348
pgbd4	22	ENSDARG00000079328	piggyBac transposable element derived 4 [Source:ZFIN;Acc:ZDB-GENE-091204-417]	0.041383	1.894831
phldb1b	15	ENSDARG00000079378	pleckstrin homology-like domain, family B, member 1b [Source:ZFIN;Acc:ZDB-GENE-121114-8]	0.043006	2.09606
pi4kab	8	ENSDARG00000062823	phosphatidylinositol 4-kinase, catalytic, alpha b [Source:ZFIN;Acc:ZDB-GENE-080220-34]	0.03349	2.201529
pid1	18	ENSDARG00000098984	phosphotyrosine interaction domain containing 1 [Source:ZFIN;Acc:ZDB-GENE-050320-72]	0.033899	3.071544
pip4k2ab	2	ENSDARG00000063544	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha b [Source:ZFIN;Acc:ZDB-GENE-070912-272]	0.043006	2.41766
pknox1.2	1	ENSDARG00000036542	pbx/knotted 1 homeobox 1.2 [Source:ZFIN;Acc:ZDB-GENE-020123-1]	0.045394	2.057834

PLCB1	17	ENSDARG00000109726	si:ch1073-140o9.2 [Source:ZFIN;Acc:ZDB-GENE- 120709-104]	0.048918	2.185045
plcl1	9	ENSDARG00000067676	phospholipase C like 1 [Source:ZFIN;Acc:ZDB- GENE-090313-194]	0.047701	2.106581
plxna1a	23	ENSDARG00000105452	plexin A1a [Source:ZFIN;Acc:ZDB-GENE-140106- 137]	0.021669	2.952333
plxna2	23	ENSDARG00000060372	plexin A2 [Source:ZFIN;Acc:ZDB-GENE-090311- 6]	0.021669	2.740609
pomgnt2	16	ENSDARG00000010941	protein O-linked mannose N- acetylglucosaminyltransferase 2 (beta 1,4-) [Source:ZFIN;Acc:ZDB-GENE-050522-242]	0.040745	2.067358
ppargc1a	7	ENSDARG00000067829	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha [Source:ZFIN;Acc:ZDB-GENE- 080505-1]	0.048918	2.309633
ppm1aa	13	ENSDARG00000032155	protein phosphatase, Mg2+/Mn2+ dependent, 1Aa [Source:ZFIN;Acc:ZDB-GENE-991102-15]	0.040888	2.357536
ppm1j	6	ENSDARG00000074690	protein phosphatase, Mg2+/Mn2+ dependent, 1J [Source:ZFIN;Acc:ZDB-GENE-091230-9]	0.043006	1.830485
ppp1r9a	19	ENSDARG00000061304	protein phosphatase 1, regulatory subunit 9A [Source:ZFIN;Acc:ZDB-GENE-060503-660]	0.036541	2.476713
ppp1r9bb	12	ENSDARG00000071709	protein phosphatase 1, regulatory subunit 9Bb [Source:ZFIN;Acc:ZDB-GENE-121214-117]	0.043006	2.273141
ppp3r1a	13	ENSDARG00000092659	protein phosphatase 3, regulatory subunit B, alpha a [Source:ZFIN;Acc:ZDB-GENE-081113-3]	0.038307	2.487292
ppp6r2b	18	ENSDARG00000069654	protein phosphatase 6, regulatory subunit 2b [Source:ZFIN;Acc:ZDB-GENE-070705-441]	0.033899	2.198932
prkacaa	3	ENSDARG00000100349	protein kinase, cAMP-dependent, catalytic, alpha, genome duplicate a [Source:ZFIN;Acc:ZDB-GENE-050114- 6]	0.033669	2.352591
PRKAR2B	25	ENSDARG00000036446	si:ch211-272n13.7 [Source:ZFIN;Acc:ZDB-GENE- 160113-22]	0.043006	2.332243
PRKCA	3	ENSDARG00000099841	si:ch73-374l24.1 [Source:ZFIN;Acc:ZDB-GENE- 141216-6]	0.033899	2.637254
prkcg	16	ENSDARG00000004561	protein kinase C, gamma [Source:ZFIN;Acc:ZDB- GENE-090206-1]	0.038307	2.831096
prkg1a	13	ENSDARG00000020656	protein kinase, cGMP-dependent, type Ia [Source:ZFIN;Acc:ZDB-GENE-040426-1308]	0.042912	2.74811
prrt1	19	ENSDARG00000062208	proline-rich transmembrane protein 1 [Source:ZFIN;Acc:ZDB-GENE-060503-686]	0.024372	3.566599
ptbp3	10	ENSDARG00000043757	polypyrimidine tract binding protein 3 [Source:ZFIN;Acc:ZDB-GENE-091116-1]	0.040745	2.221497
ptchd4	20	ENSDARG00000008249	patched domain containing 4 [Source:ZFIN;Acc:ZDB-GENE-041001-178]	0.033899	2.131973
ptprnb	6	ENSDARG00000077047	protein tyrosine phosphatase, receptor type, Nb [Source:ZFIN;Acc:ZDB-GENE-100505-1]	0.043006	2.121308
ptprt	6	ENSDARG00000097572	protein tyrosine phosphatase, receptor type, t [Source:ZFIN;Acc:ZDB-GENE-101028-4]	0.024617	2.355058
ptprub	16	ENSDARG00000101081	protein tyrosine phosphatase, receptor type, U, b [Source:ZFIN;Acc:ZDB-GENE-030131-7036]	0.033669	2.035131
purbb	10	ENSDARG00000103546	purine-rich element binding protein Bb [Source:ZFIN;Acc:ZDB-GENE-040426-1478]	0.035384	2.049682
rab3ab	11	ENSDARG00000043835	RAB3A, member RAS oncogene family, b [Source:ZFIN;Acc:ZDB-GENE-041210-268]	0.029884	3.195875
rap1gap2a	15	ENSDARG00000061551	RAP1 GTPase activating protein 2a [Source:ZFIN;Acc:ZDB-GENE-061013-179]	0.042916	2.412432
rapgefl1	3	ENSDARG00000079912	Rap guanine nucleotide exchange factor (GEF)- like 1 [Source:ZFIN;Acc:ZDB-GENE-131121-404]	0.023364	2.670178

