“Evaluating the effects of mixtures of endocrine disruptors on development and gene expression in zebrafish larvae (*Danio rerio*)”

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Te he escrito una elegía, pero he preferido dedicarte esto.
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And if you managed to get this far, thanks to you too! I hope that you are doing well, and the next part is just plain ridiculous, you may skip it. If you continue reading, do not say that I didn't warn you. This last line… I hope the last line makes you smile for its simplicity and absurdity. Kudos if you know exactly where this is coming from!

No man can eat fifty eggs (Tré Cool, 2005, Green day, Bullet in a Bible)

"*I do have reasons for hope: Our clever brains, the resilience of nature, the indomitable human spirit, and above all, the commitment of young people when they're empowered to take action*."

- Jane Goodall

Abstract

Persistent organic pollutants (POPs) and endocrine-disrupting chemicals (EDCs) represent significant environmental and health threats due to their stability, bioaccumulation, and complex interactions in biological systems. This thesis synthesizes the findings from three research publications that investigate the developmental, physiological, and behavioral effects of POP and EDC exposure using zebrafish (*Danio rerio*) as a model organism. Zebrafish larvae were exposed to a mixture of 29 POPs, at different concentrations based on what was found in the blood of Scandinavian populations, to study the effects of these chemicals on growth, skeletal development, behavior, mitochondrial function, and gene expression.

Across the studies, zebrafish larvae exposed to POP mixtures exhibited significant skeletal and craniofacial deformities. Developmental issues in cartilage and bone formation were identified, including reduced size of structures such as Meckel's cartilage and disrupted bone mineralization. The disruption of bone formation was linked to interactions with nuclear receptor pathways, such as androgen, vitamin D, and retinoic acid signaling. Exposure to the POP mixtures also led to mitochondrial toxicity, with more pronounced effects in those mixtures containing per- and polyfluorinated acids (PFAAs). Increased mitochondrial activity, indicative of oxidative stress, was observed.

POP exposure caused pronounced behavioral changes, particularly in response to light-dark transitions (startle response). Hyperactivity was observed in zebrafish exposed to mixtures containing PFAAs, and the presence of brominated (Br) and chlorinated (Cl) compounds seemed to modulate these effects. Cardiac function was also significantly affected in all treatments. Furthermore, transcriptomic analysis revealed substantial changes in gene expression following exposure to POP mixtures. Genes involved in insulin signaling, brain and eye development, and stress responses were significantly dysregulated. The most notable molecular finding was the inhibition of the condensin I complex, which is crucial for retinal development. This inhibition led to microphthalmia (small eyes) or pear-like shape in exposed zebrafish larvae, providing insights into the mechanisms underlying POP-induced developmental defects.

Finally, using the CRISPR/Cas9 technology, we generated specific mutants to explore the role of estrogen receptors (Esr1, Esr2b, and Gper1) in mediating the effects of EDCs (antagonist effects) on zebrafish development. Each receptor was found to play distinct roles in regulating skeletal, cardiac, and behavioral outcomes. Zebrafish mutants with inactivated Esr1 exhibited the most severe craniofacial deformations that were seen in 70% of the population, highlighting the role of estrogen receptors in regulating skeletal development. Zebrafish *esr2b* mutants had increased heart rates, further highlighting the role of estrogen signaling in regulating cardiovascular development and function. Finally, the *gper1* mutants displayed reduced activity and skeletal mineralization, emphasizing the complexity of estrogen receptor-mediated developmental processes.

The findings from this research highlights the significant risks posed by exposure to POP mixtures, particularly in terms of skeletal and retinal development, behavior, and endocrine disruption. Our results highlight the need for further mechanistic studies to better understand the long-term impacts of POP exposure on both environmental and human health. However, these results provide critical insights into the molecular mechanisms of POP toxicity, with important implications for environmental risk assessment and public health policy. The collective findings emphasize the importance of considering chemical mixtures rather than individual compounds, as their combined effects seemed to be often more severe and complex.

Résumé

Les polluants organiques persistants (POPs) et les perturbateurs endocriniens (EDCs) représentent des menaces environnementales et sanitaires importantes en raison de leur stabilité, de leur bioaccumulation et de leurs interactions complexes dans les systèmes biologiques. Cette thèse synthétise les résultats de trois publications de recherche qui examinent les effets développementaux, physiologiques et comportementaux de l'exposition aux POPs et aux EDCs en utilisant le poisson zèbre (*Danio rerio*) comme organisme modèle. Les larves de poisson zèbre ont été exposées à un mélange de 29 POPs, à différentes concentrations basées sur celles trouvées dans le sang des populations scandinaves, afin d'étudier les effets de ces produits chimiques sur la croissance, le développement squelettique, le comportement, la fonction mitochondriale et l'expression génique.

Dans l'ensemble des études, les larves de poisson zèbre exposées à des mélanges de POPs ont présenté des déformations squelettiques et crânio-faciales significatives. Des problèmes de développement dans la formation du cartilage et des os ont été identifiés, notamment une réduction de la taille de structures telles que le cartilage de Meckel et une minéralisation osseuse perturbée. La perturbation de la formation osseuse a été liée à des interactions avec les voies des récepteurs nucléaires, telles que la signalisation des androgènes, de la vitamine D et de l'acide rétinoïque. L'exposition aux mélanges de POPs a également conduit à une toxicité mitochondriale, avec des effets plus marqués dans les mélanges contenant des acides per- et polyfluorés (PFAAs). Une activité mitochondriale accrue, indicatrice d'un stress oxydatif, a été observée.

L'exposition aux POPs a provoqué des changements comportementaux prononcés, notamment en réponse aux transitions lumière-obscurité (réponse de sursaut). Une hyperactivité a été observée chez les poissons zèbres exposés à des mélanges contenant des PFAAs, et la présence de composés bromés (Br) et chlorés (Cl) semblait moduler ces effets. La fonction cardiaque a également été significativement affectée dans tous les traitements. En outre, une analyse transcriptomique a révélé des changements importants dans l'expression génique suite à l'exposition à des mélanges de POPs. Des gènes impliqués dans la signalisation de l'insuline, le développement du cerveau et des yeux, ainsi que les réponses au stress, ont été significativement dérégulés. La découverte moléculaire la plus notable a été l'inhibition du complexe condensine I, essentiel pour le développement rétinien. Cette inhibition a conduit à une microphtalmie (petits yeux) ou à une forme en poire chez les larves de poisson zèbre exposées, fournissant des informations sur les mécanismes sous-jacents des défauts développementaux induits par les POP.

Enfin, grâce à la technologie CRISPR/Cas9, des mutants spécifiques ont été générés pour explorer le rôle des récepteurs aux œstrogènes (Esr1, Esr2b et Gper1) dans la médiation des effets des EDC (effets antagonistes) sur le développement du poisson zèbre. Ces études ont montré que chaque récepteur—joue des rôles distincts dans la régulation des conséquences squelettiques, cardiaques et comportementales. Les mutants de poisson zèbre avec Esr1 inactivé ont présenté les déformations crânio-faciales les plus sévères, observées chez 70 % de la population, soulignant le rôle des récepteurs aux œstrogènes dans la régulation du développement squelettique. Les mutants *esr2b* de poisson zèbre présentaient des fréquences cardiaques accrues, soulignant encore une fois le rôle de la signalisation des œstrogènes dans la régulation du développement et de la fonction cardiovasculaire. Enfin, les mutants *gper1* ont montré une réduction de l'activité et de la minéralisation squelettique, ce qui souligne

la complexité des processus développementaux contrôlés par les récepteurs aux œstrogènes.

Les résultats de cette recherche soulignent les risques significatifs posés par l'exposition aux mélanges de POP, en particulier en ce qui concerne le développement squelettique et rétinien, le comportement et la perturbation endocrinienne. Nos résultats mettent en évidence la nécessité d'études mécanistiques supplémentaires pour mieux comprendre les impacts à long terme de l'exposition aux POP sur la santé environnementale et humaine. Cependant, ces résultats fournissent des informations essentielles sur les mécanismes moléculaires de la toxicité des POP, avec des implications importantes pour l'évaluation des risques environnementaux et les politiques de santé publique. Collectivement, les résultats soulignent l'importance de prendre en compte les mélanges chimiques plutôt que les composés individuels, car leurs effets combinés semblent souvent plus graves et complexes.

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List of abbreviations

Abbreviation	Definition
aa	Aminoacids
ACTH	Adreno-corticotropic hormone
ADI	Acceptable daily intake
ADME	Adsorption, distribution, metabolism, excretion
AhR	Aryl hydrocarbon receptor
AR	Androgen receptors
arh2 (gene)	Aryl-hydrocarbon receptor gene
B-TC-6	Beta-tumor cell-6
BaP	Benzo a pyrene
BMD	Benchmark dose
BMDL	Benchmark dose lower confidence limit
BMI	Body mass index
BMP	Bone morphogenetic proteins
BMS 189456	(E)-4-[2-(5,6-Dihydro-5,5-dimethyl-8-phenyl-2-naphthalenyl)ethenyl]-benzoic acid
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
Br	Mixture of brominated compounds
Br + Cl	Binary mixture of brominated and chlorinated compounds
C/EBPα	Member of the CCAAT-enhancer-binding proteins (C/EBPs) family that regulates gene expression in various tissues and cells
cAMP	Cyclic adenosine monophosphate
Cl	Mixture of chlorinated compounds
cNCC	Cranial neural crest cells
COV434	Immortalized granulosa cell line
CRH	Corticotropin-releasing hormone

CRISPR	Clustered, regulatory interspaced, short palindromic repeats
cyp19a1a (gene)	Ovarian aromatase gene
cyp19a1b	Brain Aromatase b
CYP360A8	Cytochrome P450 clan 3 gene
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
dl-PCBs	Dioxin-like pcbs
dpf	Days post-fertilization
DR-H4IIE	Rat hepatoma
DR-Hep G2	Human hepatoma
DR-T47-D	Human mammary gland carcinoma
E2	17β-estradiol
EC	European commission
ECHA	European chemicals agency
EDCs	Endocrine-disrupting chemicals
EDQM	European Directorate for the Quality of Medicines and Healthcare
EE2	17α-ethinylestradiol
EFSA	European food safety agency
EMA	European medicines agency
ER	Estrogen receptors
ERE	Estrogen response elements
FABP3	Fatty acid-binding protein 3
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GC	Granulosa cells
GFP	Green fluorescent protein
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GPCRs	G protein-coupled receptors

GPER1	G protein-coupled estrogen receptor 1
GR	Glucocorticoid receptor
HBCDD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCBD	Hexachlorobutadiene
HCH	Hexachlorocyclohexane
HeLa9903	HeLa cell line stably transfected with two constructs: the hER alpha expression construct; a firefly luciferase reporter construct bearing Estrogen-Responsive Element (ERE) driven by a mouse metallothionein (MT) promoter TATA element
HepG2	Human hepatocyte cells
hERx	Human estrogen receptor
hpf	Hours post-fertilization
HRE	Hormone response element
ICI 182,780	Fulvestrant
IGR1R	Insulin-like growth factor 1 receptor
Ins (gene)	Insulin
INS-1E	Cellosaurus INS-1E (CVCL_0351), cancer cell line
KGN	Human granulosa-like tumor cells
klf17 (gene)	Krüpple-like factor 17
LBD	Ligand binding domain
LH	Luteinizing hormone
LOAEL	Lowest observed adverse effect level
MCF-7	Breast cancer cell line, MCF-7 is the acronym of Michigan Cancer Foundation-7
mGLUR	Metabotropic glutamate receptors
MoA	Mechanisms of Action
mouse 3T3-L1	Sub clonal cell line derived from the original 3T3 Swiss albino cell line of 1962
NOAEL	No observable adverse effect level

NR	Nuclear receptors
o,p’-DDT	Isomer of dichlorodiphenyltrichloroethane, an organochlorine insecticide. 1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane is a diarylmethane
OCDD	Octachlorodibenzodioxin
OCP	Organochlorine pesticides
OECD	Organization for Economic Co-operation and Development
p,p’ DDT	Chlorophenothane
p,p'-DDE	Dichlorodiphenyldichloroethylene
PAH	Polycyclic aromatic hydrocarbon
PBDE	Polybrominated diphenyl ethers
PC12	Cell line derived from a pheochromocytoma of the rat adrenal medulla,
PCB	Polychlorinated biphenyls
PCB-118	2,3',4,4',5-Pentachlorobiphenyl is one of 209 polychlorinated biphenyls (PCBs)
PCB-138	2,2',3,4,4',5'-Hexachlorobiphenyl is one of 209 polychlorinated biphenyls (PCBs)
PCB-153	2,2',4,4',5,5'-Hexachlorobiphenyl is one of 209 polychlorinated biphenyls (PCBs)
PCDD/F	Dioxins and dioxin-like compounds
PeCB	Pentachlorobenzene
PFAA	Mixture of perfluorinated compounds
PFAA + Br	Binary mixture of perfluorinated and brominated compounds
PFAA + Cl	Binary mixture of perfluorinated and chlorinated compounds
PFAS	Per- and poly-fluoroalkylated substances
PFDA	Perfluorodecanoic acid

PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
PHT	Parathyroid hormone
POP	Persistent organic pollutants
PPAR	Peroxisome proliferator-activated receptors
PPARγ	Peroxisome proliferator-activated receptor gamma, also known as the glitazone reverse insulin resistance receptor, or NR1C3 is a type II nuclear receptor functioning as a transcription factor that in humans is encoded by the PPARG gene
PRL	Prolactin
PTFE	Polytetrafluoroethylene
RA	Retinoic acid
RAR	Retinoic acid receptors
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RfC	Reference concentration
RfD	Reference dose
RNA-Seq	RNA sequencing
RyR1	Ryanodine receptor
SC	Scientific committees
sgRNA	Guide RNA
T1D	Type 1 diabetes
T2D	Type 2 diabetes
T3	Triiodothyronine
T4	Thyroxine
TALENs	Transcription activator-like effector nucleases
TBT	Tributyltin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDI	Tolerable daily intake
TeBDE	Bde-47

TEF	Toxic equivalency factor
TILLING	Targeting induced local lesions in genomes
TLV	Tlv
TPM	Total pop mixture
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
U2OS-GR	U2OS cells expressing human glucocorticoid receptors
VDR	Vitamin D receptor
Vtg	Vitellogenin
WHO	World health organization
WHO-TEQ	WHO Toxic equivalency factor
zfERx	Zebrafish estrogen receptor

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Fig 5 Synopsis of the nuclear receptor family described in zebrafish. This figure has been taken from Schaaf (adapted from Bertrand et al & Zhao et al). Different sub-classes are shown, defined by their specific DNA-binding domains and their ligands. In blue are shown the strict steroid receptors, which bind DNA as homodimers, while the violet group represents receptors binding to more diverse ligands (TR, RAR, VDR) which predominantly bind DNA as heterodimers with RXRs. In green, receptors that bind either as homodimers or heterodimers (RXR).

Fig 6 Structural organization of nuclear receptors. Taken from Jayaprakash et al.

Fig 7 Structural regions of nuclear estrogen receptors. Taken from Yaşar and colleagues. There are 595 amino acids in human estrogen receptor α,

and 530 amino acids form ERβ. Up to 17% amino-acid identity is shared between ERs for the structurally distinct amino terminal A/B domains; their C central regions (DNA binding domain) are almost identical sharing up to 97% of identity. Regarding the D domain (hinge), there is 56% homology between ERs, this domain contains a nuclear localization signal linking the C domain to the multifunctional carboxyl terminal (E), which is responsible for binding the estrogenic compounds. Finally, the remaining short carboxyl-terminal F domain shares 18% amino-acid identity. The ERs are dimers with or without the endogenous ligand.

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1. Preface

The trajectory of human progress has been intricately entwined with our ability to harness the forces of nature. Whether it be unlocking the potential of electricity in physics, unraveling the intricacies of chemistry and biology to understand diseases and develop treatments, or delving into the vast realm of synthetic compounds, every stride forward comes with its own set of consequences. In the modern era, our journey is closely linked to the environmental challenges posed by the increasing exposure to toxicants. A staggering reality emerges when considering that the chemical industry churns out over 10 million compounds annually[1]. As a global community, we are gradually awakening to the profound impact our actions have on the world, both in the short and long term. The severity of pollution is such that contaminants permeate regions once considered pristine, highlighting the far-reaching consequences of our toxic footprint[2,3].

2. Introduction

2.1 Persistent Organic Pollutants

Persistent Organic Pollutants (POPs) are a class of toxic organic substances characterized by their remarkable resistance to environmental degradation, their capacity for bioaccumulation, and their potential for long-range atmospheric transport[4,5]. These compounds, found in various forms, exhibit a high degree of persistence, leading to their omnipresence in the environment and continuous exposure for both humans and wildlife[6,7].

These pollutants, including but not limited to polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polybrominated diphenyl ethers (PBDEs), and per- and poly-fluoroalkylated substances (PFAS), have infiltrated diverse products such as plasticizers, pharmaceuticals, pesticides, and industrial chemicals[4].

Furthermore, a significant portion of the human population is exposed to POPs through the consumption of contaminated food[8–11]. PCBs, HCH, PBDEs and other POPs have been repeatedly found in food in different regions around the world at

different concentrations, some below the safety thresholds like the values reported of DDTs (25.6 pg/g wet wt), HCHs (13 pg/g wet wt), and PCBs (468 pg/g wet wt) in goose liver in Hong Kong[8] others exceeded the safety levels established by the local authorities, as was the case in Belgium back in 1999, where 50 kg of PCBs contaminated with 1 g of dioxins were accidentally added to animal food precursors resulting in contamination of meat and other products (Belgium had established values on the range 100, 200 and 1000 ng/g in milk, meat and animal feed respectively of PCDD/F)[12], or the dioxins detected in Baltic Sea fish in Estonia where authors reported values in 7.5-year-old herring and 5.3 year-old sprat at 9.65 pg WHO-TEQ/g fresh weight (EU limit is 8.0 pg WHO-TEQ/g fresh weight)[10]. These events show that even banned compounds, such as PCBs, continue to impact the environment through leakage from outdated equipment and building materials.

Ongoing use of certain OCPs in specific regions, coupled with the inadvertent production of chemicals like hexachlorobenzene (HCB) during manufacturing processes, adds to the complexity of the POP landscape. The production of building materials, for instance, has become the main source of PCBs and HCBs in places like Odessa, Ukraine, and the main source of air pollution in the area[13]. Also, brick and zinc production, as well as waste incineration can produce polychlorinated dibenzo-*p*-dioxins/furans (PCDD/Fs), dioxin-like PCBs (dl-PCBs), HCB and pentachlorobenzene (PeCB). Recently, PCDD/Fs and dl-PCBs have been found in unintentional emissions from a production plant in Northern Vietnam, at concentrations ranging from 0.034 to 50.55 pg/Nm^3 TEQs (Toxic Equivalents to dioxin) in flue gas samples and 1.43 to 440 pg/g TEQs in fly ash samples, respectively[14].

Notably, the consequences of POP exposure extend beyond the immediate health impacts, with emerging evidence pointing to their role as endocrine-disrupting chemicals (EDCs). In brief, this designation signifies their ability to interfere with the endocrine system, potentially causing adverse health effects in both animals and humans. Early-life exposure to EDCs is particularly concerning,

given its association with long-term impacts on cardiovascular, metabolic, or reproductive functions, as well as behavior and more[15–19]. Further details will be discussed later.