rasgrf2b	5	ENSDARG00000002816	Ras protein-specific guanine nucleotide- releasing factor 2b [Source:ZFIN;Acc:ZDB-GENE-060504-1]	0.021669	3.977892
rbm25a	13	ENSDARG00000039266	RNA binding motif protein 25a [Source:ZFIN;Acc:ZDB-GENE-090312-142]	0.033899	2.197043
rc3h1a	22	ENSDARG00000062550	ring finger and CCCH-type domains 1a [Source:ZFIN;Acc:ZDB-GENE-050208-702]	0.025746	3.04076
rc3h2	10	ENSDARG00000063050	ring finger and CCCH-type domains 2 [Source:ZFIN;Acc:ZDB-GENE-060503-694]	0.029884	3.050228
rgl2	16	ENSDARG00000007727	ral guanine nucleotide dissociation stimulator-like 2 [Source:ZFIN;Acc:ZDB-GENE-010131-1]	0.025806	2.253161
rhbdl1	24	ENSDARG00000087040	rhomboid, veinlet-like 1 (Drosophila) [Source:ZFIN;Acc:ZDB-GENE-120529-2]	0.021669	2.842924
rhobtb1	12	ENSDARG00000079468	Rho-related BTB domain containing 1 [Source:ZFIN;Acc:ZDB-GENE-130530-871]	0.028922	3.845695
rims3	19	ENSDARG00000062305	regulating synaptic membrane exocytosis 3 [Source:ZFIN;Acc:ZDB-GENE-060503-788]	0.025806	3.930239
rnf19b	19	ENSDARG00000060926	ring finger protein 19B [Source:ZFIN;Acc:ZDB- GENE-060503-281]	0.02814	3.251527
rnf217	20	ENSDARG00000060944	ring finger protein 217 [Source:ZFIN;Acc:ZDB- GENE-060503-400]	0.024617	2.26414
rnf38	1	ENSDARG00000062055	ring finger protein 38 [Source:ZFIN;Acc:ZDB- GENE-030131-8693]	0.048236	2.080719
rnf44	14	ENSDARG00000068582	ring finger protein 44 [Source:ZFIN;Acc:ZDB- GENE-060929-604]	0.039987	1.97531
rtn2a	15	ENSDARG00000016088	reticulon 2a [Source:ZFIN;Acc:ZDB-GENE- 060420-1]	0.046367	2.466006
rtn4rl2a	1	ENSDARG00000052012	reticulon 4 receptor-like 2 a [Source:ZFIN;Acc:ZDB-GENE-040310-4]	0.025806	1.875274
sbno2a	2	ENSDARG00000098801	strawberry notch homolog 2a [Source:ZFIN;Acc:ZDB-GENE-100629-3]	0.030406	2.590462
sc5d	21	ENSDARG00000044642	sterol-C5-desaturase [Source:ZFIN;Acc:ZDB- GENE-040912-56]	0.044042	2.072382
scai	5	ENSDARG00000060865	suppressor of cancer cell invasion [Source:ZFIN;Acc:ZDB-GENE-060526-57]	0.043006	2.844109
scamp5a	25	ENSDARG00000018743	secretory carrier membrane protein 5a [Source:ZFIN;Acc:ZDB-GENE-030131-3188]	0.036465	2.830502
scap	16	ENSDARG00000018096	SREBF chaperone [Source:ZFIN;Acc:ZDB-GENE- 040107-41]	0.036572	2.406363
scn2b	15	ENSDARG00000101713	sodium channel, voltage-gated, type II, beta [Source:ZFIN;Acc:ZDB-GENE-070920-1]	0.02814	2.889949
scn3b	15	ENSDARG00000062359	sodium channel, voltage-gated, type III, beta [Source:ZFIN;Acc:ZDB-GENE-070920-2]	0.047559	2.003571
scube3	23	ENSDARG00000011490	signal peptide, CUB domain, EGF-like 3 [Source:ZFIN;Acc:ZDB-GENE-060717-1]	0.038307	2.307866
sema3ab	18	ENSDARG00000042210	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3Ab [Source:ZFIN;Acc:ZDB-GENE-991209-6]	0.029875	3.862469
sema4ba	18	ENSDARG00000074414	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4Ba [Source:ZFIN;Acc:ZDB-GENE-070705-31]	0.023364	3.796013
sept5b	10	ENSDARG00000036031	septin 5b [Source:ZFIN;Acc:ZDB-GENE-040808- 43]	0.043294	2.67348
sfxn3	13	ENSDARG00000057374	sideroflexin 3 [Source:ZFIN;Acc:ZDB-GENE- 070112-2092]	0.043006	2.35292
sgcb	20	ENSDARG00000052341	sarcoglycan, beta (dystrophin-associated glycoprotein) [Source:ZFIN;Acc:ZDB-GENE-030131-6695]	0.043006	2.064503
SGCZ	1	ENSDARG00000106258	sarcoglycan zeta [Source:HGNC Symbol;Acc:HGNC:14075]	0.042916	2.449612
sh3glb2a	8	ENSDARG00000008983	SH3-domain GRB2-like endophilin B2a [Source:ZFIN;Acc:ZDB-GENE-061201-8]	0.048468	2.206702

sh3pxd2aa	1	ENSDARG00000060148	SH3 and PX domains 2Aa [Source:ZFIN;Acc:ZDB- GENE-060503-271]	0.023237	2.731423
SH3RF3	9	ENSDARG00000102334	si:ch211-124n19.2 [Source:ZFIN;Acc:ZDB-GENE- 081104-105]	0.02814	2.715923
shank1	3	ENSDARG00000060539	SH3 and multiple ankyrin repeat domains 1 [Source:ZFIN;Acc:ZDB-GENE-130530-691]	0.031615	2.693619
shank3a	18	ENSDARG00000063332	SH3 and multiple ankyrin repeat domains 3a [Source:ZFIN;Acc:ZDB-GENE-060503-369]	0.024617	2.603181
shisa7a	16	ENSDARG00000062462	shisa family member 7a [Source:ZFIN;Acc:ZDB- GENE-100922-37]	0.023324	2.886297
shisa9b	3	ENSDARG00000052642	shisa family member 9b [Source:ZFIN;Acc:ZDB- GENE-081022-105]	0.044308	2.199716
shoc2	22	ENSDARG00000040853	SHOC2 leucine-rich repeat scaffold protein [Source:ZFIN;Acc:ZDB-GENE-050208-523]	0.047579	1.740992
si:cabz0102 9535.1	7	ENSDARG00000105432	si:cabz01029535.1 [Source:ZFIN;Acc:ZDB-GENE- 160114-82]	0.021669	3.376527
si:ch211- 120g10.1	3	ENSDARG00000090930	si:ch211-120g10.1 [Source:ZFIN;Acc:ZDB-GENE- 130531-56]	0.041341	2.470316
si:ch211- 166g5.4	12	ENSDARG00000063555	si:ch211-166g5.4 [Source:ZFIN;Acc:ZDB-GENE- 121214-319]	0.03349	2.518554
si:ch211- 168b3.1	2	ENSDARG00000056478	si:ch211-168b3.1 [Source:ZFIN;Acc:ZDB-GENE- 081104-34]	0.04738	1.730269
si:ch211- 188f17.1	24	ENSDARG00000100958	si:ch211-188f17.1 [Source:ZFIN;Acc:ZDB-GENE- 131120-111]	0.021669	2.32751
si:ch211- 194b1.1	3	ENSDARG00000102436	si:ch211-194b1.1 [Source:ZFIN;Acc:ZDB-GENE- 131126-2]	0.033355	2.446298
si:ch211- 210g13.5	3	ENSDARG00000062575	si:ch211-210g13.5 [Source:ZFIN;Acc:ZDB-GENE- 030131-5675]	0.043006	2.35538
si:ch211- 271d10.2	16	ENSDARG00000104269	si:ch211-271d10.2 [Source:ZFIN;Acc:ZDB-GENE- 141212-343]	0.025297	2.191705
si:ch73- 127m5.1	12	ENSDARG00000060680	si:ch73-127m5.1 [Source:ZFIN;Acc:ZDB-GENE- 130531-25]	0.024372	2.661593
si:ch73- 233f7.7	14	ENSDARG00000099487	si:ch73-233f7.7 [Source:ZFIN;Acc:ZDB-GENE- 141215-50]	0.035384	3.386849
si:ch73- 287m6.1	21	ENSDARG00000104101	si:ch73-287m6.1 [Source:ZFIN;Acc:ZDB-GENE- 100920-2]	0.04738	1.58028
si:ch73- 362m14.2	21	ENSDARG00000090164	si:ch73-362m14.2 [Source:ZFIN;Acc:ZDB-GENE- 120215-235]	0.042916	2.908599
si:dkey- 151g10.3	23	ENSDARG00000021611	si:dkey-151g10.3 [Source:ZFIN;Acc:ZDB-GENE- 090312-81]	0.04738	2.233725
si:dkey- 175m17.7	21	ENSDARG00000078317	si:dkey-175m17.7 [Source:ZFIN;Acc:ZDB-GENE- 091204-18]	0.040888	2.297766
si:dkey- 206p8.1	17	ENSDARG00000096971	si:dkey-206p8.1 [Source:ZFIN;Acc:ZDB-GENE- 131127-460]	0.029884	2.210769
si:dkey- 222b8.1	19	ENSDARG00000045094	si:dkey-222b8.1 [Source:ZFIN;Acc:ZDB-GENE- 060503-248]	0.02518	2.997567
si:dkey- 263f15.2	8	ENSDARG00000074828	si:dkey-263f15.2 [Source:ZFIN;Acc:ZDB-GENE- 081105-67]	0.02293	4.376863
si:dkey- 38k9.5	21	ENSDARG00000079102	si:dkey-38k9.5 [Source:ZFIN;Acc:ZDB-GENE- 091204-100]	0.039673	2.651376
si:dkey- 92j12.5	1	ENSDARG00000094677	si:dkey-92j12.5 [Source:ZFIN;Acc:ZDB-GENE- 090313-354]	0.045766	2.169073