In essence, POPs exemplify a complex amalgamation of compounds that persist in the environment, presenting challenges for comprehensive research on mixture toxicity and emphasizing the critical need for a holistic understanding of their diverse effects on human and environmental health.

2.2 History

The utilization of technologies for human progress has been a constant since the earliest stages of civilization. While the manufacturing of Persistent Organic Pollutants (POPs) is a relatively recent development, their purpose can be traced back to ancient compounds produced by alchemists such as wood vinegar and Tar as pest control[20], making them almost as old as the early stages of civilization and tightly linked to agricultural societies.

The intricate history of POPs unfolds from the late 19th century to the present day, from the discovery of chlorinated chemicals like polychlorinated biphenyls (PCBs) and their widespread manufacturing from the 1930s[21] to the banning of many of these compounds in the 1970s and 1980s due to their detrimental health effects. The narrative of POPs reveals a persistent struggle to balance the benefits of technological innovation with its environmental impacts. The inclusion of brominated compounds and perfluoroalkyl and polyfluoroalkyl substances (PFAS) in the list of POPs further emphasizes the ongoing challenges in managing these pollutants.

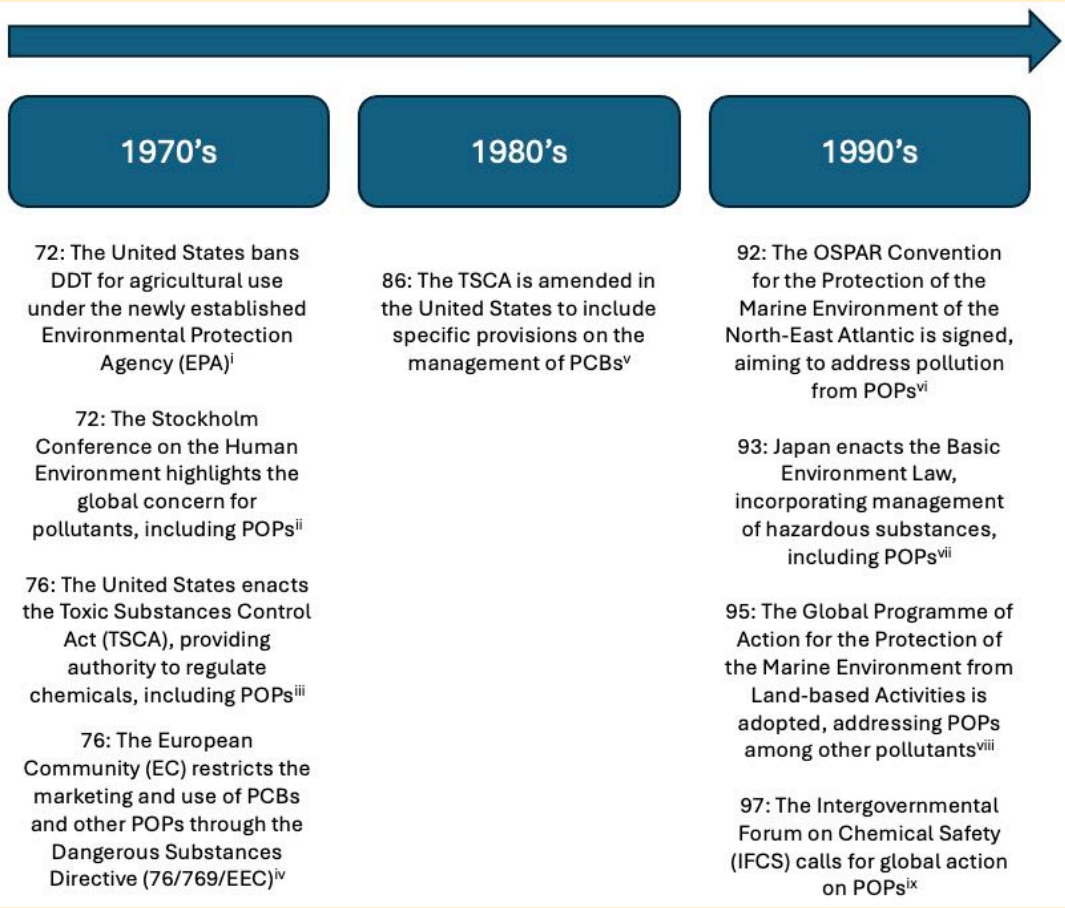
2.3 Regulation

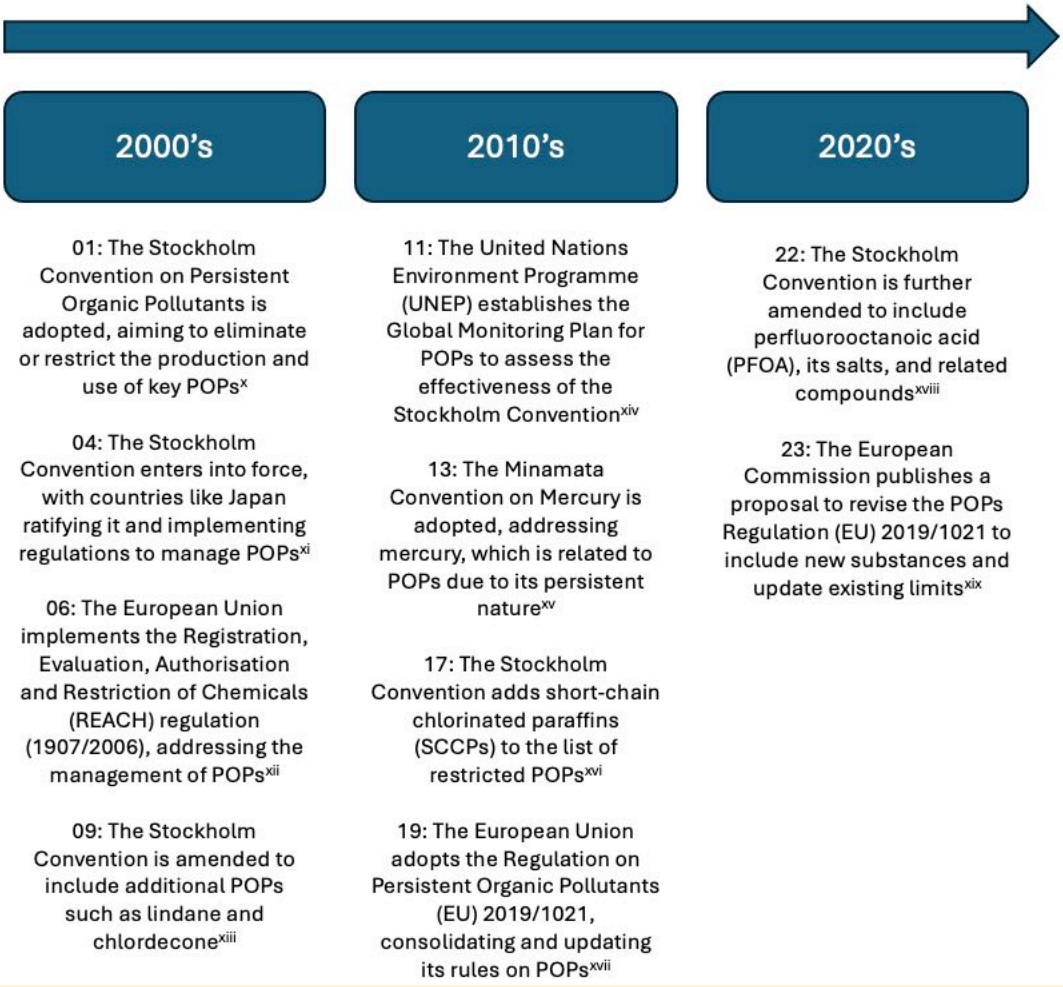
In today's world, such are the impacts caused by these chemicals that treaties, conventions, and other kinds of regulations have been implemented to either stop their production and use entirely, or to regulate their usage under specific criteria. This is the case of the 20 years old, iconic "Stockholm Convention" on

persistent organic pollutants, published in May 2001 and entered into force in May 2004. Several amendments have been made throughout the years, to consider new findings and to add new products developed by industry. The original Stockholm Convention listed a set of 12 compounds that were known as the "Dirty Dozen" (see: https://chm.pops.int/TheConvention/ThePOPs/tabid/673/Default.aspx).

Throughout the decades, international cooperation and agreements, completing and extending the Stockholm Convention, have played a significant role in shaping the regulation of POPs globally (Fig. 1). These regulations continue to evolve as new scientific evidence emerges and more countries impose stricter regulations to the production, banning and control of these substances[22–24]. Nevertheless, despite regulatory efforts to curtail the production of certain POPs, recent studies indicate their continued usage and presence in specific regions, underscoring the enduring nature of these environmental contaminants[25].

Figure 1. Timeline with (some) milestones that aided the development of POP policies on a global and regional scale, in order to mitigate the risks posed by these hazard chemicals.





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In the European Union, several organizations collaborate to create a comprehensive regulatory chemical safety framework. In a nutshell, the guidelines and frameworks to toxicology and safety of chemicals, pharmaceuticals, and other products are generated by the European Medicines Agency (EMA, see: https://www.ema.europa.eu/en/homepage), European Chemicals Agency (ECHA), European Food Safety Agency (EFSA, see: https://www.echa.europa.eu/), European Commission (EC, see: https://commission.europa.eu/energy-climate-change-environment_en), Scientific Committees (SC, see: https://health.ec.europa.eu/scientific-committees_en), European Directorate for the Quality of Medicines and Healthcare (EDQM, see: https://www.edqm.eu/en/), as well as competent authorities in Member States. Together (not necessarily all contributing to it), they have set up a handful of important concepts within the framework of regulatory toxicology (Table 1).

Toxicity Equivalency Factor (TEF)

Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) has been used as a reference to create this benchmark. It is used in risk assessment to estimate the toxicity of single compounds and complex mixtures, most commonly mixtures of chlorinated dibenzo-p-dioxins, furans, and biphenyls (Aroclors), relative to that of dioxin[26]. TEFs strictly apply only to dioxins and dioxin-like compounds, but they could be theoretically applied to other groups of chemicals whose actions can be represented by a reference compound.

No Observable Adverse Effect Level (NOAEL)

This concept defines the highest exposure level at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects[26,27]

Lowest Observed Adverse Effect Level (LOAEL)

This concept defines the lowest exposure level at which there are no-statistically nor biologically significant increases in the frequency or severity of adverse effects[26,27]

Acceptable daily intake (ADI)

Another important concept that defines the maximum amount of a chemical that can be ingested daily over a lifetime without compromising health[26,27]

Tolerable daily intake (TDI)

This is similar to the above ADI, but applied to pollutants[26]

Benchmark dose (BMD) and Benchmark Dose Lower Confidence Limit (BMDL)

A dose that produces a predefined change in responses of an adverse effect compared to background. BMDL is the lower confidence limit of this dose[26,28].

Threshold limit values (TLV)

These are guidelines to exposure limits in workers. Strictly speaking, this is a concept of the American Conference of Governmental Industrial Hygienists (ACGIH)[26]

Reference dose (RfD) and reference concentration (RfC)

Daily exposure that is likely to be without appreciable risk of harmful effects[26]

Table 1. Non-exhaustive list of examples of the regulatory concepts applied to POPs

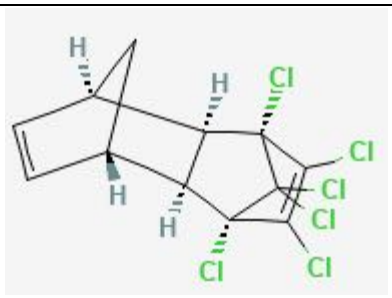
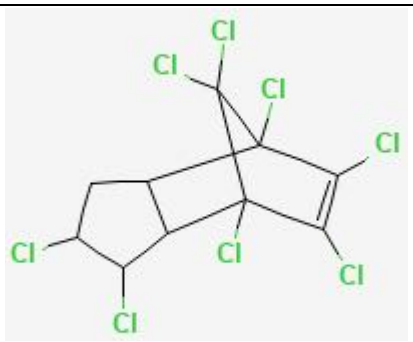
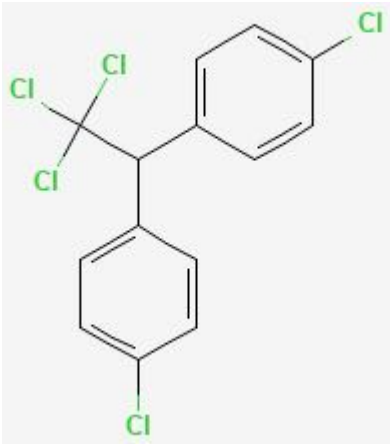
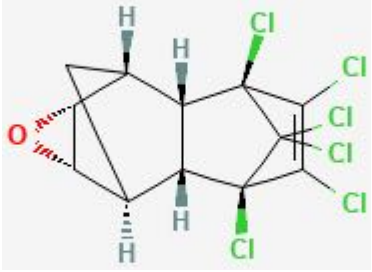
Regulatory concept	**Chemical example / value**	**Ref**
TEF	Pentachlorodibenzofuran 1,2,3,7,8-Cl5DF: (0.01 **WHO-TEQ$_{DFP}$**)	WHO[29]
NOAEL and LOAEL	Aldrin: NOAEL is 25.5 mg/kg/day and LOAEL is 6.1 mg/kg body weight/day	ATSDR[30]
ADI	DDT and metabolites: 10,000 ng/kg body weight/day	IPCS/WHO[31]
TDI	PFOS: 0.02 µg/kg	NHMRC[32]
BMDL	BDE-47: Lowest BMDL$_{10}$ for reproductive effects is: 0.023 mg/kg body weight per day	EFSA[33]

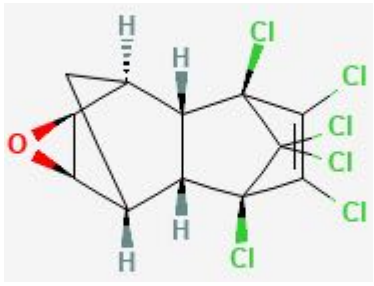
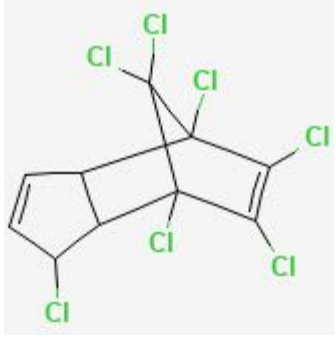
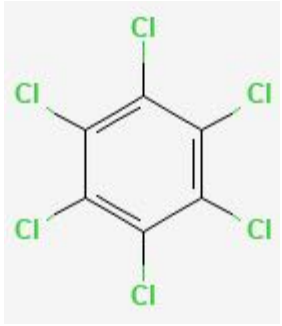
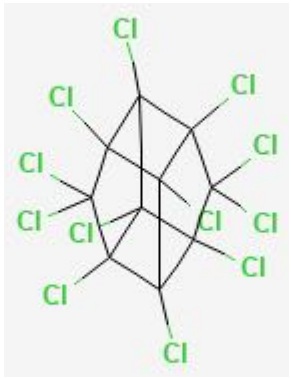
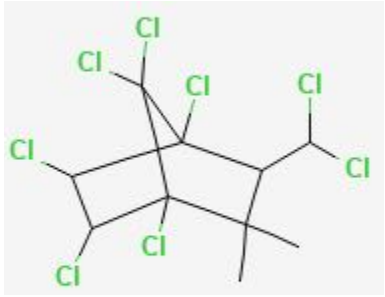
2.4 Diversity and categories of POPs

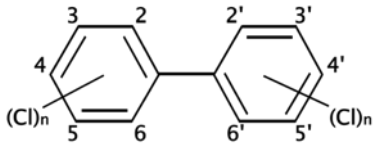
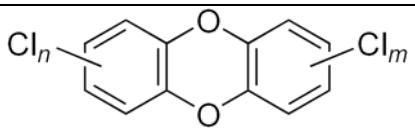
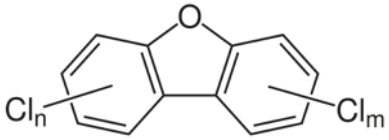
POPs are usually divided into three categories: Pesticides, Industrial and Unintentional[4,5].

Pesticides are "*any substance, or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest, or regulating plant growth*". In the Stockholm convention (Table 2), 16 chemicals have been listed in Annex A, thus, parties that signed the treaty in 2001 have pledged to eliminate them. Some of these compounds are Chlordane, Dieldrin, α-Hexachlorocyclohexane, β-Hexachlorocyclohexane, Lindane, Hexachlorobenzene, etc[34].

Table 2. Original list of the “dirty dozen”.

Name	Structure	CAS	Molecular formula
Aldrin		309-00-2	$C_{12}H_8Cl_6$
Chlordane		57-74-9	$C_{10}H_6Cl_8$
DDT		50-29-3	$C_{14}H_9Cl_5$
Dieldrin		60-57-1	$C_{12}H_8Cl_6O$

Endrin		72-20-8	$C_{12}H_8Cl_6O$
Heptachlor		76-44-8	$C_{10}H_5Cl_7$
Hexachlorobenzene (HCB)		118-74-1	C_6Cl_6
Mirex		2385-85-5	$C_{10}Cl_{12}$
Toxaphene		8001-35-2	$C_{10}H_{10}Cl_8$

Polychlorinated biphenyls (PCB)		Various	$C_{12}H_{10-x}Cl_x$
Polychlorinated dibenzo-p-dioxins (PCDD)		Various	$C_{12}H_xCl_xO_2$
Polychlorinated dibenzofurans (PCFD)		Various	$C_{12}H_xCl_xO$

Similarly, the United States Code in its 2012 edition describes a pesticide as "a substance or mixture of substances with the intention to prevent, destroy, repel, or mitigate any kind of pest". This includes combatting insects and other predators, but also plant regulators, defoliants, desiccants, or nitrogen stabilizers (see https://tinyurl.com/yfvm5b8f). Pesticides are composed of active and inert ingredients. An "active" ingredient is basically the compound that gives the pesticide its desired properties. Any other ingredient within the formulation is considered an "inert" ingredient; they are important for the product to enhance their performance and usability.

The next category is the Industrial POPs, such as flame retardants, plastics, polymers, composites, solvents, etc.[4] (Table 3). Chemicals such as PFOS, HBCDD, PBDEs are catalogued in this category. They are thus man-made, hazardous, and toxic chemicals with several purposes and plenty of applications[4].

Finally, Unintentional POPs are the last category. As the name implies, these derive from anthropogenic sources and are emitted during incomplete combustion processes where chemicals involving chlorine and organic matter are involved. Some of these unintentional POPs have debuted as an intentional production (e.g., PCBs, HCB, HCBD, etc.) serving as coolants, pesticides, or

solvents for chlorine-containing compounds[4,5]. Within this category, dioxins and furans are of particular interest for their toxic properties, they have been involved in many scandals across the world[34].

Table 3. Examples of POPs that can be used as part of synthetic polymers.