si:dkey-93n13.3	3	ENSDARG00000078736	si:dkey-93n13.3 [Source:ZFIN;Acc:ZDB-GENE- 091118-107]	0.038307	3.216366
si:dkey-94l16.4	12	ENSDARG00000090142	si:dkey-94l16.4 [Source:ZFIN;Acc:ZDB-GENE- 121214-291]	0.048236	2.227981
si:dkeyp-113d7.10	19	ENSDARG00000092359	si:dkeyp-113d7.10 [Source:ZFIN;Acc:ZDB-GENE- 061207-80]	0.043294	2.282217
sipa1l3	18	ENSDARG00000061699	signal-induced proliferation-associated 1 like 3 [Source:ZFIN;Acc:ZDB-GENE-050420-109]	0.038922	2.438478
slc12a5a	6	ENSDARG00000075815	solute carrier family 12 (potassium/chloride transporter), member 5a [Source:ZFIN;Acc:ZDB-GENE-120927- 3]	0.029884	2.600688
slc16a1b	8	ENSDARG00000068572	solute carrier family 16 (monocarboxylate transporter), member 1b [Source:ZFIN;Acc:ZDB-GENE-030515- 5]	0.043006	2.678771
slc17a6a	25	ENSDARG00000001127	solute carrier family 17 (vesicular glutamate transporter), member 6a [Source:ZFIN;Acc:ZDB- GENE-050105-4]	0.025806	3.946379
slc1a3b	10	ENSDARG00000043148	solute carrier family 1 (glial high affinity glutamate transporter), member 3b [Source:ZFIN;Acc:ZDB- GENE-090708-3]	0.039987	2.451697
slc23a2	8	ENSDARG00000017365	solute carrier family 23 (ascorbic acid transporter), member 2 [Source:ZFIN;Acc:ZDB-GENE-090521- 3]	0.037211	2.615652
slc24a3	13	ENSDARG00000006760	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3 [Source:ZFIN;Acc:ZDB-GENE-090312-67]	0.023364	3.889733
slc25a22a	25	ENSDARG00000020718	solute carrier family 25 member 22a [Source:ZFIN;Acc:ZDB-GENE-110408-61]	0.028922	2.919583
slc25a23b	3	ENSDARG00000024708	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23b [Source:ZFIN;Acc:ZDB-GENE-040724-220]	0.04738	2.135831
slc2a1a	23	ENSDARG00000001437	solute carrier family 2 (facilitated glucose transporter), member 1a [Source:ZFIN;Acc:ZDB-GENE-030131- 3158]	0.021669	3.398694
slc43a2a	15	ENSDARG00000036848	solute carrier family 43 (amino acid system L transporter), member 2a [Source:ZFIN;Acc:ZDB-GENE-040426- 964]	0.02814	2.989673
SLC45A4 (1 of many)	19	ENSDARG00000104981	solute carrier family 45 member 4 [Source:HGNC Symbol;Acc:HGNC:29196]	0.023224	3.627351
slc48a1a	23	ENSDARG00000026907	solute carrier family 48 (heme transporter), member 1a [Source:ZFIN;Acc:ZDB-GENE-040718-121]	0.039987	2.490252
slc4a10b	9	ENSDARG00000060303	solute carrier family 4, sodium bicarbonate transporter, member 10b [Source:ZFIN;Acc:ZDB-GENE-081105- 62]	0.029884	2.707324
slc4a3	9	ENSDARG00000104005	solute carrier family 4 (anion exchanger), member 3 [Source:ZFIN;Acc:ZDB-GENE-141006-1]	0.023364	2.938328
slc6a15	18	ENSDARG00000062821	solute carrier family 6 (neutral amino acid transporter), member 15 [Source:ZFIN;Acc:ZDB-GENE-050420- 93]	0.02518	2.591682
slc7a4	8	ENSDARG00000068286	solute carrier family 7, member 4 [Source:ZFIN;Acc:ZDB-GENE-030919-2]	0.021669	3.546894
slc7a5	25	ENSDARG00000099265	solute carrier family 7 (amino acid transporter light chain, L system), member 5 [Source:ZFIN;Acc:ZDB-GENE-080818-4]	0.04738	2.911087
slc8a1b	17	ENSDARG00000043406	solute carrier family 8 (sodium/calcium exchanger), member 1b [Source:ZFIN;Acc:ZDB-GENE-060110-3]	0.045766	2.109854
slc8a3	13	ENSDARG00000004931	solute carrier family 8 (sodium/calcium exchanger), member 3 [Source:ZFIN;Acc:ZDB-GENE-050810-1]	0.046987	2.211904
slc8a4a	21	ENSDARG00000055154	solute carrier family 8 (sodium/calcium exchanger), member 4a [Source:ZFIN;Acc:ZDB-GENE-060110-2]	0.025414	2.842893
slc8b1	7	ENSDARG00000105584	solute carrier family 8 (sodium/lithium/calcium exchanger), member B1 [Source:ZFIN;Acc:ZDB-GENE-130530-900]	0.033899	2.512954

slco5a1b	2	ENSDARG00000086104	solute carrier organic anion transporter family member 5A1b [Source:ZFIN;Acc:ZDB-GENE-070912-587]	0.043006	2.260328
soat1	8	ENSDARG00000062297	sterol O-acyltransferase 1 [Source:ZFIN;Acc:ZDB-GENE-081105-177]	0.046367	2.250619
sobpa	13	ENSDARG00000054253	sine oculis binding protein homolog (Drosophila) a [Source:ZFIN;Acc:ZDB-GENE-090313-313]	0.016296	4.214959
soga3b	23	ENSDARG00000075455	SOGA family member 3b [Source:ZFIN;Acc:ZDB- GENE-081022-198]	0.049786	1.886848
sorbs2a	1	ENSDARG00000003046	sorbin and SH3 domain containing 2a [Source:ZFIN;Acc:ZDB-GENE-070308-2]	0.035202	2.327224
soul4	22	ENSDARG00000101008	heme-binding protein soul4 [Source:ZFIN;Acc:ZDB-GENE-110718-1]	0.039987	1.917747
sox9a	12	ENSDARG00000003293	SRY (sex determining region Y)-box 9a [Source:ZFIN;Acc:ZDB-GENE-001103-1]	0.040888	2.087045
spina	10	ENSDARG00000058949	spindlin a [Source:ZFIN;Acc:ZDB-GENE-161230- 18]	0.033669	2.251309
sptlc2a	17	ENSDARG00000018976	serine palmitoyltransferase, long chain base subunit 2a [Source:ZFIN;Acc:ZDB-GENE-050522-23]	0.037536	1.545971
srebf1	3	ENSDARG00000067607	sterol regulatory element binding transcription factor 1 [Source:ZFIN;Acc:ZDB-GENE-090812- 3]	0.033899	2.592695
srgap3	6	ENSDARG00000060309	SLIT-ROBO Rho GTPase activating protein 3 [Source:ZFIN;Acc:ZDB-GENE-060524-4]	0.049903	1.922432
sstr3	3	ENSDARG00000014477	somatostatin receptor 3 [Source:ZFIN;Acc:ZDB- GENE-061009-35]	0.038307	2.513877
st3gal2	18	ENSDARG00000112898	ST3 beta-galactoside alpha-2,3- sialyltransferase 2 [Source:ZFIN;Acc:ZDB-GENE-050419-181]	0.042046	2.070391
st6gal1	21	ENSDARG00000044514	ST6 beta-galactosamide alpha-2,6- sialyltranferase 1 [Source:ZFIN;Acc:ZDB-GENE-060322-3]	0.048468	2.258727
st6galnac6	21	ENSDARG00000036913	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta- galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6- sialyltransferase 6 [Source:ZFIN;Acc:ZDB-GENE-060322-7]	0.036465	2.955304
st8sia3	21	ENSDARG00000045301	ST8 alpha-N-acetyl-neuraminide alpha-2,8- sialyltransferase 3 [Source:ZFIN;Acc:ZDB-GENE-060228-4]	0.030314	2.407322
stk40	16	ENSDARG00000098135	serine/threonine kinase 40 [Source:ZFIN;Acc:ZDB-GENE-030131-4831]	0.039939	2.414973
strn	11	ENSDARG00000100011	striatin, calmodulin binding protein [Source:ZFIN;Acc:ZDB-GENE-070112-1172]	0.039767	2.049268
sv2c	5	ENSDARG00000059997	synaptic vesicle glycoprotein 2C [Source:ZFIN;Acc:ZDB-GENE-060526-233]	0.047701	2.71652
svopa	5	ENSDARG00000056833	SV2 related protein a [Source:ZFIN;Acc:ZDB- GENE-060526-336]	0.029467	2.887026
syndig1l	20	ENSDARG00000042390	synapse differentiation inducing 1-like [Source:ZFIN;Acc:ZDB-GENE-041001-116]	0.025806	3.247122
syngap1a	19	ENSDARG00000063713	synaptic Ras GTPase activating protein 1a [Source:ZFIN;Acc:ZDB-GENE-060503-370]	0.043006	2.271659
syt14a	13	ENSDARG00000010934	synaptotagmin XIVa [Source:ZFIN;Acc:ZDB-GENE- 090313-200]	0.033899	3.838162
syt17	3	ENSDARG00000060741	synaptotagmin XVII [Source:ZFIN;Acc:ZDB-GENE- 090527-1]	0.038558	3.261455
SYT2	6	ENSDARG00000014169	synaptotagmin 2 [Source:HGNC Symbol;Acc:HGNC:11510]	0.038307	3.275228
syt2a	23	ENSDARG00000025206	synaptotagmin IIa [Source:ZFIN;Acc:ZDB-GENE- 060503-315]	0.039566	2.454364
syt3	3	ENSDARG00000075830	synaptotagmin III [Source:ZFIN;Acc:ZDB-GENE- 090601-1]	0.037604	1.965598
tacc1	10	ENSDARG00000073753	transforming, acidic coiled-coil containing protein 1 [Source:ZFIN;Acc:ZDB-GENE-090521-5]	0.035384	2.311093