Name	Found in	Ref
PCBs	Plasticizers in rubber, plastic, paint, hydraulic and electrical equipment	[35]
PBDEs	Flame retardants used in a variety of polymers, including electronics, textiles, and plastics	[36]
PFAS	Polytetrafluoroethylene (PTFE or Teflon)	[37]
Chlorinated Paraffins	Plasticizers, flame retardants, and lubricants (short-chain chlorinated paraffins)	[38]

3. Deleterious health effects

Evidence for deleterious effects on human health results from different types of studies. The most relevant, but also the most difficult to obtain, are obviously results from studies on human subjects. We can identify at least three types of studies:

1) Correlational studies where the presence of a particular compound in the diet, or in the blood, coincides with an increased incidence of a particular condition. These require large cohorts, extensive control of co-variables (gender, age, health status, compound mixtures, etc.), and appropriate statistical tools, while they may often fail to definitively prove causation, as they tend to focus and measure the prevalence of a specific condition rather than the mechanisms causing it[39–43].

2) Observation and recording of catastrophic events, where exposure of an entire population to a specific compound leads to a dramatic increase of certain conditions relative to unexposed populations, or to the same population before

the event, thereby allowing to directly link the condition to the exposure. We are not short in examples of such events. In the record we can find iconic cases such as the mercury poisoning in the Minamata bay. This tragic episode was the consequence of the discharge of methylmercury, a by-product of acetaldehyde, in the water streams by the Chisso and Showa Denko companies. Eventually, it reached the coasts and the bay of Minamata were fish and seashells were contaminated and eventually consumed by people. It is estimated that more than 2500 people presented symptoms of the Minamata disease[44]. This condition is a neurological disorder characterized by e.g. the disturbance in control of distal portions of limbs, ataxia, and concentric contractions of visual field. The first case was reported in 1956 and by the 70s the Japanese government declared the outbreak as finished. More in the realm of the POPs, another such a tragic episode was described in the Love canal, New York (1970s). This has been labelled by Eckardt C. Beck as – "*one of the most appalling environmental tragedies in American history"*. In brief, the Hooker Chemical company used the Love Canal as a landfill site where it stored around 20,000 tons of chemical waste including products such as caustic, alkaline, fatty acid and chlorinated hydrocarbons. A total of 82 different compounds, 11 of them suspected carcinogens (*e.g.*, benzene and dioxins) that affected the health and livelihoods of at least 100 homes and a public school built on the banks of the canal. Some of the health consequences ranged from acute exposure (kids playing at the playground coming back home with chemical burns in their hands) to more long-lasting consequences such as nervous disorders and several types of cancer[45].

In Europe, we found cases such as the Seveso disaster in Italy (1976) where dioxins were released to the environment following an explosion and creating the highest known exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[46]. The population (particularly children) exposed to this suffered from chloracne, decreased male and female fertility as well as increased risk for cancers and cardiometabolic disorders. The latter has been reviewed in Eskenazi and colleagues (2018)[47]. Other examples of dioxin-related cases worldwide come from the usage by the U.S. military of Agent Orange during the Vietnam war (primarily dioxins)[48,49] and more recently cases such as the Belgian Dioxin Crisis

(a.k.a. Dioxin affair) in 1999[50,51], where a mixture of PCBs contaminated with dioxins was accidentally added to fat that would be used to produce animal feeds. This episode of contamination is traceable in blood plasma and, although the exposure was significant, no conclusive adverse public health effects have been found.

3) Finally, experimental approaches consisting of exposing biological systems to a controlled amount of a compound are required to prove causation and to gain a deeper understanding of the mechanisms involved, but they are obviously not possible to perform on humans. Therefore, organs or cell cultures (which can be of human origin) are used to study more specific effects and to investigate detailed mechanisms involved, while lethality and whole organism effects are explored in animal models (see also below).

POPs have a variety of effects and Mechanisms of Action (MoA) (Table 4). They have been linked to many diseases and conditions, many of which are of major concern in our ageing population.

Table 4. This non-exhaustive table shows some of the different diseases and conditions that have been linked to exposure to any of the known POPs.

Substance	**Health problems**	**Refs**
PCBs (99, 118, 105, 138, 153 and 180), OCDD and PBDE-47	Cardiovascular problems, including increased levels of cholesterol	52–55
PCB congeners and organochlorine pesticides	Increased blood pressure	56
PCBs, DDE, HCB, TeBDE, chlordane, PAHs and phtalates	Several types of cancer (Testicular, kidney, prostate, breast, uterine and ovarian)	57–62
PCB congeners 153, 138, 180 and 170, organochlorine pesticides, DDE, beta-HCH, dioxins, furans and PBDEs	Diabetogenic obesogenic effects (obesity, insulin resistance, type 2 diabetes)	42,63–66
Oxychlordane & p,p' DDT, PCDDs, PBDEs, PCBs, DDE, DDT and o,p'-DDT.	Endocrine disrupting effects (weight gain, advanced puberty, thyroid hormone signaling)	67–72

3.1 Diabetes

Studies have positively linked POPs to an increased risk of type 2 diabetes (T2D). Research indicates that individuals with higher concentrations of POPs in their serum are more likely to develop diabetes, suggesting a dose-response relationship[73]. High incidence of diabetes upon exposure to POPs, specifically to DDE (*dichlorodiphenyldichloroethylene*), have been found in a cohort of fish consumers among sport fishers of the Great Lakes that was followed from 1990 to 2005[42]. POPs influence diabetes through mechanisms such as disrupting insulin signaling pathways and glucose metabolism, inducing oxidative stress, and altering lipid metabolism. These effects contribute to insulin resistance, a hallmark of type 2 diabetes[74]. When murine Beta-Tumor Cell-6 (B-TC-6) pancreatic beta cells were exposed to DDE, the findings suggest that the insulin (*Ins*) gene transcription was not affected, but that prohormone convertase levels were increased, suggesting that the insulin protein maturation might have been affected by DDE. Though beta cell dysfunction caused by DDE is affecting insulin secretion and regulation, these authors did not consider it as a major culprit of causing T2D following exposure to DDE[75]. In contrast, in a more recent study, researchers did find that type 1 diabetes (T1D) could be caused by exposure to POPs such as PCB-153 and p,p'-DDE. These two compounds impaired the production and secretion of insulin in response to glucose, as they caused decreased levels of *Ins1* and *Ins2* mRNA levels at environmentally relevant concentrations in INS-1E rat pancreatic β-cells[43].

3.2 Obesity

Organochlorine pesticides and polychlorinated biphenyls (PCBs) disrupt endocrine function and adipose tissue regulation, linking them to obesity. Exposure to these pollutants increases the body mass index (BMI) and waist circumference, underscoring their role as environmental obesogens[41]. The mechanisms through which POPs influence obesity include dysregulation of adipogenesis regulators like PPARγ and C/EBPα (CCAAT-enhancer binding

protein α), binding to nuclear receptors, epigenetic effects, and proinflammatory activities. Such is the case of the polybrominated diphenyl ethers (DE-47, BDE-47 and BDE-99) that induced adipocyte differentiation in mouse 3T3-L1 preadipocyte cells[76,77]. These mechanisms contribute to adipose tissue dysfunction and obesity[41].

3.3 Cancer

Several types of cancers have been associated with POPs. For example, in breast cancer, significant amounts of POPs in serum and adipose tissues are linked to adverse prognostic markers[40]. In colorectal cancer, concentrations of specific organochlorine pesticides and PCBs in serum show a significant association with increased risk. These findings indicate a dose-response relationship, underscoring the potential role of POPs in promoting colorectal carcinogenesis[39]. Another epidemiological study found that exposure to POPs has preliminarily been linked to an overall increased cancer risk in adults. For instance, in a cohort study from Southern Spain, PCB 153 exposure in males was positively associated with total cancer risk[40].

3.4 Other effects

Research has expanded its reach and is showing that POPs may even play a role in depression. Researchers found associations between maternal depressive symptoms and compounds such as PBDE congeners. The study followed a cohort of mothers from pregnancy (~16 ± 3 weeks) to 8 years postpartum[78].

Toxic neurological effects, such as impaired recognition memory observed in 5- and 7-months old babies, as well as neurobehavioral deficits like increased startle response, low motor reflexes, and neuromuscular function depression, have also been documented. These effects include hypoactive reflexes, impaired visual recognition, and short-term memory deficits observed at around 7 months of age. Further deficits, including weight gain issues, reduced responsiveness, and decreased visual recognition, were evident at 4 years of age[79].

Also, changes in neurobehavioral activities have been observed in offspring of monkeys and rats exposed to dioxins and PCBs [80–83]. Additionally, non-dioxin like PCB congeners and PFOS can bind to ryanodine receptor (RyR1)[84] inducing hyperactivity through alteration of the calcium signaling in zebrafish[85].

3.5 Endocrine system and disruption

The endocrine system actively manages maturation, growth, development, and regulates the body's functions[86–88]. Various glands, including the pituitary, pancreas, adrenals, ovaries, and testes, secrete different hormones to control bodily processes (Fig. 2). The endocrine system oversees the production and function of hormones. Hormones are molecules that are produced and released into the bloodstream following environmental cues or triggered by specific stimuli. Some hormones are extremely specific when targeting specific cells. Their interaction triggers a domino-effect of biochemical reactions that leads to change in the function and/or activity of their target cells[89].

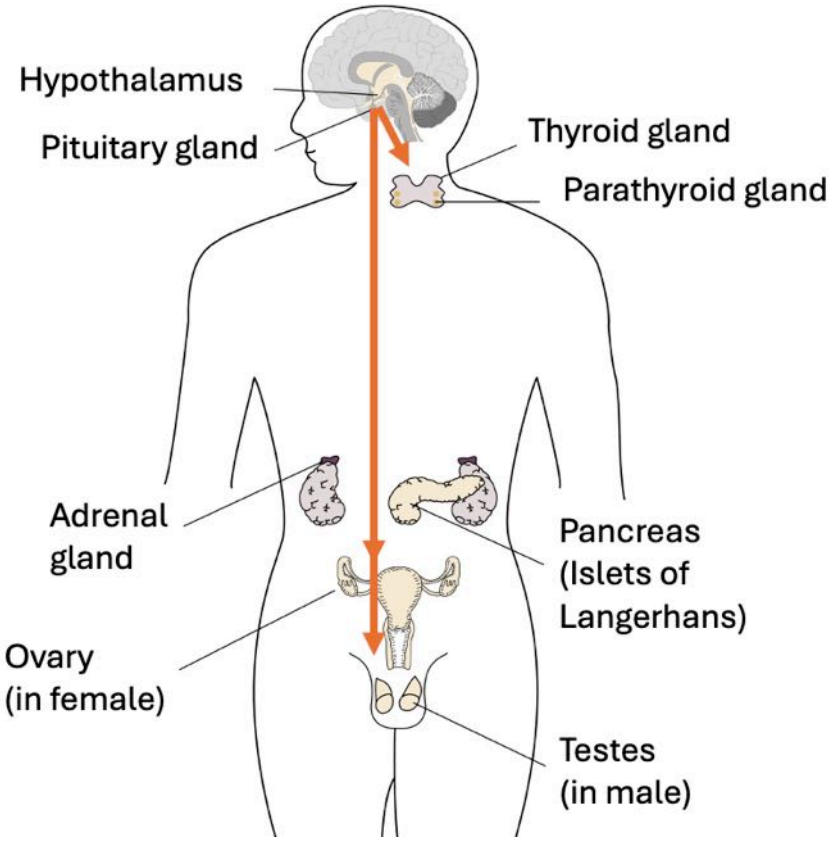


Figure 2. Endocrine system. Scheme showing the location of the major hormone-producing organs in the body. Illustrations and information were adapted and taken from Hiller-Sturmhöfel and Bartke[89] and Halmos and colleagues[90].

The hypothalamus is a small region located within the brain that produces pivotal hormones controlling bodily functions, such as eating and drinking, sexual functions and behaviors, heart rate and blood pressure, body temperature maintenance, body biorhythms and emotional states. The major releasing and inhibiting hormones produced by the hypothalamus are: corticotropin-releasing hormone (CRH) that regulates part of the carbohydrate, protein and fat metabolism as well as the response to stress, gonadotropin-releasing hormone (GnRH) that aides with the control of sexual and reproductive functions, thyrotropin-releasing hormone (TRH) that helps controlling metabolic processes of cells, energy balance, thermogenesis, and triggering the TSH in the pituitary gland, and finally growth hormone-releasing hormone (GHRH), somatostatin and dopamine.

As such, the hypothalamus is linking the brain to the endocrine system by acting on the pituitary gland, also known as hypophysis, which consists of two parts, anterior and posterior. The anterior pituitary produces hormones that stimulate other target glands or organs directly. These hormones are Adreno-corticotropic hormone (ACTH), gonadotropins, thyroid-stimulating hormone (TSH), growth hormone (GH), and prolactin (PRL). The gonadotropins regulate the production of female and male sex hormones respectively in the ovaries and testes, these hormones are luteinizing (LH) and follicle-stimulating hormones (FSH). Growth hormone is required for general and skeletal growth, while prolactin controls ion homeostasis and lactation in females. The posterior pituitary does not really produce its own hormones, rather it stores and releases vasopressin and oxytocin that are produced by neurons in the hypothalamus.

The thyroid gland is responsible for producing, under the control of TSH, two structurally similar hormones, thyroxine (T4) and triiodothyronine (T3), which are collectively known as the thyroid hormones (Fig. 3). Thyroid hormones increase

the metabolism of almost all body tissues and play an important role in the development of the central nervous system during late fetal and early postnatal developmental stages. They also play an important role in normal development of the bone growth and maturation and the development of teeth, skin, or hair follicles.

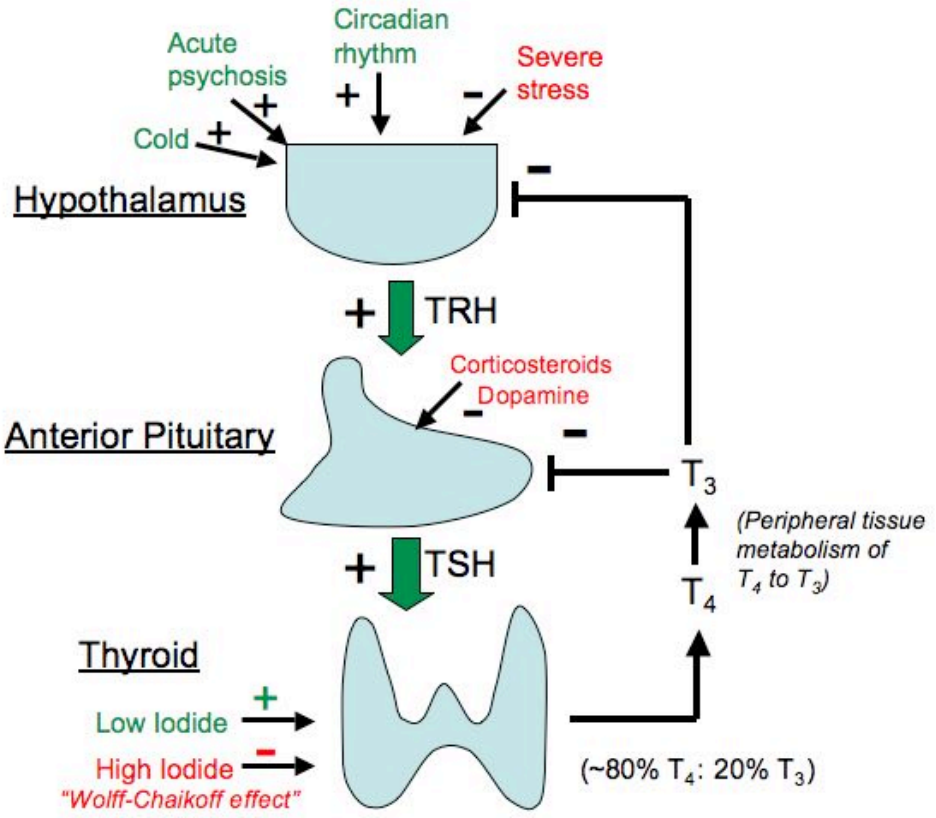


Figure 3. Scheme representing the regulatory pathway and negative feedback loop for thyroid hormones. Figure adapted from Holt & Hanley[91].

The parathyroid produces parathyroid hormone (PTH) which is responsible for increasing calcium levels in the blood, with important consequences in maintaining bone quality and an adequate supply of calcium.

The adrenal glands secrete numerous hormones, primarily corticosteroids. These are divided into glucocorticoids and mineralocorticoids, the main glucocorticoid in humans is cortisol and the primary mineralocorticoid produced is aldosterone. Cortisol manages how the body uses carbohydrates (increases glucose), fats and proteins, regulates blood pressure, boosts energy to handle

stress, while aldosterone regulates salt (to manage blood pressure) and water in the body.
The pancreas has a double function: first as an exocrine organ by producing various digestive enzymes, and second as an endocrine organ producing mainly two hormones, insulin and glucagon (in the Islets of Langerhans) that control the glucose levels in the blood.

Finally, the gonads have two major tasks, the first one is to produce germ cells such as ova in the ovaries and spermatozoa in the testes, secondly the gonads synthesize steroid sex hormones that are necessary for development and function of both female and male reproductive organs and secondary sex characteristics. Three types of hormones can be synthesized by gonads: estrogens, progestogens and androgens[89].

Disturbances in the endocrine system can lead to various diseases and health issues. Due to the complexity of the endocrine system and the diversity of the involved substances, there are many levels where EDCs can interfere. Compounds can act on the synthesis, secretion, transport, metabolism or elimination of endogenous hormones[92,93]. Endocrine disruptors are thus chemicals that interfere with the endocrine system, have adverse effects on reproductive, neurological, and immune systems, both in humans and in wildlife. As an example for adverse effects on the reproductive system in wildlife, tributyltin (TBT) induces imposex (imposition of male sex organs in female prosobranchs) in snails (*Nucella lapillus*)[94]. Other reproductive effects described upon exposure to TBT are a slower rate of egg-laying behavior and increased frequency of polyembryony[95]. Similarly, in fish, exposure to EDCs can cause intersex and males producing vitellogenin (a protein normally synthesized by female fish producing eggs), thus indicating feminization or hermaphroditism[96,97]. Alligators have been an example where DDT and its metabolites, such as DDE, caused abnormal sex hormone levels, feminized males with reduced phalli size and displaying intersex characteristics[98]. Atrazine causes feminization and hermaphroditism in male American leopard frogs (*Rana*

pipiens) and African clawed frogs (*Xenopus pipiens*)[99,100]. In birds, compounds such as Di-(2-ethylhexyl) phthalate (a plasticizer) induce developmental abnormalities in ovaries of Japanese quail[101]. Additionally, EDCs can alter sexual dimorphism by disrupting the normal patterns of plumage colors, for instance, the circulating carotenoids and skin carotenoids in captive American kestrels were disrupted following exposure to mixtures 1:1 Aroclor 1254, 1248 and 1260[102]. Another example of PCBs causing deleterious effects in wildlife comes from the belugas at the St Lawrence River and their exposure to high levels of PCBs and polycyclic aromatic hydrocarbons (PAHs), which have been found linked to high rates of cancers in reproductive tracts of this marine mammal species[103–105].