taok2b	12	ENSDARG00000100620	TAO kinase 2b [Source:ZFIN;Acc:ZDB-GENE- 110603-3]	0.023224	2.511349
tecpr1a	12	ENSDARG00000062515	tectonin beta-propeller repeat containing 1a [Source:ZFIN;Acc:ZDB-GENE-070112-1792]	0.046367	2.230658
tet1	13	ENSDARG00000075230	tet methylcytosine dioxygenase 1 [Source:ZFIN;Acc:ZDB-GENE-090312-88]	0.033669	2.709254
tgfbr1b	24	ENSDARG00000042259	transforming growth factor, beta receptor 1 b [Source:ZFIN;Acc:ZDB-GENE-091027-1]	0.038743	2.653082
thsd7ab	16	ENSDARG00000090496	thrombospondin, type I, domain containing 7Ab [Source:ZFIN;Acc:ZDB-GENE-130530-999]	0.039987	2.392967
tlnrd1	7	ENSDARG00000079017	talin rod domain containing 1 [Source:ZFIN;Acc:ZDB-GENE-130103-2]	0.036572	1.906602
TMEM164	21	ENSDARG00000113609	transmembrane protein 164 [Source:HGNC Symbol;Acc:HGNC:26217]	0.038307	2.815253
TMEM179 (1 of many)	17	ENSDARG00000090064	transmembrane protein 179 [Source:HGNC Symbol;Acc:HGNC:20137]	0.037971	2.432503
tmem200a	20	ENSDARG00000031540	transmembrane protein 200A [Source:ZFIN;Acc:ZDB-GENE-040724-81]	0.035384	3.25933
tmem8a	1	ENSDARG00000032795	transmembrane protein 8A [Source:ZFIN;Acc:ZDB-GENE-090312-158]	0.044042	2.076669
tmtc2b	18	ENSDARG00000035273	transmembrane and tetratricopeptide repeat containing 2b [Source:ZFIN;Acc:ZDB-GENE-081031-100]	0.046367	2.224964
tnr	2	ENSDARG00000020771	tenascin R (restrictin, janusin) [Source:ZFIN;Acc:ZDB-GENE-030804-1]	0.025806	2.233693
tns2a	23	ENSDARG00000076292	tensin 2a [Source:ZFIN;Acc:ZDB-GENE-090312- 163]	0.036572	2.571398
tomm20a	13	ENSDARG00000103163	translocase of outer mitochondrial membrane 20 [Source:ZFIN;Acc:ZDB-GENE-040426-1976]	0.029884	2.453486
traf2b	5	ENSDARG00000101036	Tnf receptor-associated factor 2b [Source:ZFIN;Acc:ZDB-GENE-030131-5345]	0.048376	1.596438
trhde.1	4	ENSDARG00000109358	thyrotropin releasing hormone degrading enzyme, tandem duplicate 1 [Source:ZFIN;Acc:ZDB-GENE- 170406-2]	0.048376	2.676737
trim2a	1	ENSDARG00000031817	tripartite motif containing 2a [Source:ZFIN;Acc:ZDB-GENE-050327-99]	0.02815	2.919746
trim35-19	3	ENSDARG00000099639	tripartite motif containing 35-19 [Source:ZFIN;Acc:ZDB-GENE-070424-178]	0.042916	-3.04136
TRIM67	20	ENSDARG00000108787	tripartite motif containing 67 [Source:HGNC Symbol;Acc:HGNC:31859]	0.025806	3.633135
trps1	19	ENSDARG00000104082	trichorhinophalangeal syndrome I [Source:ZFIN;Acc:ZDB-GENE-030131-8404]	0.045766	2.175044
tspan5b	1	ENSDARG00000070449	tetraspanin 5b [Source:ZFIN;Acc:ZDB-GENE- 041212-12]	0.046112	2.166691
ttbk2a	17	ENSDARG00000005670	tau tubulin kinase 2a [Source:ZFIN;Acc:ZDB- GENE-120928-3]	0.033899	2.43136
ube2ql1	19	ENSDARG00000079276	ubiquitin-conjugating enzyme E2Q family-like 1 [Source:ZFIN;Acc:ZDB-GENE-030131-8137]	0.037211	2.816916
uhrf1bp1	23	ENSDARG00000077011	UHRF1 binding protein 1 [Source:ZFIN;Acc:ZDB- GENE-090312-82]	0.032687	2.515567
unc5db	5	ENSDARG00000061541	unc-5 netrin receptor Db [Source:ZFIN;Acc:ZDB-GENE-060531-162]	0.033899	3.33612
unc80	6	ENSDARG00000098290	unc-80 homolog (C. elegans) [Source:ZFIN;Acc:ZDB-GENE-140106-167]	0.02814	2.268086
unm_sa1261	3	ENSDARG00000031618	un-named sa1261 [Source:ZFIN;Acc:ZDB-GENE- 120411-10]	0.033899	3.35371
usp2b	5	ENSDARG00000074120	ubiquitin specific peptidase 2b [Source:ZFIN;Acc:ZDB-GENE-100208-2]	0.037104	3.167489
vat1	3	ENSDARG00000056481	vesicle amine transport 1 [Source:ZFIN;Acc:ZDB-GENE-030616-178]	0.047064	1.862766
vdrb	6	ENSDARG00000070721	vitamin D receptor b [Source:ZFIN;Acc:ZDB- GENE-080403-10]	0.04869	3.127142

wbp1la	13	ENSDARG00000013245	WW domain binding protein 1-like a [Source:ZFIN;Acc:ZDB-GENE-030131-1961]	0.025927	2.409108
wdr47a	2	ENSDARG00000078136	WD repeat domain 47a [Source:ZFIN;Acc:ZDB- GENE-070216-3]	0.04738	2.313052
WDR7	21	ENSDARG00000100274	WD repeat domain 7 [Source:HGNC Symbol;Acc:HGNC:13490]	0.047701	2.397464
wwc1	21	ENSDARG00000076041	WW and C2 domain containing 1 [Source:ZFIN;Acc:ZDB-GENE-091118-8]	0.025927	2.904033
xkr5a	20	ENSDARG00000103017	XK related 5a [Source:ZFIN;Acc:ZDB-GENE- 160113-27]	0.037536	2.835559
xylt1	3	ENSDARG00000061248	xylosyltransferase I [Source:ZFIN;Acc:ZDB- GENE-111017-2]	0.043006	2.388446
xylt2	3	ENSDARG00000059557	xylosyltransferase II [Source:ZFIN;Acc:ZDB- GENE-081022-125]	0.02293	3.329557
zbtb22b	19	ENSDARG00000003251	zinc finger and BTB domain containing 22b [Source:ZFIN;Acc:ZDB-GENE-010110-4]	0.04738	2.58305
zc3h12b	5	ENSDARG00000062463	zinc finger CCCH-type containing 12B [Source:ZFIN;Acc:ZDB-GENE-060526-290]	0.025806	2.919102
zgc:103755	17	ENSDARG00000104663	zgc:103755 [Source:ZFIN;Acc:ZDB-GENE-041010- 101]	0.035384	2.295806
zgc:113278	23	ENSDARG00000037813	zgc:113278 [Source:ZFIN;Acc:ZDB-GENE-050327- 66]	0.038522	3.956078
zgc:122979	21	ENSDARG00000004187	zgc:122979 [Source:ZFIN;Acc:ZDB-GENE-051127- 45]	0.00943	2.594613
zgc:162872	15	ENSDARG00000012184	zgc:162872 [Source:ZFIN;Acc:ZDB-GENE-081022- 1]	0.047559	2.04712
zgc:165508	15	ENSDARG00000104296	zgc:165508 [Source:ZFIN;Acc:ZDB-GENE-070719- 4]	0.033899	2.485048
zmat3	11	ENSDARG00000080021	zinc finger, matrin-type 3 [Source:ZFIN;Acc:ZDB-GENE-130530-881]	0.030646	2.815121
zmp:0000001168	7	ENSDARG00000076697	zmp:0000001168 [Source:ZFIN;Acc:ZDB-GENE- 140106-128]	0.016296	3.369014
znf385a	23	ENSDARG00000009899	zinc finger protein 385A [Source:ZFIN;Acc:ZDB-GENE-090313-255]	0.025297	3.253546
znf704	19	ENSDARG00000061718	zinc finger protein 704 [Source:ZFIN;Acc:ZDB- GENE-060503-775]	0.024013	3.076908

PFOS70

si:ch211-93g21.1	21	ENSDARG00000093602	si:ch211-93g21.1 [Source:ZFIN;Acc:ZDB-GENE- 060503-703]	0.000159	11.91576
anxa1a	5	ENSDARG00000026726	annexin A1a [Source:ZFIN;Acc:ZDB-GENE-030131- 6664]	0.049262	-3.03023

POP10

egr1	14	ENSDARG00000037421	early growth response 1 [Source:ZFIN;Acc:ZDB- GENE-980526-320]	6.27E-05	1.742813
npas4a	14	ENSDARG00000055752	neuronal PAS domain protein 4a [Source:ZFIN;Acc:ZDB-GENE-060616-396]	0.007075	3.952055
ier2a	3	ENSDARG00000099195	immediate early response 2a [Source:ZFIN;Acc:ZDB-GENE-030131-9126]	0.033076	2.466302
egr2b	12	ENSDARG00000042826	early growth response 2b [Source:ZFIN;Acc:ZDB-GENE-980526-283]	0.033076	2.118679
si:dkey-172k15.4	4	ENSDARG00000097086	si:dkey-172k15.4 [Source:ZFIN;Acc:ZDB-GENE- 131127-210]	0.040572	2.726539

POP70

nr4a1	23	ENSDARG00000000796	nuclear receptor subfamily 4, group A, member 1 [Source:ZFIN;Acc:ZDB-GENE-040704-11]	1.41E-05	4.972721
npas4a	14	ENSDARG00000055752	neuronal PAS domain protein 4a [Source:ZFIN;Acc:ZDB-GENE-060616-396]	0.001161	3.851462
egr1	14	ENSDARG00000037421	early growth response 1 [Source:ZFIN;Acc:ZDB- GENE-980526-320]	0.003404	1.563269
sik1	9	ENSDARG00000058606	salt-inducible kinase 1 [Source:ZFIN;Acc:ZDB- GENE-030131-9446]	0.008434	1.744598
adgrb1b	19	ENSDARG00000078529	adhesion G protein-coupled receptor B1b [Source:ZFIN;Acc:ZDB-GENE-081031-45]	0.009049	1.633312
anxa1a	5	ENSDARG00000026726	annexin A1a [Source:ZFIN;Acc:ZDB-GENE-030131- 6664]	0.011559	-3.01277
fosl1a	14	ENSDARG00000015355	FOS-like antigen 1a [Source:ZFIN;Acc:ZDB- GENE-061207-7]	0.011559	2.240446
egr2a	17	ENSDARG00000044098	early growth response 2a [Source:ZFIN;Acc:ZDB-GENE-030723-6]	0.011559	2.039245
zgc:122979	21	ENSDARG00000004187	zgc:122979 [Source:ZFIN;Acc:ZDB-GENE-051127- 45]	0.011559	2.806659
c3b.2	22	ENSDARG00000001818	complement component c3b, tandem duplicate 2 [Source:ZFIN;Acc:ZDB-GENE-030131-3063]	0.011559	1.980835
si:dkey-79d12.5	16	ENSDARG00000040284	si:dkey-79d12.5 [Source:ZFIN;Acc:ZDB-GENE- 131127-429]	0.014356	2.003098
egr2b	12	ENSDARG00000042826	early growth response 2b [Source:ZFIN;Acc:ZDB-GENE-980526-283]	0.031529	2.140015
rtn4rl2a	1	ENSDARG00000052012	reticulon 4 receptor-like 2 a [Source:ZFIN;Acc:ZDB-GENE-040310-4]	0.043069	2.032351

Table S4. Genes common between conditions. FC, fold change

	PFOS10 FC	PFOS70 FC	POP10 FC	POP70 FC
Common genes in PFOS10 and POP10				
ier2a	2.10		2.47	
Common genes in PFOS10, POP10 and POP70				
egr2b	1.93		2.12	2.14
npas4a	3.27		3.95	3.85
Common genes in POP10 and POP70				
egr1			1.74	1.56
Common genes in PFOS70 and POP70				
anxa1a		-3.03		-3.01
Common genes in PFOS10 and POP70				
fosl1a	1.80			2.24
rtn4rl2a	1.88			2.03
egr2a	1.88			2.04
zgc:122979	2.59			2.81
nr4a1	3.48			4.97