In humans, EDCs can induce hypospadias (in which the opening of the urethra is located on the underside of the penis instead of the tip, one of the most common congenital abnormalities) that affects approximately 1/231 live male births[106]. Although evidence around the mechanisms linking this defect and exposure to EDCs is limited, epidemiological studies such as Bonde et al[107] and Raghavan et al[108], have found that exposure to compounds such as pharmaceuticals, DDT, HCB, and phthalates have strong associations with hypospadias risk.
Cryptorchidism describes the failure of one or both testes to descend into the scrotum and has been linked to exposure to pesticides with antiandrogenic and/or estrogenic effects. Although the evidence is weak due to confounding effects of other factors such as hypothalamo-pituitary causes in individuals with Kallman, Prader-Willi syndromes and pituitary hypoplasia for instance, pesticides may still play a role[109,110].

Premature thelarche is a condition where the breast starts developing in girls before 8 years of age. Some evidence has been found that links the excessive ingestion of phytoestrogens as the trigger of this condition[111–113]. Also, Bisphenol A has been suggested as one EDC that could trigger premature thelarche[114].

Each one of the dirty dozen POPs are endocrine disruptors that can affect hormone functions[115]. Indeed, we can find several examples on the endocrine disrupting effects induced by POPs[116]. In addition, it has been reported that the effects of POPs during fetal development may persist throughout life[117]. Most endocrine-disrupting pesticides belong to the group of organochlorine pesticides, such as DDT, dieldrin, toxaphene, chlordane, mirex, and endosulfan. These pesticides act as estrogens, altering sex organs and reproductive systems. Using the stable prostatic cell line PALM that has a human androgen receptor expression vector and the reporter MMTV-luciferase, compounds like DDT and methoxychlor were found to antagonize androgen receptors, thus suggesting a role in male sexual differentiation disorders (hypospadias and cryptorchidism)[118]. Though endosulfan (as other POPs) act primarily as a neurotoxic by blocking the gamma-aminobutyric acid (GABA)-gated chloride channel, it has been also linked to deleterious effects in ovarian tissue and oocyte blockade in several fish species, as reviewed in Senthilkumaran (2015)[119].

Another example comes from exposure to PCBs and their hydroxylated metabolites. They can interfere with the thyroid system at multiple levels, hydroxylated PCBs compete with thyroxin (T4) for binding to transthyretin[120].

4. Defining an endocrine disrupting chemical

The concept of an endocrine disrupting chemical has been under scrutiny and the debate is still ongoing as to which compound can be classified as an actual endocrine disruptor. However, it is generally accepted that any one of the following characteristics is deemed as key to label a chemical as EDC (Fig. 4) [121]:

1) Interacts with or activates hormone receptors;
2) Antagonizes hormone receptors;
3) Alters hormone receptor expression;
4) Alters signal transduction in hormone-responsive cells;
5) Induces epigenetic modifications in hormone-producing or hormone-responsive cells;
6) Alters hormone synthesis;

7) Alters hormone transport across cell membranes;
8) Alters hormone distribution or circulating levels of hormones;
9) Alters hormone metabolism or clearance

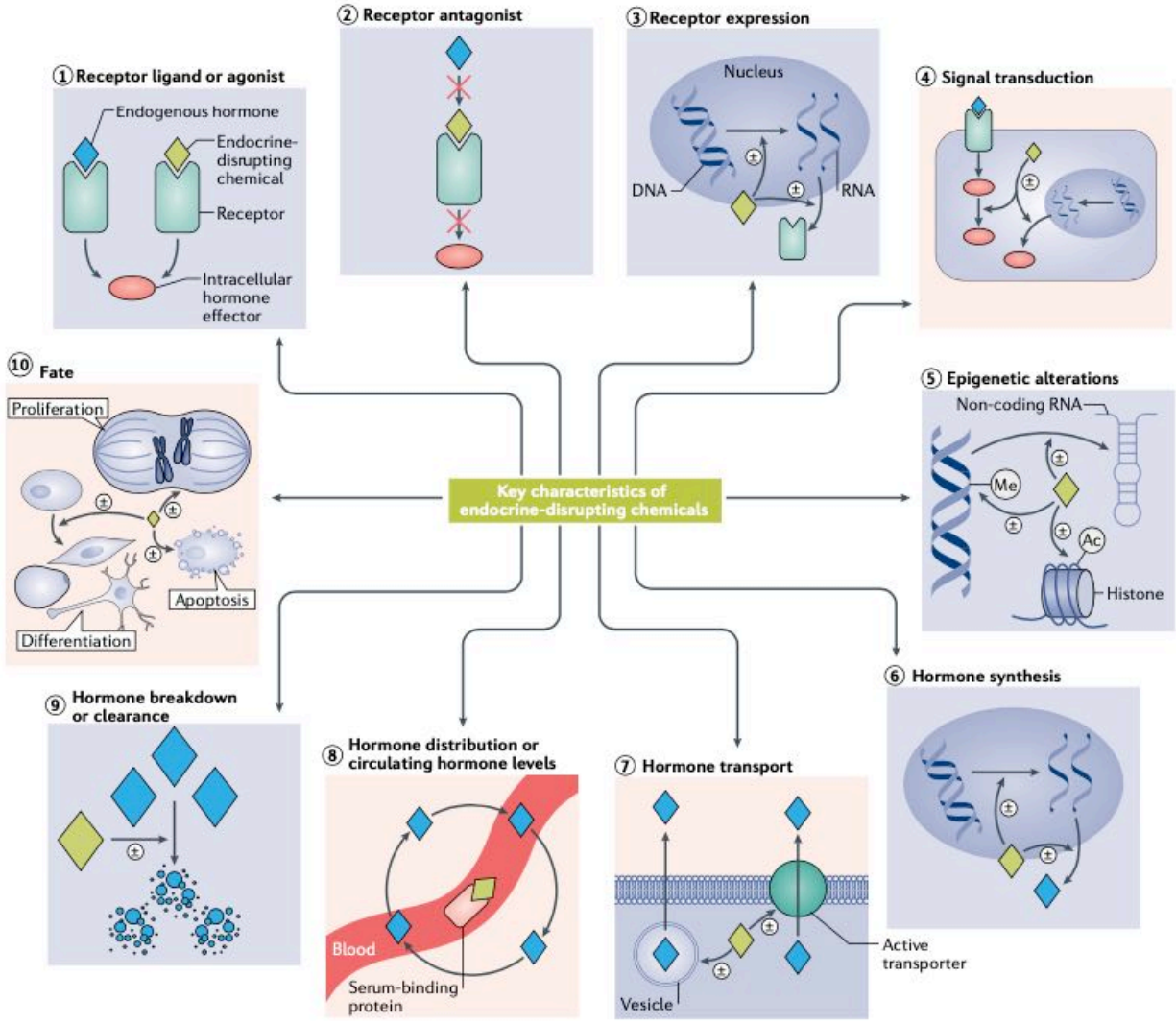


Figure 4. Key characteristics deeming a compound as EDC. Taken from "Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification" in Merrill et al[121].

5. Nuclear receptors

Among the most prominent, and the most studied EDC targets of the POPs are the receptors that can directly bind this type of compounds, namely the dioxin receptor mediating the effects of dioxin-like compounds, and the larger family of

nuclear receptors that regulate transcription upon binding to a variety of small organic molecules. The superfamily of nuclear receptors (NR) is the largest family of transcription factors in heterotrophic eukaryotic organisms. For example, 73 genes are encoding for NRs within the genome of the zebrafish (Fig. 5).

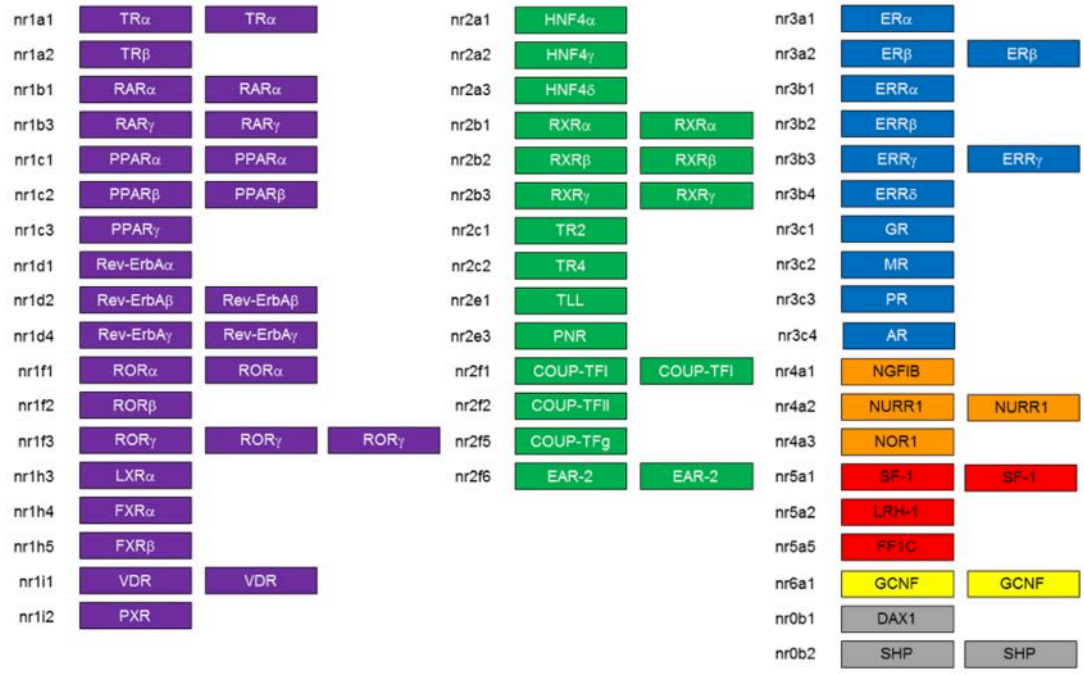


Figure 5. Synopsis of the nuclear receptor family described in zebrafish. This figure has been taken from Schaaf[122] (adapted from Bertrand et al[123] & Zhao et al[124]). Different sub-classes are shown, defined by their specific DNA-binding domains and their ligands. In blue are shown the strict steroid receptors, which bind DNA as homodimers, while the violet group represents receptors binding to more diverse ligands (TR, RAR, VDR) which predominantly bind DNA as heterodimers with RXRs. In green, receptors that bind either as homodimers or heterodimers (RXR).

They regulate reproduction, development, metabolism and the immune system[122,125].

Nuclear receptors all share a modular domain structure. All have an N-terminal domain that varies in length, a small DNA-binding domain that is well-conserved and a fairly conserved and larger C-terminal ligand-binding domain (Fig. 6). The DNA-binding domain of the nuclear receptors is a characteristic two Zinc-finger domain which, as a dimer, binds specifically to a hormone response element

(HRE) located in the target gene to regulate its transcription. These common traits helped in the identification of nuclear receptors in genomic sequences[126,127].

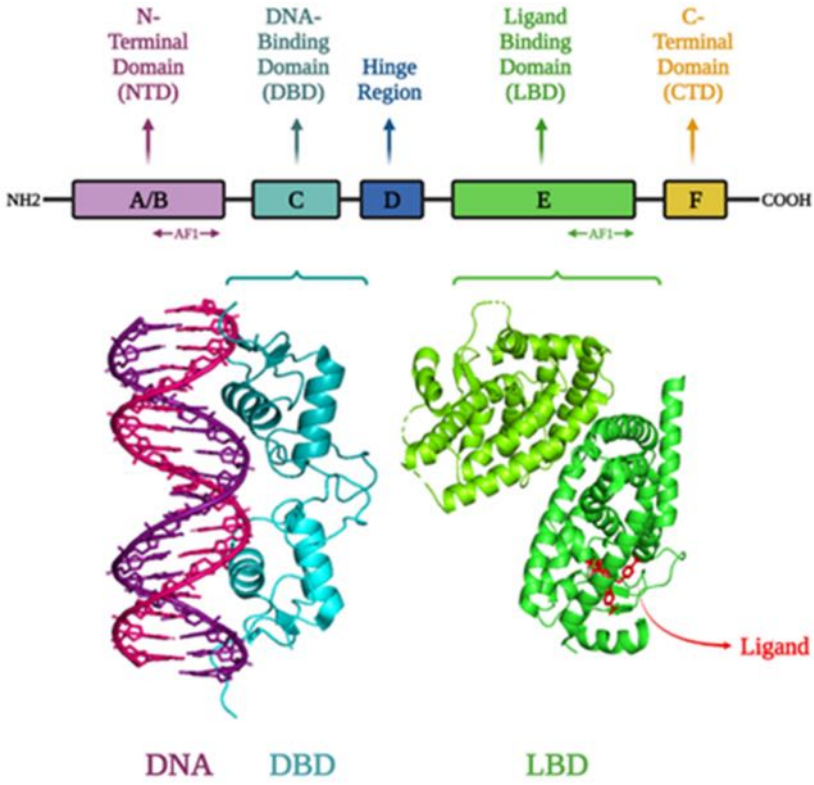


Figure 6. Structural organization of nuclear receptors. Taken from Jayaprakash et al.[128]

5.1 Retinoic Acid receptors (RAR)

An important group of nuclear receptors are those mediating the effects of retinoic acid, the active metabolite of vitamin A. Vitamin A plays an important role in development, studies have shown that vitamin A is required for normal organogenesis, growth, adequate immune response, appropriate gastrointestinal activity and vision[129].

5.2 Peroxisome proliferator-activated receptors (PPAR)

PPARs are involved in signaling pathways such as prostaglandins and leukotrienes. They are activated by saturated and unsaturated fatty acids and are involved in physiological processes regulating energy metabolism and adipocyte differentiation[130]. Three PPARs have been identified in vertebrates. They are encoded by three different genes: PPARα, PPARβ (also called PPARδ) and PPARγ[131].

5.3 Androgen receptors (ARs)

ARs are important for development and maintenance of male characteristics, spermatogenesis, muscle and bone growth, and even the central nervous system with consequences over libido, mood and cognitive functions.

ARs are activated by androgenic steroids such as testosterone and dihydrotestosterone[132].

5.4 Vitamin D receptor (VDR)

Vitamin D collectively designates a group of lipophilic steroids, comprising mainly vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol), which regulate genes involved in calcium, magnesium, and phosphate absorption in the intestine and reabsorption in the kidneys, suppresses cancerous cells and aids in normal growth, proliferation and differentiation of various cell types. It helps modulating innate and adaptive immune responses, and it is essential for bone health and mineralization. Further, it is also involved in cardiovascular health by regulating blood pressure[133,134].

5.5 Estrogen receptors (ER)

Estrogen receptors mediate the effects of estrogens, which are vital hormones crucial for reproductive and various physiological processes, thereby linking their function to the body's homeostasis and reproductive health. They have a tissue-specific distribution (Table 5). ERα for instance, is mainly found in reproductive tissues, whereas ERβ is found in the immune, central nervous and cardiovascular systems, lung, colon, and kidney, in addition to male reproductive organs and ovary[135].

Table 5. Characteristics of estrogen receptors. Adapted from Luo and Liu[136]

	ERα	ERβ	GPER1
Category	Nuclear steroid hormone receptor superfamily		G protein-coupled receptor superfamily
Location	Nucleus		Membrane-associated
Size	595 aa	530 aa	375 aa
Numbers of isoforms	3	5	1
Chromosome region	6q25.1	14q23.2	7p22.3
Structure	DNA-binding domain, ligand-binding domain, N-terminal domain		7 transmembrane α-helical regions, 4 extracellular and 4 4 cytosolic segments
Distribution in tissues	Hypothalamus, hippocampus, testes, ovary, endometrium, uterus, prostate, kidney, liver, breast, epididymis, muscle, adipose tissue	Testes, ovary, prostate, vascular endothelium, bladder, colon, adrenal gland, pancreas, muscle, adipose tissue	Central and peripheral nervous system, uterus, ovary, mammary glands, testes, pancreas, kidney, liver, adrenal and pituitary glands, cardiovascular system, adipose tissue

Similar to the other nuclear receptors, ERs are ligand-activated transcription factors encoded by different genes. Their protein structure is mainly characterized by their functional domains, just as the other NRs (Fig. 7).

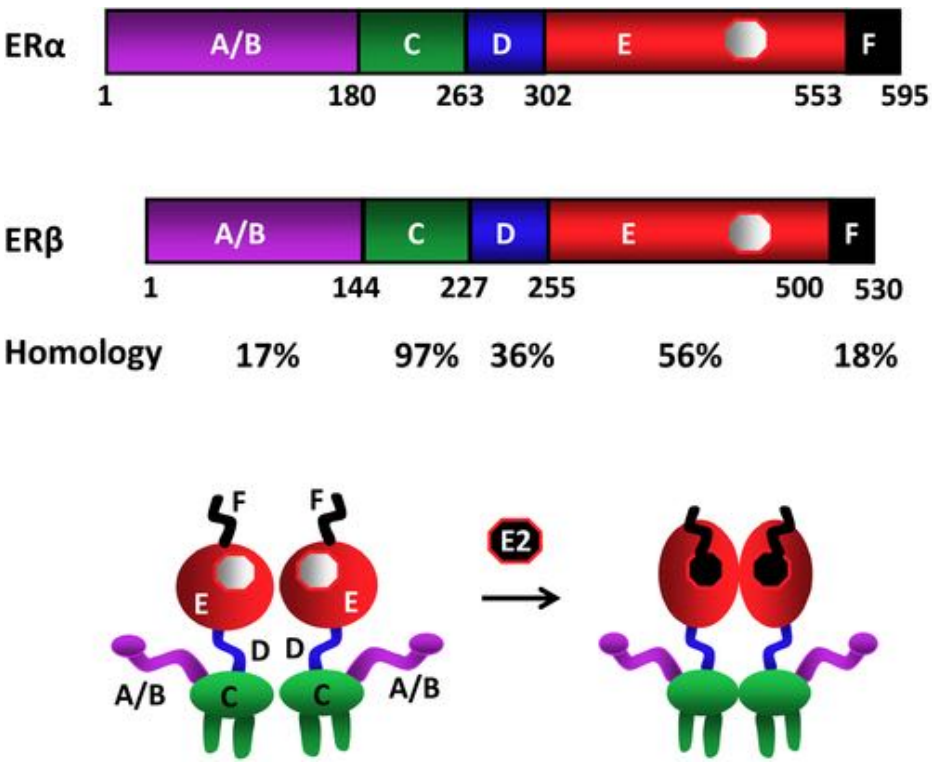


Figure 7. Structural regions of nuclear estrogen receptors. Taken from Yaşar and colleagues[135]. There are 595 amino acids in human estrogen receptor α, and 530 amino acids form ERβ. Up to 17% amino-acid identity is shared between ERs for the structurally distinct amino terminal A/B domains; their C central regions (DNA binding domain) are almost identical sharing up to 97% of identity. Regarding the D domain (hinge), there is 56% homology between ERs, this domain contains a nuclear localization signal linking the C domain to the multifunctional carboxyl terminal (E), which is responsible for binding the estrogenic compounds. Finally, the remaining short carboxyl-terminal F domain shares 18% amino-acid identity. The ERs are dimers with or without the endogenous ligand.