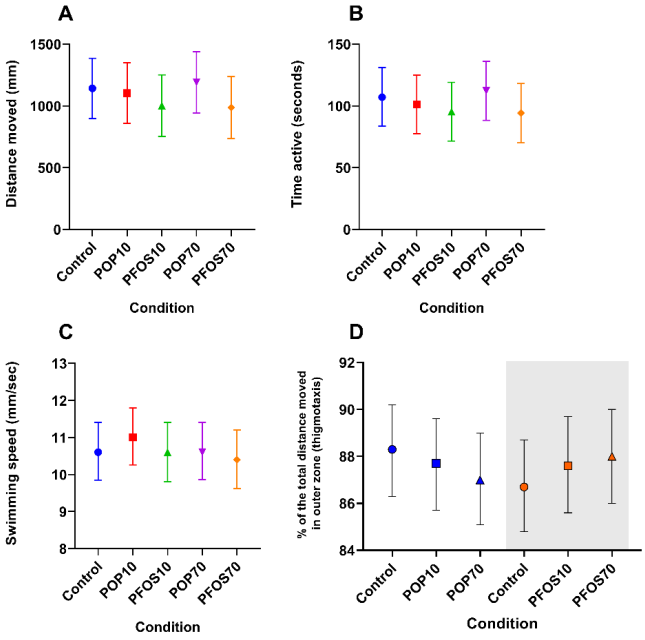

Figure S5. Responses following the light-dark transition (A-C), and the thigmotaxis test (D) of F larval zebrafish originating from POP10, POP70, PFOS10 and PFOS70 F0 adults. **A)** Distance moved, **B)** Time spent active **C)** Swimming speed **D)** percent of total distance moved in outer zone. Data shown are least square means ± 95% CI

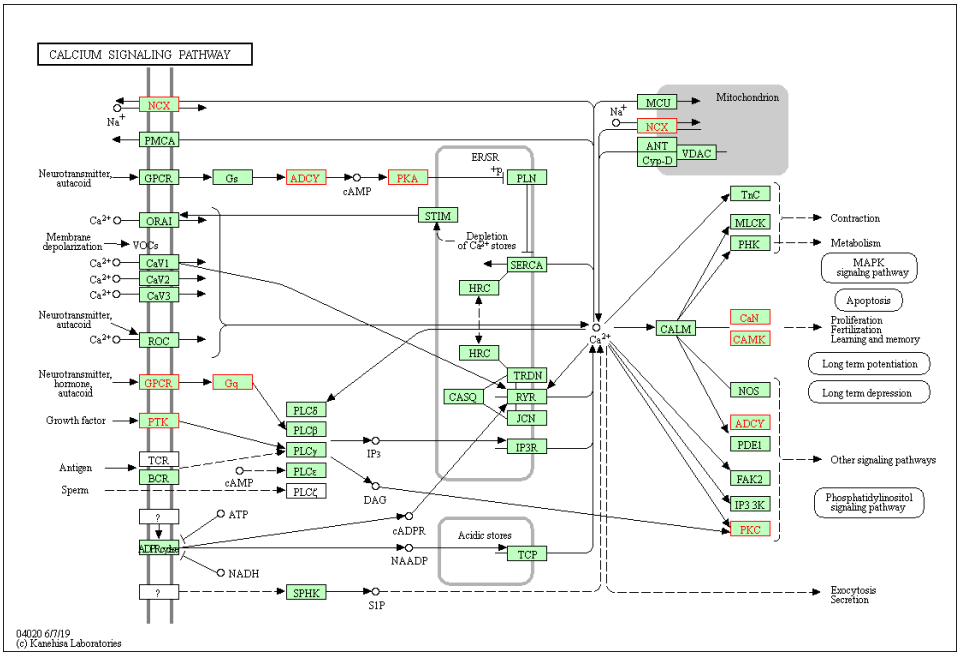

Figure S6. Enrichment of calcium signaling pathway in the brains of PFOS10 adults. Components in red represent DEG in the PFOS10 dataset. Pathway was created in KEGG

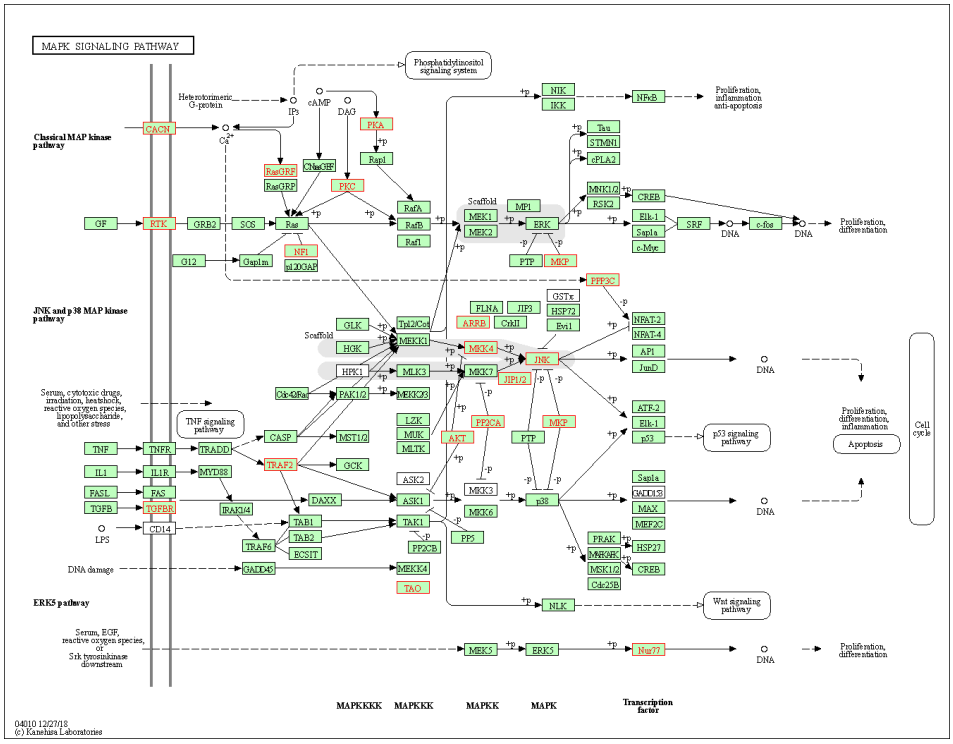

Figure S7. Enrichment of MAPK signaling pathway in the brains of PFOS10 adults. Components in red represent DEG in the PFOS10 dataset. Pathway was created in KEGG

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

108233 / ANDVORDGRAFISK.NO