Nuclear ERs are dimers on DNA that regulate transcription by binding sequences upstream of their target genes that are called Estrogen Response Elements (EREs). The prototypic ERE is a palindromic consensus sequence of AGGTCA motifs separated by a 3-base spacer. EREs are specific DNA sequences that bind to ERs once activated by estrogen. They play a role at regulating gene transcription as they are the docking sites of ERs[137,138]. Although there are specific differences between ERα and ERβ binding sites (Fig. 8), it has been reported that significant overlapping is also present[139].

6. Membrane receptors for steroids: the GPER1

The GPER1 belongs to the family of the G protein-coupled receptors (GPCRs). The members of this family are characterized by their seven transmembrane domain structure[140]. GPER1 is a transmembrane estrogen receptor that exerts estrogenic effects in cellular and physiological processes as it is expressed in many tissues within the body[141]. It is a fast response, non-genomic receptor that is gaining attention due to its role in several physiological processes. It is considered an orphan receptor because its endogenous ligand remains unknown; however, it is responsive to estrogens, to the selective agonist G1[142], and even to persistent organic pollutants such as 2,2-Dichlorodiphenyldichloroethylene (p,p'-DDE), Hexachlorobenzene (HCB), Perfluorooctanoate (PFOA), and Perfluorooctane Sulfonate (PFOS) known for their estrogenic activity[143]. After ligand binding, the GPER1 was shown to act on cAMP levels through Gαs or Gαi proteins, other mechanisms have however been suggested as well[136] (Fig. 8).

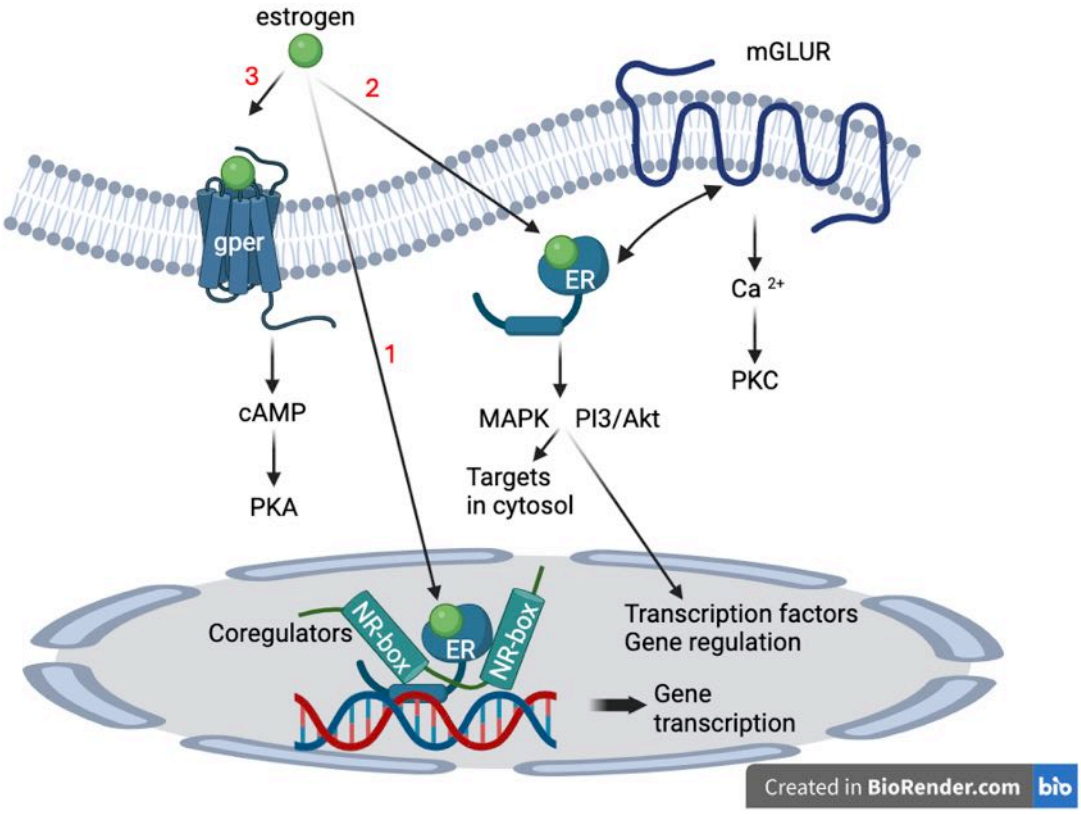


Figure 8. Representation of estrogen receptors within the cell and the various intracellular pathways related to estrogen signaling. Three main pathways can be found: 1) Classical role of

estrogen receptors as nuclear transcription factors (slower genomic response). 2) Estrogen signaling involving cytoplasmic or membrane-localized ER triggering crosstalk with membrane-associated receptors (e.g., mGLUR) and cytoplasmic signal transduction pathways. 3) Transmembrane binding to GPER that leads to signal transduction via various cytoplasmic kinases[144].

Thus, estrogens and estrogenic EDCs exert their effects through both nuclear receptor-mediated transcriptional regulation and through membrane receptor-mediated intracellular signaling pathways.

7. Zebrafish (*Danio rerio*)

7.1 Advantages (Biology, ecology and genome)

Endemic to South Asia, the zebrafish is a small fish species of 3 to 5 cm of size when fully mature (Fig. 9). Its origins can be traced to rivers located at Northern India, Northern Pakistan, Nepal, Bhutan and Bangladesh. It is a teleost fish, meaning that it belongs to the bony fish class within the *Cyprinidae* family[145].

It inhabits freshwaters with pH ranges of 6 to 8 in tropical temperatures of 18 to 24ºC. These can be streams, canals, ditches, ponds and beels, however it can occur in slow-moving to stagnant waters. Out in the wild, its diet consists of worms, small crustaceans and insect larvae. It can breed all year round, but it is primarily an annual spawner. The spawning season corresponds to just before the onset of the monsoon. The spawning is induced by temperature and food availability[145,146].

This species is easy to keep in aquariums, as the natural conditions are relatively easy to mimic. Thus, the zebrafish is a low-cost, high fecundity, easy to maintain species, with a well-known genome that allows us to modify it rather easily and life stages well-studied that makes them an ideal model system[147,148].

Figure 9. Photo of an adult zebrafish. Picture from www.istockphoto.com

7.2 As Model Organism

Within the European Union, the zebrafish is foreseen as a good model in the upcoming years. Policies are changing and many animal experiments will be reduced. From REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) to academia, the usage of animals will be limited and will be under thorough scrutiny. There is a huge movement to phase out animal experimentation at all levels. The latter seems unfeasible at the moment although it will certainly reduce the number of the tested individuals, and the use of non-experiment data will be prioritized[78]. In this context, the zebrafish is available to occupy a position that traditionally has been filled with data coming from mammalian models (mainly mice and rats). It is well-suited to respond to the requirements of the 3R-principle, standing for Replacement, Reduction and Refinement, that is becoming paramount in research worldwide[149]. In particular, studying larvae until the age of 5 days is not covered by animal experimentation legislation (Directive 2010/63/EU[150]), although many organs and processes are already in place at these stages to be investigated.

The zebrafish has a large degree of gene conservation with mammals, about 71% of human genes are represented by at least one zebrafish ortholog, whereas 69%

of the genes in zebrafish have a human equivalent[74]. Similarly, zebrafish proteins resemble those found in humans. For instance, the glucocorticoid receptor is about 54% similar to the one described in humans, but the ligand binding domain and its pharmacological properties are about 74% identical. Furthermore, there are anatomical and physiological similarities, the zebrafish has organs analogous to humans, performing the same functions. In some cases, the zebrafish may be a better model (*e.g.* cardiac electrophysiology) than rodents, while the physiological mechanisms are well conserved at the molecular and cellular levels[151]. Moreover, the zebrafish shares vision, olfaction, taste, touch, balance and hearing senses with humans, and displays a complex and extensive behavioral repertoire, comprising simple stimulus-response behaviors, sleep, pain, affective and depressive-like behavior, locomotion, social interactions and cognitive behaviors[152]. In brief, as model, zebrafish is a well-suited analogue since it possesses key advantages such as: a) completely sequenced genome[148], b) available and optimized gene-editing tools[153] and, c) larvae are optically transparent, thus visualization and staging are rather easy across developmental stages[147,154].

For all these reasons, the zebrafish has now been established as a vertebrate model system not only for developmental biology, but also for physiology and for a wide variety of pathologies.

7.3 Early development of zebrafish

One of the many advantages of zebrafish as model is its rapid development. Within the first 10 days post fertilization (dpf), the fertilized zebrafish egg undergoes significant morphological and physiological change (Fig. 10).

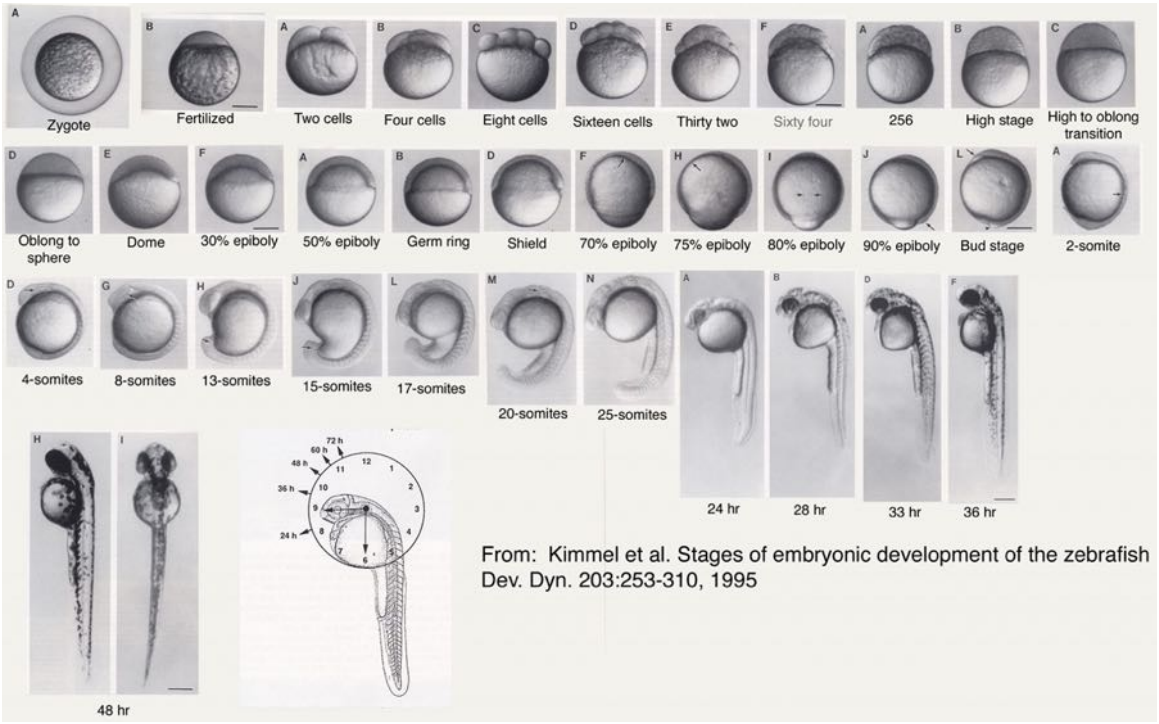


Figure 10. Developmental stages of zebrafish from 0 hpf to 48 hpf. Taken from Kimmel et al.[154] .

Early development of zebrafish can be subdivided in several stages:

1) Fertilization and cleavage (0-6 hpf)

After fertilization, the two-cell zygote is rapidly formed (40 min), followed by a series of rapid cell divisions without growth, the blastula stage ends with completion of the epiboly where the embryonic cells migrate to cover nearly the entire egg periphery, at around 6 hpf.

2) Blastula to gastrula (6-10 hpf)

During this period, the three embryonic layers are formed: ectoderm, mesoderm and endoderm. This is due to morphogenetic movements during gastrulation, which also lead to formation of the body axis to lay out the body plan.

3) Segmentation (10-24 hpf)

The most visible feature is the formation of the somites, which are precursors to the vertebral column and associated muscles. During this phase, organogenesis of the heart, eyes and other major organs also starts.

4) Pharyngula period (24 -48 hpf)

During this period, development of the pharyngeal arches, brain, and sensory organs takes place. First beats of the heart occur, and the blood circulation is established.

5) Hatching period (48 -72 hpf)

The embryo hatches and some feeding might start, although the larva will still primarily rely on the yolk for its growth. This is the beginning of the free-swimming phase.

6) Larval period (from 72hpf and up to 10 dpf)

Post-hatching, the larvae continue to develop with exponential growth in the nervous and muscular systems, as well as sensory organs. The thymus is colonized by lymphoblasts, and the immune system starts developing[155]. Eye morphogenesis and the development of sensory hair cells in the inner ear is completed at around 3 to 4 dpf[156].

7.3.1 Bone and cartilage

Bones are formed by osteoblast and osteocytes while cartilage is formed by chondroblasts and chondrocytes (Fig. 11).

1) Initial bone formation

Between 14 and 48 hpf, cNCC (cranial neural crest cells) start migrating from hindbrain rhombomeres towards their ventral location in the pharyngeal arches[157]. At this stage, some of the factors involved in cartilage development are: Dlx2 (marker that leads to pharyngeal cartilage formation), Sox9a (marker for first differentiation of cartilaginous cells and Egr1 (zinc finger transcription factor), fibroblast growth factors through their receptors Fgfr1 and Fgfr2, Runx3 and Sox9b, working together for morphogenesis of pharyngeal cartilage development[158–160].

Bone morphogenetic proteins (BMPs) will also play a crucial role in early development of the skeleton[161]. In early development, BMP signaling is essential

for dorso-ventral patterning of the embryo and for initial cartilage formation and patterning.

2) Osteoblast differentiation and function

Sox9 expression is required for differentiation of chondrocytes and osteoblasts. During the pre-osteoblast phase, factors such as Sp7/Osx and Runx2(a and b) trigger a mechanism that leads to the formation of osteoblasts[162]. BMP signaling is further important after the first 48 hpf for promoting osteoblast function and bone mineralization. BMP inhibition will lead to a significant reduction of bone formation[161].

3) Mineralization and structural development

Bone mineralization starts at around 3-5 dpf. The bony components are divided in three modes: intramembranous, perichondral, or endochondral. The first one occurs directly from mesenchyme, without a preformed cartilage matrix *e.g.* in the cleithrum, opercle, and later in the cranial roof (i.e. skull). Perichondral ossification occurs in the perichondrium around preexisting cartilage elements *e.g.* in hyomandibulars, ceratohyals and Meckel's cartilage. Finally, endochondral ossification occurring within a preformed cartilage element is rare in teleosts, but it occurs in the ceratohyal and the radials of the pectoral fin (reviewed in Tonelli et al[162] and Apschner et al[163]).

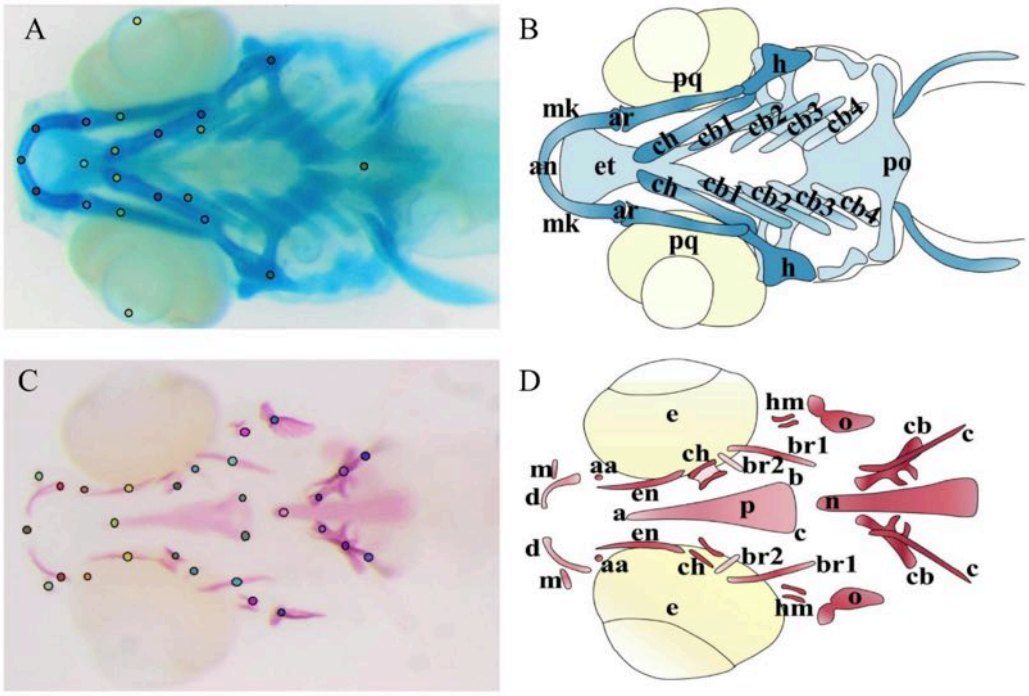


Figure 11. Cartilage and bone elements of the skull in 10 dpf zebrafish (Taken from Aceto et al[164]). A) Cartilage elements of the head stained with Alcian blue; B) Schematics of cartilage elements: anterior limit (an), articulation (ar), ceratobranchial pairs 1 to 4 (cb1-4), ceratohyal (ch), ethmoid plate (et), hyosymplectic (h), Meckel's cartilage (mk), palatoquadrate (pq), posterior limit (po). C) Bone elements of the head stained with Alizarin red; D) Schematics of bone elements: anguloarticular (aa), anterior (an), branchiostegal ray1 (br1), branchiostegal ray2 (br2), entopterygoid (en), maxilla (m), notochord (n), opercle (o), parasphenoid (p), cleithrum (c), ceratobranchial 5 (cb), ceratohyal (ch), dentary (d), hyomandibular (hm). The parasphenoid is a triangular structure defined by an anterior summit (a) and two posterior summits (b, c).

7.3.2 Eye

1. Initial morphogenesis

This takes place at 16 hpf, when the optic vesicle starts forming as the precursor of the eye. At 24 hpf, the optic vesicle transforms into the eye cup, comprising the neural retina and the pigmented epithelium[165].

2. Cell proliferation and differentiation

The eye cup undergoes a remarkable cell proliferation between 24 and 36 hpf. All the cells within the eye primordium are proliferative, with a cell cycle length of approximately 10 hours[165]. At 48 hpf, differentiation of the different layers of the retina starts to take place, also the lens starts to form as a solid sphere of cells, eventually separating from the developing cornea[166].

3. Development of visual functions

First evidence of startle responses linked to a functional visual system starts somewhere between 48 and 72 hpf. Startle response is induced by changes in light intensity and optokinetic responses such as eye movement due to moving visual stimuli[167]. Further differentiation of the lens and the retina continues to develop its layered structure[166].

4. Functional maturation

The retinal image is clearer at 72 hpf, also functional extraocular muscles are formed to allow for eye movements and tracking behaviors. Between 81 and 96 hpf, some spontaneous saccadic eye movement can be observed. Optokinetic responses are refined[167].

5. Environmental and genetic factors

The development of the eye and retina can be disrupted by environmental pollution. Exposure to heavy metals and POPs for instance can impact visually-guided behaviors[168] and compromise eye morphology[169]. Also, genetic factors play a role, it has been demonstrated that disruption in thyroid hormone signaling leads to alterations in development and function[170].

7.3.3 Lateral line

1) Early morphogenesis

The origin of the lateral line is a specific placode, called the lateral line placode, that is located at the posterior part of the otic vesicle[171,172]. Placodes are focal thickening of the surface ectoderm which, together with neural crest, generate the peripheral nervous system of the vertebrate head. These placodes form at around 17hpf during the 16-somite stage. They derive from the pre-placodal region where genes such as *six1b* and *eya1* are uniformly expressed[171–173]. Then, the primary posterior lateral line placode produces a migrating sensory primordium connecting with afferent neurons at around 24 hpf. In order to have proper development, this process requires retinoic acid signaling[172].

2) Neuromast formation and migration

Between 24 and 73 hpf, the neuromasts, which are the sensory organs of the lateral line, are formed after the sensory primordium migrates and deposits clusters of precursor cells. The formation of initial neuromasts occurs along the horizontal myoseptum. Between 7 to 8 neuromasts are formed at the end of the embryogenesis[174] .

3) Functional maturation

The primary neuromasts are fully formed and their detection of water movement capabilities starts to function at 72 hpf. At this stage, the hair cells within the neuromasts are also fully developed, they will be sensitive to mechanical stimuli[175].

4) Environmental and genetic factors

Several factors can compromise the normal development of the neuromasts in the lateral line. For instance, acidic water conditions can impair their development[176]. Also, genetics play a role. Mutations in the Krüpple-like factor 17 (*klf17*) result in abnormal neuromast deposition with consequences leading to high mortality without hatching[177].

7.4 The zebrafish as a model organism for (eco)toxicological studies.

A plethora of advantages makes the zebrafish a promising and powerful model that is gaining more and more popularity (Fig. 12).

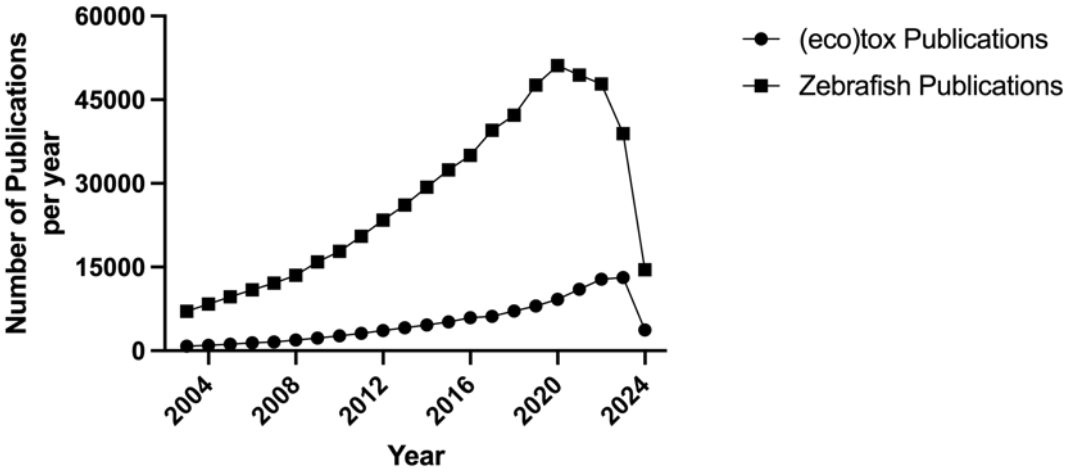


Figure 12. Publications found in a quick search using terms such as "zebrafish" and "POPs" and "(eco)toxicology". These are the number of publications found in web repositories that are using zebrafish as model system since 2003. The popularity of this fish species has been increasing through time due to its many advantageous characteristics. To date it is possible to find more than 694,000 publications across disciplines only for zebrafish where around 14% of the publications (99,300) are about (eco)toxicology as disciplines. Data from Google Scholar using the terms such as *"zebrafish" AND ("ecotoxicology" OR "toxicology")* with Boolean operators.

Researchers use zebrafish as bioindicator models to analyze environmental quality, particularly in evaluating the toxicity of textile effluents. Conducting embryonic and larval analyses with zebrafish yields robust results inexpensively and quickly, meeting international standards such as the OECD 236 guidelines[178]. Zebrafish enable rapid and reproducible toxicological profiling, especially in early life-stage embryos. They have served in investigating the mechanisms of action via, among others, the aryl hydrocarbon receptor (AhR) in toxicological studies[179]. Zebrafish have been applied in medium-throughput toxicity and activity screening of medicinal plant extracts, capitalizing on their status as embryotoxicity model and aiding in new drug research[180,181]. Using zebrafish, researchers have evaluated the sub-lethal toxicity of pollutants such as silver ions, nanoparticles, or mixtures of persistent organic pollutants, in addition extensively profiling locomotion to gain insights into neurodevelopmental and behavioral impacts of the pollutants at environmentally relevant concentrations[169,182]. Combining zebrafish embryo

bioassays with high-resolution mass spectrometry enables the assessment of water bodies' ecotoxicological status, showcasing the efficacy of this approach in real-time environmental monitoring[183].

Toxic effects in zebrafish can be observed and measured at different levels (molecular, structural, systems, etc.), and molecular changes can be linked to phenotypic effects[184,185]. Zebrafish has a clear advantage over cell-based assays since it can provide ADME (adsorption, distribution, metabolism, excretion) outcomes and thus is not limited to toxicokinetic studies[151]. Toxicological screening studies can be set up easily, as exposure tests can be performed by direct addition of the compounds of interest to the raising media of zebrafish and can yield large datasets as results from automatization by high-throughput screening approaches using robots and multi-well plates[186].

8. Genome editing technologies

Harnessing the power of nature sometimes requires introducing modifications. Recent progress in our ability to modify the genome of organisms has opened new possibilities to study the functions of specific genes not only under normal conditions, but also in pathologies and in the responses to environmental challenges.

In zebrafish, morpholino antisense RNAs have been widely used to transiently influence the expression of a specific target gene during early stages of development[187], while novel genome editing technologies such as TILLING (targeting induced local lesions in genomes)[188] or TALENs (transcription activator-like effector nucleases)[189] have increased, simplified, and accelerated the possibilities to generate mutations in selected genes. Among these, the CRISPR/Cas9 (clustered, regulatory interspaced, short palindromic repeats)[190,191] approach has become the method of choice to manipulate the zebrafish genome.

8.1 CRISPR/Cas9

The CRISPR/Cas system was discovered as a defense mechanism in bacteria and archaea against infections by plasmids or viruses[192] . The system relies on small RNAs for sequence-specific detection of foreign nucleic acids. These RNAs are transcribed from a specific region in the genome characterized by clustered, regulatory interspaced, short palindromic repeats (CRISPR), where short sequences from previous invasions by foreign genetic elements have been incorporated. After transcription, these sequences are processed into small RNAs, which then recruit and guide a protein complex (the CAS complex) to recognize and cleave the targeted genetic material[192–194].

This system has been adapted as a tool for genome editing by microinjecting a synthetic, well designed guide RNA (the sgRNA) into a one- to two-cell fertilized egg, along with a purified Cas9 protein to induce double strand breaks at the targeted genomic locus. This lesion is then repaired (Fig. 13), eventually leading to the generation of an indel mutation that may inactivate the target gene.

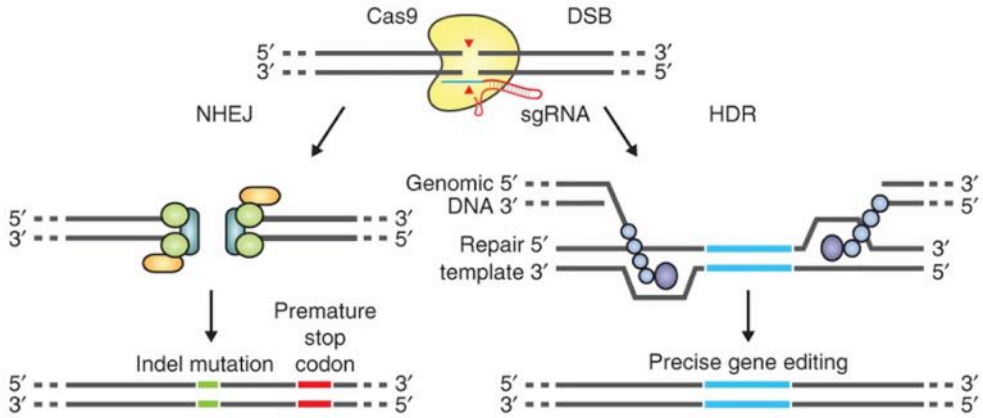


Figure 13. “Double Strand Breaks (DSBs) induced by Cas9 (yellow) can be repaired in one of two ways. In the error-prone Non-Homologous End Joining (NHEJ) pathway, the ends of a DSB are processed by the endogenous DNA repair machinery and rejoined, which can result in random indel mutations at the site of junction. Indel mutations occurring within the coding region of a gene can result in frameshifts and the creation of a premature stop codon, resulting in gene knockout. Alternatively, a repair template in the form of a plasmid or single stranded DNA Oligonucleotides (ssODNs) can be supplied to leverage the Homology Directed Repair (HDR) pathway, which allows high fidelity and precise editing. Single-stranded nicks to the DNA can also induce HDR” taken from Ran et al. [195]

This genome editing tool has been successfully applied in *Daphnia magna* to inactivate the CYP360A8 (cytochrome P450 clan 3 gene) to test the toxic properties of paraquat, which turned out to be more toxic in the mutants than in their wildtype counterparts, thus indicating that CYP360A8 is involved in degrading the compound[196]. Similarly, the aryl-hydrocarbon receptor gene (*arh2*) has been inactivated in zebrafish to test the role of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in development[197].

9. Nuclear estrogen receptors in zebrafish: Esr1, Esr2a & Esr2b and the G protein-coupled Estrogen Receptor 1 (Gper1)

Teleost fishes express several subtypes of nuclear estrogen receptors (ESR), including Esr1, Esr2, occasionally a third type, Esrγ and transmembrane receptors such as the Gper1[198,199]. These receptors exhibit distinct distribution patterns and can bind estrogens with varying affinities, indicating their diverse roles in fish biology. The presence of multiple ER types suggests a specific evolutionary adaptation in teleost fishes that potentially enhances their ability to respond to environmental estrogens[198,200].

Given the increasing concerns about EDC issues, the ERs are possibly the most studied nuclear receptors in zebrafish, with three identified genes encoding, respectively Esr1 and two Esr2a and Esr2b variants[201]. These receptors are expressed in the brain, pituitary, liver, gonads, and especially in the neuromasts of the lateral line[202].

As described above, the zebrafish is an advantageous model to represent human conditions due to its level of genetic conservation. In particular, the E/F domain responsible for binding estrogenic or endocrine disrupting compounds reveal a strikingly high degree of conservation, suggesting that their responses may be very similar to those of the human ERs (Fig. 14).

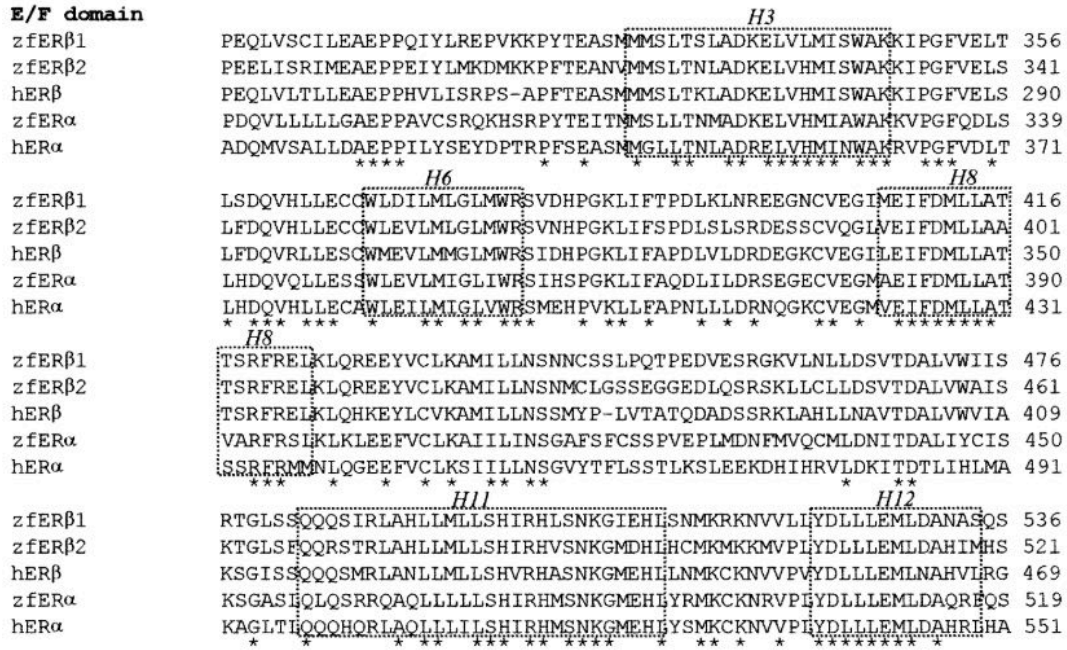


Figure 14. Aligned sequences showing similarities and differences between E/F domains of the ERs from human (hERx) and zebrafish (zfERx) (Taken from Menuet et al[203]). Homology modeling studies have found that there is great degree of conservation amongst species, thus suggesting functional similarities in how receptors bind estradiol and other ligands. The LBD in ERα ranges from 60 to 80% similarity between species, key amino acids involved in ligand binding and receptor activation are conserved[203,204]. The C (DNA binding) and E domains of ERβ1 are highly conserved in vertebrate species, the zebrafish and human share up to 90% (86% compared to goldfish[205]) of similarity.

Genetic experiments have been carried out to investigate specific functions of each of the estrogen receptors. Morpholino knockdown of *esr1* led to severe developmental defects and early death[206]. Transient knockdown of *esr2b* using morpholino oligonucleotides led to disrupted neuromast development, linked to Notch signaling pathway issues[207]. An Esr2b mutant line (8 additional amino acids) showed skewed sex ratios, altered testicular morphology, and increased hormone (testosterone and 17β-estradiol) levels, alongside a reduced immune response[208].

Interestingly, mutation of the membrane receptor *gper1* using TALENs in zebrafish resulted in a fertility rate of female zebrafish reduced by 41% and an increase in the number of early vitellogenic follicles compared to wildtype fish. It

also reduced the concentration of vitellogenin in plasma and the expression of epidermal growth factor receptor. Thus, a role in control of ovarian follicle development can be attributed to Gper1[209]. In addition, Gper1 was shown to play a role as regulator of the embryonic heart rate by altering maternal estrogen levels and embryonic T3 levels[210]. Furthermore, Gper is a hepatic estrogen sensor that is involved in regulating the growth of the liver during development in both humans and zebrafish[211].

The zebrafish has been commonly used to identify endocrine-disrupting chemicals (EDCs) and assess their risks to humans and wildlife[147]. Research aims to develop screening assays to detect EDC activity and understand their mechanisms. About half of the zebrafish ER research focuses on endocrine-disrupting chemicals (EDCs)[120,199]. Such estrogenic EDCs include natural and synthetic hormones, phytoestrogens, pesticides, PCBs, BPA, phthalates, flavonoids, and polycyclic musks, primarily acting through ER agonistic or antagonistic interactions[212,213].

The most used biomarker for detecting ER-activating compounds is the concentration of vitellogenin (Vtg), an egg yolk precursor protein produced in the liver, or the levels of the *vtg* mRNAs coding for it. Vtg protein levels are hormonally controlled and can be measured in various tissues. In male zebrafish, which normally do not produce Vtg at all, its production can be induced by estrogens within 24 hours, making it useful for short-term EDC screening. While Vtg induction indicates ER activation, it can also be influenced by Ar, Pr and Gr activation[214]. Other phenotypic endpoints commonly used to track EDC activity in partial and full life-cycle studies are growth, sexual differentiation, sex ratio, egg production, and fertilization success[147,214].

Transcriptomic analyses have also been conducted to identify additional molecular biomarkers for ER activity[215]. These studies involved exposing adult male and female zebrafish to EE2 (17α-ethinylestradiol) and analyzing liver and telencephalon transcriptomes via microarray[216,217]. Another study analyzed

whole-body transcriptomes of male adults exposed to E2 that revealed a striking similarity between estrogen-responsive genes in male zebrafish and their homologs in human cancer cell lines[218]. Microarray studies were also performed by exposing zebrafish to estrogens during early developmental stages (1-4 days post-fertilization). In addition to the expected ER target genes, such as *vtg1, vtg3, vtg4,* and *esr1*, these analyses identified many metabolic genes and genes involved in cell cycle and DNA repair as regulated by ER[216–219].

Transgenic reporter fish have been generated for rapid screening of EDCs, where an ER-responsive promoter is linked to a reporter gene like luciferase or the Green Fluorescent Protein GFP. Legler and colleagues created a transgenic zebrafish line containing a luciferase gene controlled by a single estrogen response element (ERE) that can measure estrogenic activity after 96 hours of EDC exposure by measuring the luciferase activity in tissue homogenates. This assay is effective in both adult zebrafish and 30-day post-fertilization (dpf) juveniles, with the latter being more responsive during gonad differentiation[220]. Additionally, two GFP-based transgenic zebrafish lines were generated, in which the activity of the reporter gene can be easily observed in the living animal by microscopic observation[221]. In the first line, Tong et al linked GFP to the brain aromatase b (*cyp19a1b*) gene promoter expressed specifically in radial glial cells near the brain ventricles and strongly induced by estrogenic compounds[222]. In a second zebrafish transgenic line, a promoter containing five EREs drives GFP expression already in early embryonic and larval stages in the brain, liver, and pancreas upon estrogen exposure[219,221]. GFP was also induced in the ventral fin, olfactory organ, and heart, an effect that could be inhibited by the ER antagonist fulvestrant (ICI 182,780)[221]. Tissue-specific GFP responses were observed with EDCs like EE2, BPA, and genistein, with notable differences in adults; males showed no liver GFP expression, while females did, and ovaries and pituitaries exhibited GFP without exogenous estrogen exposure.

To gain some insight into which receptor may specifically be involved in specific effects of estrogenic compounds, knocking down genes using morphants has

also been coupled with exposure to chemicals to elucidate their EDC mechanism of action. For instance, a study by Bertotto and colleagues demonstrated that bifenthrin (a pyrethroid insecticide) exerts antiestrogenic effects in embryos, but estrogenic effects in juveniles leading to increased plasma concentrations of 17β-estradiol (E2) and altered expression of dopaminergic pathway components[223]. However, these effects were still present in *esr1* morphants, suggesting that Esr1 was not responsible for these observations[224]. In a similar approach, various effects (on body length, optic vesicle size, swim bladder inflation, pericardial and abdominal edema, and incidence of normal tail shape) of the anti-estrogenic compound Benzo(*a*)pyrene (BaP) were shown to be similar to those observed in Cyp19a1b-morphant zebrafish larvae or larvae treated with the Cyp19a1b inhibitor fadrozole. In each case, the effects could be relieved by adding E2, but most importantly the exposure to BaP caused a decrease of *cyp19a1a* expression at 96 hpf, suggesting that BaP may mainly act through decreasing Cyp19a1a expression leading to decreased concentrations of endogenous E2[225]. An alternative approach is the use of receptor-specific compounds, such as the Gper1 binding agonistic compound G1 who caused altered gene expression, cardiac edema, as well as other developmental abnormalities upon exposure[226]. Environmental pollutants can also target specific receptors such as the GPER1. The latter was proven by Gogola and colleagues where they concluded that POPs such as p,p'-DDE, HCB, PFOA, and PFOS can exert their estrogenic effects through GPER1 signaling in two human GC tumor-derived cell lines (COV434 and KGN)[227].

10. Mixtures of POPs and their various effects

POPs have been traditionally assessed using a reductionist and risk-based approach. In this regard, their effects are linked to a specific exposure to a specific chemical. However, in real life, exposure to a single chemical is rather the exception than the rule.

The main concern, and unsolved question, related to these mixtures is to know how these different chemicals will interact in causing a specific outcome: - will their effects simply add up; -will there even be synergism, causing stronger effects in combination than the sum of each compound alone; - will effects be mitigated by the combined presence of agonistic and antagonistic compounds for a specific target[228–231]. Answers will depend on the outcome considered, the targets (receptors) involved, and the nature of the mixture[232].

Several approaches (*in vitro, in vivo, in silico,* etc.) have been applied to test the capabilities of POPs as mixtures and acting as endocrine disruptors. In the frame of the PROTECTED consortium, and for this thesis, a human-relevant POP mixture (Total POP mix, TPM henceforth) as well as several sub-mixtures (6 in total) thereof were designed and generated by Berntsen and colleagues[233], based on the average levels of chemicals found in human blood and/or food and breastmilk of Scandinavian people (more details on this POP mixture within the research paper chapters)[234–236]. This mixture has been used by members of the consortium to test their effects on various biological systems. To characterize their effect through *in utero* and lactational exposure on the female mammary gland, ovarian folliculogenesis and liver function, CD-1 pregnant female mice were fed 3 g feed/day either containing, either a low or a high dose of the POP mixture. These exposures resulted in altered development of the mammary gland and suppressed ovarian (primary and antral) follicle maturation in the offspring, thus suggesting a significant endocrine disruption[237]. Using the same POP mixture, McComb and colleagues, have proven antagonistic effects following exposure to this mixture on androgen receptor transactivation and translocation,

with potential implications in the development of prostate cancer[238]. Another example of testing this mixture was portrayed in Wilson et al, where using high content analysis in recombinant U2OS cells expressing human glucocorticoid receptors (U2OS-GR), the authors concluded that the POP mixture did not exert effects at the GR translocation or produce any glucocorticoid agonistic effect[239]. However, specific POPs (*p,p'*-DDE (0.005 mg/ml)) within the mixture interfered with the activity of this nuclear receptor when tested alone.

In a different study using dioxin-responsive cells such as DR-H4IIE, DR-Hep G2 and DR-T47-D (rat hepatoma, human hepatoma and human mammary gland carcinoma, respectively), Doan and colleagues demonstrated that the same POP mixture can inhibit the transactivation activity of the aryl-hydrocarbon receptor and that, within such a complex mixture, only some compounds are responsible for the mixture's activity[240]. Also, the same mixture was tested on human hepatocyte HepG2 cells in a high content screening approach, where Wilson and colleagues[241], demonstrated that some of the sub-mixtures (Brominated and Chlorinated mixtures) caused Reactive Oxygen Species production but not apoptosis[241].

In A-498 human kidney cells, this POP mixture and some sub-mixtures influenced the cell count, nuclear area and mitochondrial membrane potential[242], while it enhances the nerve-growth-factor-induced neurite outgrowth in PC12 cell at high concentration[243].

The presence of POPs as mixtures has also been linked to the disruption of thyroid hormone levels in newborn infants. This has been tested in human populations, where relationships between the concentrations measured of multiple POPs in mothers' breast milk correlated with a disbalance of the concentrations of thyroid hormones[244,245].

POP mixtures can exert their effects at different potencies, targeting sometimes unexpected receptors. Gogola and colleagues investigated tumorigenesis in granulosa cells (GC), the steroidogenic supportive cells surrounding the developing oocyte follicle (immature egg cell). The researchers demonstrated that POPs such as p,p'-DDE, HCB, PFOA, and PFOS may stimulate cell

proliferation in two human GC tumor-derived cell lines (COV434 and KGN). The effects of individual compounds and of the mixture were mediated mainly by the insulin-like growth factor 1 receptor IGF1R or GPER1, as shown using specific inhibitors. Interestingly, their findings point to a weaker mixture effect than would be predicted by a synergistic or additive model of the effects of each compound tested individually and that the estrogenic effects were not induced by interaction with the NR, but GPER1, thus suggesting a non-genomic endocrine effect [227]. This example highlights the complexity of studying chemical mixtures and their effects.

11. Aim of this work

This work was integrated into the framework of the larger European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Innovative Training Network (ITN) program PROTECTED (Grant agreement No. 722634) which was designed to develop specialized knowledge and protection capabilities against the so-called endocrine disruptors (EDs), with a particular focus on the impact due to the real-life exposure to various potentially deleterious compounds.

To that end, our contribution falls within the *in vivo* testing category, thus, we added the zebrafish embryo toxicity testing to the set of endpoints that were tested using the POP mixtures available in the consortium. In our study, we performed a comprehensive panel of biological tests to screen for harmful effects following exposure to the POP mixture, including lethality, teratogenicity in eye, bone, cartilage, presence of edemas and other commonly assessed malformations, changes in swimming behavior, cardiotoxicity, and development of the posterior lateral line.
To evaluate the potential for endocrine disruption of these POPs, we determined the changes in whole body, whole genome transcriptome to define which genes or developmental processes would be affected.

Finally, we decided to focus on the most studied endocrine disrupting effect, interfering with estrogen signaling. Considering the complexity of this pathway and the difficulties to assign specific effects to specific receptors, we generated mutant zebrafish lines that lack individual estrogen receptors (ERs) to explore the potential antagonistic effects on development, providing insights into the role of estrogen signaling in mediating these effects.

12. Structure of the thesis

The information generated during this research project has been published in three peer-reviewed papers. Each of the sections in the following chapter present first the aims of each research paper, then the published article and finally a section with the highlights.

On the first part (Research paper 1), using a battery of biological tests, we assessed the effects of an environmentally relevant Persistent Organic Pollutants mixture (POP mixture) on zebrafish larvae and overall development. Furthermore, using RNAseq, it is explained how some mechanisms are disrupted and biologically plausible links between effect, adversity and exposure are established.

The second part (Research paper 2) delved into the role of this POP mixture in bone and cartilage development. More specifically, we explored the adverse effects following exposure on osteotoxicity.

Finally, the last part (Research paper 3) analyzed the antagonism of chemicals using a novel approach. Here the CRISPR/Cas9 technique was applied to knock out genes that express estrogen receptors., with the aim to mimic total antagonism. Then, the same battery of biological tests described in papers 1 and 2 was used to assess the same previously studied endpoints.

Details of published work:

Research paper 1

- A Realistic Mixture of Persistent Organic Pollutants Affects Zebrafish Development, Behavior, and Specifically Eye Formation by Inhibiting the Condensin I Complex.

  Gustavo Guerrero-Limón, Renaud Nivelle, Nguyen Bich-Ngoc, Dinh Duy-Thanh, and Marc Muller

Toxics, 2023, 11, 357, https://doi.org/10.3390/toxics11040357

Research paper 2

- A realistic mixture of ubiquitous persistent organic pollutants affects bone and cartilage development in zebrafish by interaction with nuclear receptor signaling

  Gustavo Guerrero-Limón, Jérémie Zappia, Marc Muller

PLoSONE, 19(3): e0298956, https://doi.org/10.1371/journal. pone.0298956

Research paper 3

- Exploring Estrogen antagonism using CRISPR/Cas9 to generate specific mutants for each of the receptors.

  Gustavo Guerrero-Limón, Marc Muller

Chemosphere, 364, https://doi.org/10.1016/j.chemosphere.2024.143100

13. Main results

a. Chapter 1 – Research paper "A Realistic Mixture of Persistent Organic Pollutants Affects Zebrafish Development, Behavior, and Specifically Eye Formation by Inhibiting the Condensin I Complex"

Aim

The objective of this research is to employ the zebrafish larvae as a model organism to study the adverse developmental effects of exposure to a realistic mixture of persistent organic pollutants (POPs) and specific sub-mixtures. The study aims to investigate how these mixtures impact zebrafish development, behavior, and particularly eye formation by examining the mechanisms at play, including the role of the condensin I complex. Additionally, this study includes RNA-seq analysis to elucidate potential molecular mechanisms behind the observed developmental and behavioral changes.

toxics

MDPI

Article

A Realistic Mixture of Persistent Organic Pollutants Affects Zebrafish Development, Behavior, and Specifically Eye Formation by Inhibiting the Condensin I Complex

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Abstract: Persistent organic pollutants (POPs) are posing major environmental and health threats due to their stability, ubiquity, and bioaccumulation. Most of the numerous studies of these compounds deal with single chemicals, although real exposures always consist of mixtures. Thus, using different tests, we screened the effects on zebrafish larvae caused by exposure to an environmentally relevant POP mixture. Our mixture consisted of 29 chemicals as found in the blood of a Scandinavian human population. Larvae exposed to this POP mix at realistic concentrations, or sub-mixtures thereof, presented growth retardation, edemas, retarded swim bladder inflation, hyperactive swimming behavior, and other striking malformations such as microphthalmia. The most deleterious compounds in the mixture belong to the per- and polyfluorinated acids class, although chlorinated and brominated compounds modulated the effects. Analyzing the changes in transcriptome caused by POP exposure, we observed an increase of insulin signaling and identified genes involved in brain and eye development, leading us to propose that the impaired function of the condensin I complex caused the observed eye defect. Our findings contribute to the understanding of POP mixtures, their consequences, and potential threats to human and animal populations, indicating that more mechanistic, monitoring, and long-term studies are imperative.

Keywords: SVHC; persistent organic pollutants; POP; PFOS; zebrafish; development; behavior; condensin I

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1. Introduction

Sixty years ago, Rachel Carson started raising awareness about persistent organic pollutants (POPs) in her book "Silent Spring". She documented the deleterious effects caused by the indiscriminate use of DDT. Ever since, research has proven and continues to prove her point; POPs have been listed in the "Stockholm Convention on Persistent Organic Pollutants" [1]. The European Union and the United Nations Environmental Program define the persistent organic pollutants (POPs) as "chemical substances that are hard to degrade, with a tendency to bioaccumulate, transfer through the food web rather easily, transport across international boundaries, and having long half-lives" [1,2]. They have been extensively linked to adverse health effects [3–7]. POPs, though very relevant for modern life, are normally studied in a reductionist approach, where a single compound is targeted and tested. Nevertheless, POPs are rarely found as stand-alone compounds in nature [8–10]. Mixtures are the rule [11], and their effects have not been widely described yet. To understand potential threats resulting from this exposure, studies have been carried out using chemical mixtures with different approaches ranging from molecular biology to transgenerational studies, using in vitro, in vivo, and in silico techniques. Studies recently focused on a constructed mixture of POPs that was designed based on the levels found in the

blood of a Scandinavian human population [12]. Using cell reporter assays, this mixture was found to antagonize the androgen receptor transactivation and nuclear translocation [13], to inhibit the transactivation activity of the aryl hydrocarbon receptor [14], and to induce cytotoxicity while it enhances nerve-growth-factor-induced neurite outgrowth in PC12 cells at high concentrations [15]. Microscopic high content analysis (HCA) revealed that some sub-mixtures affected cell number, nuclear area, and mitochondrial membrane potential in A-498 human kidney cells [16].

In this study we have chosen zebrafish larvae due to their many technical and practical advantages. To name a few, the zebrafish (*Danio rerio*) shares a non-negligible amount of genetic pool with humans (up to 80%), small size, and ease of maintenance in captivity, high fecundity and short times till adulthood and reproduction, amongst many others [17]. In this context, research using zebrafish has shed some light on the toxicological features of chemical compounds. Therefore, the aim of this research was to employ zebrafish larvae as a model organism to describe the many adverse developmental effects caused by realistic doses of the POP mixture and of specific sub-mixtures. Furthermore, we performed RNA-seq analysis on the larvae exposed to the POP mixture, aiming at elucidating the mechanism of action for specific observations.

2. Materials and Methods

2.1. Zebrafish Husbandry and Ethical Considerations

Adult wild-type zebrafish of the AB strain and the transgenic line *Tg(kdrl-mls:GFP)* [18] were obtained from breeding facilities at the GIGA-Institute, Liege, Belgium. Fish maintenance, breeding conditions, and egg production were described in detail [19,20] and are in accordance with internationally accepted standards. Animal care and all experimentation were conducted in compliance with Belgian and European laws (Authorization: LA1610002 Ethical commission protocol ULg19-2134 and Ulg19-2135).

2.2. Chemicals and POP Mixture

Dimethyl sulfoxide (DMSO, >99.9%, CAS number 67-68-5) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The stock solutions for the total POP mixture and six sub-mixtures were designed and prepared by the Norwegian University of Life Sciences, Oslo, Norway [12] as indicated in Supplementary Table S1. Briefly, the total POP mixture was designed to represent a mixture of 29 compounds at 1,000,000-fold the mean concentrations found in the blood of a Scandinavian population, while the sub-mixtures consisted of either one single class of these compounds (PFAA, Br, Cl) or of two combined (PFAA + Br, PFAA + Cl, Br + Cl) classes. Stock solutions of POP mixtures and their sub-mixtures were prepared in DMSO and stored at −20 until the day of testing. For all treatments, we used the stock solution (1,000,000×) that was further diluted on the testing day in E3 zebrafish raising media [21]. Next, the concentration of DMSO was corrected to achieve 0.1% in all cases, including the control groups.

2.3. Exposure Tests

Exposure tests were performed in 6 well-plates, with 25 fertilized eggs per well in 4 mL of E3 medium supplemented or not with the test compounds. For each experiment, 150 fertilized eggs were selected, 50 as controls and 100 for the specific treatment, to ensure a sufficient number of treated individuals for the tests. Each treatment was repeated at least three times in independent experiments. To keep stable chemical concentrations, we used a static-renewal approach where at least 90% of the media was refreshed every 24 h. Exposure started between 0 to 6 h post fertilization (hpf); the larvae were treated for at least 96 h. Finally, following the guidelines of the OECD Test number: 236, we tested 8 different concentrations (1×, 5×, 25×, 75×, 125×, 250×, 500×, 1000×) to estimate the median lethal concentration LC_{50}.

2.4. Morphological Observations

A set of morphological features was recorded, including presence of edemas, inflation of the swim bladder, eye malformations, etc. Pictures of treated and untreated larvae at different stages were taken. All observations were made with a stereomicroscope Leica M165 FC (Leica Microsystems©, Leica, Wetzlar, Germany). Standard length was estimated in fish at 5- and 10-days post fertilization (dpf) using FIJI line tool for measurement (ImageJ2, v. 2.3.0/1.53f).

2.5. Mitochondrial Toxicity

Estimation of the mitochondrial integrity in the blood vessels was conducted using the transgenic zebrafish line *Tg(kdrl-mls:GFP)*, which expresses the fluorescent protein GFP fused to a peptide targeting it to the mitochondria (MLS) under the control of the endothelial cell-specific promoter sequence of the zebrafish *kdrl* gene. Heterozygous parents for the transgene were crossed, offspring carrying the transgene were selected based on fluorescence at 24 hpf and separated in control and treated groups. Then, the exposure test was carried on as described above. Fluorescence intensity was observed, and pictures were taken at 120 hpf using the epifluorescence stereomicroscope Leica M165 FC (Leica Microsystems©). Then, fluorescence was quantified using FIJI. Since the transgene is expressed in all blood vessels (head, heart, etc.), to avoid overestimation of the intensity, values were obtained from sectioning the body in a lateral view and using only the tail, from the opening of the anus to the caudal peduncle. Each intensity value was determined using the corrected total fluorescence (CTF) [22] and expressed as RFU (relative fluorescence units).

2.6. Heart Rate

Heartbeats were counted manually using an inverted Nikon Eclipse TS100 microscope and a counter for 15 s. To obtain the beats per minute (BPM), measurements were multiplied by 4. The heart rate was estimated on 96 hpf larvae that were acclimated to the lighting conditions for no less than 5 min prior to counting; the larvae were not immobilized by anesthetics or other means. Each larva was observed sequentially at least three times. Ten larvae were observed per treatment and each experiment was performed at least in triplicate.

2.7. Behavior

Behavioral tests were conducted on zebrafish larvae at 98~120 hpf and every test was performed between 10:00 and 13:00 to maintain a constant position in the circadian cycle. During the entire exposure period to the chemicals, special care was taken to avoid the interference of environmental factors. Exposed larvae were shielded from loud noises, changes in the temperature of the incubator (27–28 °C) and the raising media (~26° at the time of testing), changing light conditions and activities in the room by putting them in a specific enclosure harboring its own, constant dark/light regime. Prior to each behavioral test, the zebrafish larvae were inspected under a stereomicroscope to select and transfer to the testing plates only individuals devoid of any malformation that might interfere with mobility outcome (e.g., yolk sac or pericardial oedemas, spinal aberrations, aberrations in pigmentation, and/or loss of equilibrium, etc.). The larvae were placed individually in a well of a 96-well plate and observed using a ViewPoint® Zebrabox system and its tracking software (ViewPoint Life Sciences, Lyon, France). The light level was set to 20% on the ViewPoint software (7.45 klux, TES 1337 light meter), while infrared light (850 nm) was used to track larval activity. We applied a light–dark cycle that lasted for a total of 1 h and consisted of 20 min of light, allowing for the larvae to acclimate to the situation and discarded from the analysis, followed by 10 min of darkness, 10 min of light, 10 min of darkness and 10 min of light. The video and tracking software were used to screen larval locomotion behavior for 10 s intervals; the distance travelled, and the time spent

active were determined and, from these parameters, the mean swimming speed was also calculated by dividing the cumulated distance travelled by the total time spent active.

2.8. Injection of Antisense Oligonucleotide Morpholino

As previously described [23,24], one cell-stage embryos were injected with a concentration of 100 µM of MO^{p53} (MO, Gene Tools Inc., Philomath, OR, USA). The morpholino was diluted in Danieau buffer and 0.5% tetramethylrhodamine dextran (Invitrogen, Merelbeke, Belgium). To assess the effects of morpholino injection, 150 individuals were microinjected in two independent experiments, followed by exposure to chemicals as described above. Sequence of the morpholino oligonucleotide:

MO^{p53}: 5′-GACCTCCTCTCCACTAAACTACGAT-3′

2.9. RNA Extraction

RNA was extracted from pools of 65 larvae at 5 dpf using the RNA mini extraction kit (Qiagen, Hilden, Germany). Samples were lysed in RLT+ buffer with β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and homogenized at least 10 times with a 26-gauge needle in a 1 mL syringe. An amount of 22 µL of RNAse free water was used to resuspend total RNA. RNA extract was treated with DNAseI (Qiagen, Hilden, Germany) to avoid DNA contamination. Quantity (ng/µL) and quality (260/280 and 260/230 ratios) of each extract was assessed by nanodrop spectrophotometer measurements. Poor quality (260/280 < 2; 260/230 < 2) samples were subsequently purified by lithium chloride precipitation, followed by 2 times pellet washing with 70% ethanol, and resuspended in 51 µL of RNAse-free water and stored at −80 °C. The integrity of total RNA extracts was assessed with BioAnalyzer analysis and provided RIN (RNA integrity number) scores for each sample (Agilent, Santa Clara, CA, USA).

2.10. RNAseq

cDNA libraries were generated from 100 to 500 ng of extracted total RNA using the Illumina Truseq mRNA stranded kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. cDNA libraries were then sequenced on a NovaSeq sequencing system, in 1 ×100 bp (single end). Approximatively 20–25 M reads were sequenced per sample. The sequencing reads were processed through the Nf-core rnaseq pipeline 3.0 [25] with default parameters and using the zebrafish reference genome (GRCz11) and the annotation set from Ensembl release 103 (www.ensembl.org; accessed 1 May 2020). Differential gene expression analysis was performed using DESeq2 pipeline [26]. Pathway and biological function enrichment analysis was performed using the WEB-based "Gene SeT AnaLysis Toolkit" (http://www.webgestalt.org; accessed on 10 November 2022) based on the integrated GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes) [27,28], Panther, and WikiPathways databases (all accessed on 10 November 2022 via http://www.webgestalt.org). An additional database was constructed using the Gene-mutant/Phenotype database from zfin (zfin.org; accessed on 6 March 2023). The cut-off values were set for the false discovery rate (FDR) to "adjusted p value < 0.05" and the fold change > 1.5.

2.11. Data and Statistical Analysis

For the estimation of the lethal concentration (LC_{50}), data were transferred to R (4.0.2) [29] and the command "*dose.p*" used in the library "MASS" [30]. Morphological and fluorescence data were transferred to Prism 9.0.0 (v86) (Graphpad, San Diego, CA, USA). Each data set was tested for normality (e.g., using a visual cue (QQ plot), D'Agostino–Darling and Shapiro–Wilk tests) and equal variances (Bartlett's test). Thus, parametric or non-parametric tests were performed, as indicated in each case in each figure.

Raw behavioral data sets consisted of tables holding the positions of each larva in each video frame (30 frames/second). This table was first trimmed to eliminate very

short, oscillating, and likely artefactual movements, and then aggregated into 10-s periods for further analysis. These data were transferred to R version 4.0.2 to analyze motility during the dark and light phases. To assess behavior, we used linear mixed effect (LME) models within the "nlme" package [31]. Three dependent variables were used, either the "mean time spent active" (seconds), the "mean distance travelled" (mm), or the "mean swimming speed" (calculated as the mean distance travelled/mean time spent active) within each 10 s period, with "compound" and "time" as the categorical and continuous independent variables, and "batch" as a random effect. The "*Anova*" command within the "*car*" library [32] was used to extract the results for the main effects whereas the "*lsmeans*" command [33] 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 [34]. Type II sum of squares was used for the model. Two kinds of analyses were performed (see also below in results): the "startle" response including the 10 s prior to change of phase (light to dark, or dark to light) with a length of 50 s and the values obtained for 560 s after the spike (the remaining time of the phase). Confidence was assigned at $\alpha = 95\%$ and a p-value of ≤ 0.05 was considered as significant, $p \leq 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***).

3. Results and Discussion

3.1. LC_{50}–Chronic Exposure to Total POP Mix Is Lethal at Relatively High Doses

In a preliminary range-finding experiment, we exposed AB zebrafish fertilized eggs to eight concentrations (1×, 5×, 25×, 75×, 125×, 250×, 500×, 1000× the mean human blood concentration) of the total POP mixture and we monitored survival at 24, 48, 72, and 96 hpf (Figure 1A) compared to untreated controls. The median lethal concentration (LC_{50}) for this mixture was calculated at 386-fold the human blood concentration (386×). Consequently, in the following experiments, we limited the concentration range to 75×, 125×, and 250× the mean human blood concentration. This may seem high considering a normal population; however, we must consider that the chorion is a protective layer, which can be crossed easily by molecules with a size below 4000 Da [35]. The POPs studied here are hydrophobic and of small size; thus, they are potentially able to cross the chorion and to exert their effects right after adding the solutions to the media. Previous experiments revealed that only about 10% of, e.g., PFOS could be found in zebrafish larvae exposed (continuously, i.e., without medium change) for 96 hrs to the compound [36], while between 0 and 16% of the nominal amounts were found in larvae exposed to the POP mixture [37]. Given their high bioconcentration values (BCF factor used to estimate the potential to bioaccumulate) and the persistent nature of these chemicals [38,39], the harmful concentrations used here may be, eventually, reached in individuals that are constantly exposed, exerting their effects in later stages in life while continuing to accumulate through the many pathways of exposure [40–42]. Thus, we can assume that, in our experiments, larvae are exposed to concentrations of the POP mix that may be reached in exposed populations [43].

3.2. The POP Mix Significantly Reduces the Standard Length of Zebrafish Larvae

We first evaluated the effect of the POP mixture on general growth by measuring the standard length of the larvae at 5 dpf after continuous exposure to the POP125× (125× human blood concentration) mix. In parallel, we also tested equivalent concentrations of the different sub-mixtures. At 5 dpf, the average size of the larvae was significantly affected, fish treated with the total POP125× mix were significantly smaller by about 10% (from 3.4 to 3.1 mm) (Figure 1C). Among the single sub-mixes, only the PFAA mix resulted in a significantly decreased standard length, similar to but slightly less than the POP125× mix. Cl and Br mixes alone did not cause a clear effect. In line with these observations, only the binary mixtures containing PFAA (PFAA + Cl, PFAA + Br) caused a similar effect on standard length comparable to PFAA alone. Cl + Br had no effect. Thus, only those mixtures containing PFAA affected the size of zebrafish larvae at 5 dpf.

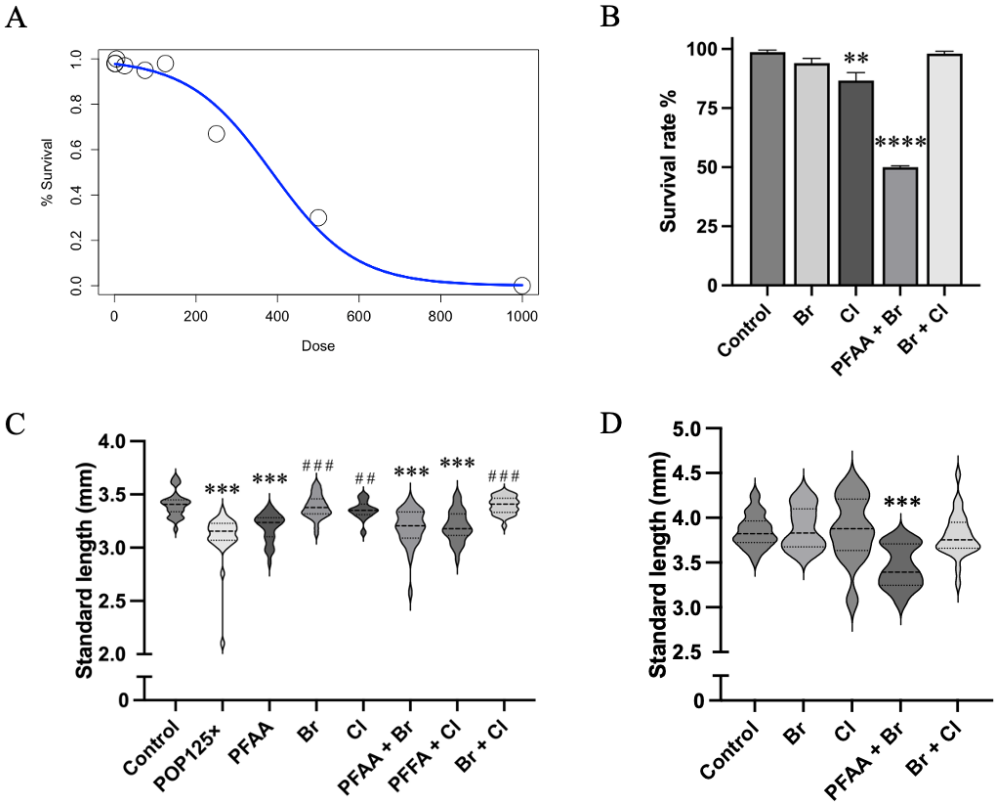


Figure 1. (**A**) Survival after 96 h of exposure to POP mixture. Survival decreases drastically at 386×. (**B**) Survival rate for POP mixtures and sub-mixtures at 10 dpf; ordinary one-way ANOVA and Dunnett's multiple comparisons test. (**C**) Standard length of fish at 5 dpf. Data presented as median with higher and lower quartiles for each treatment. Asterisks (*) indicate when significant differences were found compared to control, hash sign (#) when differences were found relative to PFAA alone. (**D**) Fish measured at 10 dpf. Missing groups due to high mortality rates were not included. Kruskal–Wallis and Dunn's multiple comparison tests, $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). In short, PFAA < Total Mix < Cl = Br = Control.

Previously, exposure to PFOA 4 ng/mL has been found to decrease the body length of 3 dpf zebrafish larvae, but not at 40 or 400 ng/mL [44]. In contrast, other PFAS (PFBA, PFHxA) did cause decrease of size at 40 and 400 ng/mL. Another study revealed that PFOS or PFOA decreased total body length at 200 or 2000 ng/mL, respectively [45]. Here, we used 217 ng/mL in the 125× POP mix for PFOA, in addition to PFOS and PFHxS, to analyze the effect on standard length at 5 dpf. Taken together, there is clear evidence that PFAS affects larval growth.

After halting the exposure (at 96 hpf) we kept the larvae to grow in normal E3 medium, free of POPs until 10 dpf. We observed a significant lethality during this period, which was not assessed in the preliminary experiment (Figure 1B). Among the surviving larvae, only those exposed to PFAA + Br were significantly smaller (Figure 1D), while all other treatments including PFAA, as well as the total mix, left no survivor. PCB and PBDE congeners were previously shown to impact survival of zebrafish larvae at concentrations around 1–5 µg/mL [46,47]; we did not observe a significant lethality induced by the Cl and Br mixtures here, suggesting that the congeners present in the POP mix are indeed less toxic.

3.3. Common Developmental Toxic Effects Such as Edemas and Non-Inflated Swim Bladder Were Commonly Found following Exposure to POP Mix

We also looked for other developmental defects induced by the different treatments, according to the recommendations for the Zebrafish Embryotoxicity test [48]. The most

striking features observed were the presence of edemas and non-inflated swim bladder. Even at the lowest concentration of the total mix (POP 75×), a relatively large proportion of the population had edemas (~70%). Using the swim bladder as another phenotypical endpoint to assess developmental retardation, a high number of fish (close to 100%) presented developmental impairment of their swim bladders after 96 hpf compared to only 50% in the controls at this stage (Figure 2). While edemas are commonly observed in toxicity assays, they may have been caused in our experiments (whether yolk sac or pericardium) by the presence of PBDE47 [49]. The lack of inflation of the swim bladder is less common; it was previously reported as being affected by exposure to PFOA at 4.7 ng/mL [50] by interfering with thyroid hormone signaling. Other chemicals present in the mixtures may in addition contribute to disruption of thyroid hormone action [50]. Underdevelopment of the swim bladder would have important ecological consequences, impeding the normal swimming of the larvae at a crucial age.

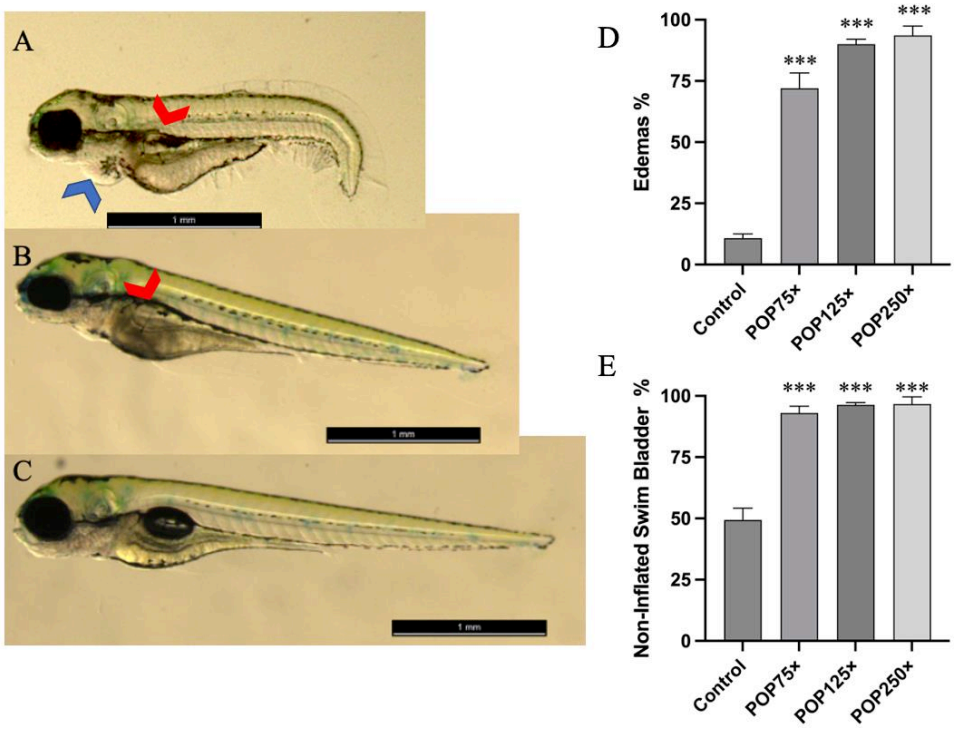


Figure 2. Examples of fish treated and untreated with the POP mix. Several malformations and size differences are striking. Pictures were cropped purposefully to enhance the differences found. (**A**) Fish treated with POP250x, red arrow pointing at the non-inflated swim bladder, blue arrow pointing at a pericardial edema. (**B**) Fish treated with POP125×. (**C**) Control, size bar = 1 mm. (**D**) Edema, as percentage of the population having this malformation. (**E**) Non-inflated swim bladder at 96 hpf, as percentage of population. Data presented as mean percentage of population having either malformation and standard deviation; ordinary one-way ANOVA and Tukey's multiple comparison test, $n = 100$, $p \leq 0.001$ (***).

The deleterious effects we describe here were only seen after 72 hpf. In a preliminary experiment (data not shown), we tested the capabilities of early developmental disruption of the POP mix and could not find a clear effect at stages earlier than 72 hpf, almost three full days of continuous exposure. A similar observation was made previously when testing pharmaceutical pollutants, some of which exerted their effects mainly on 72 and 96 hpf larvae [21]. We hypothesized that, though the chemicals would cross the chorion and be taken up by the embryos, the absence of some targeted molecules at these early stages would make them impervious to the POPs' effects. That would be the case of thyroid follicles that start developing after 96 hpf [51]; hence, some PBDEs would not be exerting their effects through this pathway until a later stage.

3.4. Striking Eye Malformation in Fish Treated with Any of the POP Mix and Its Sub-Mixes

One outstanding feature we observed was the pear-like shaped eyes, with dents in the polar regions on the eyeball of the fish treated with the POP mix (Figure 3A–C). Compared to the control group (~7%), treatment with the POP75× mix affected about 50% of affected larvae, while POP125× affected about 70% and POP250× close to 90% of the fish (Figure 3D). When we tested the sub-mixtures at 125× the mean human blood concentration, we observed that each of the single mixes caused a slightly lower fraction of affected individuals (Figure 3E) compared to the total POP125×. However, the dual combinations PFAA + Br or PFAA + Cl reached similar levels to POP125×; addition of either Br or Cl significantly increased the incidence of this malformation relative to PFAA alone, indicating that each sub-mixture contributed to various degrees to the effect caused by the POP mix.

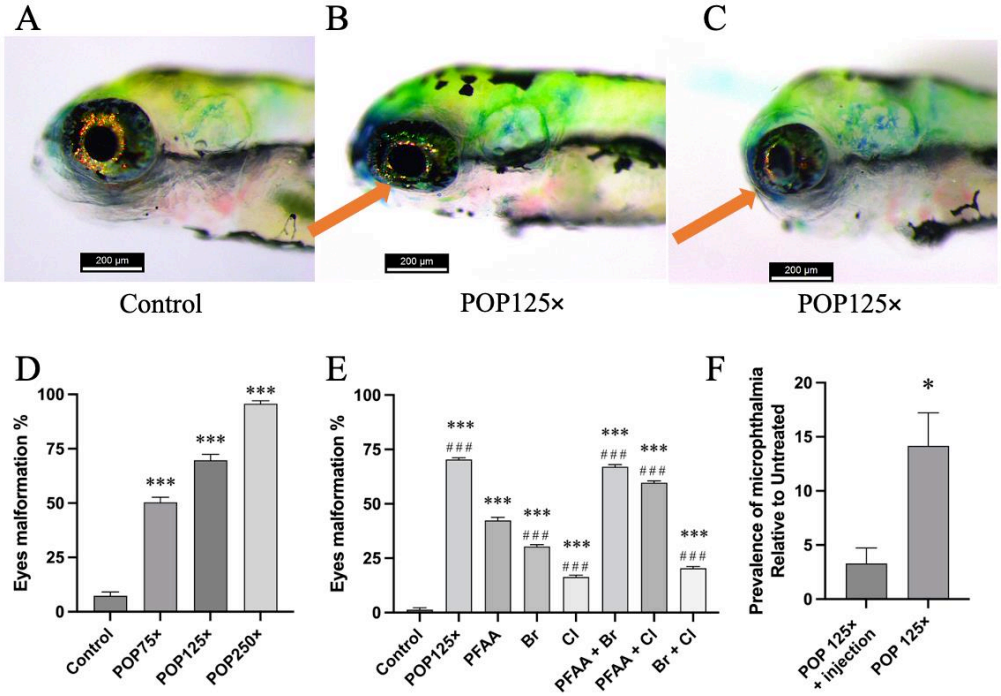


Figure 3. Example of eye malformation. (**A**) Untreated larva 4 dpf; (**B**) flattened eye with dents at both the upper and lower side of the eye and (**C**) eye hypoplasia of treated larvae with POP125×; (**D**) dose-based prevalence of eye malformation; (**E**) prevalence of eye malformation in larvae upon treatment with the different POP sub-mixtures. Data is presented as mean percentage of population having eye malformation and standard deviation; ordinary one-way ANOVA and Šidák's multiple comparison test, $n = 100$, $p \leq 0.001$ (***). Asterisks indicate when significant differences were found compared to control, hash sign (#) when differences were found compared to PFAA. (**F**) Prevalence of eye malformation present in fish exposed to POP125× relative to untreated individuals. Columns represent the effect of POP125 treatment in larvae previously injected with Mo^{p53} (POP125× + injection) or not (POP125×). A Fisher's exact test revealed the difference with a significance of $p = 0.055$ (*).

This malformation of the eyes was one of the most striking and unexpected effects. Previous studies have shown a link between eye malformation and certain compounds or the suppression of expression of certain genes. Two main eye malformations are described in the literature: either eyes were absent (anophthalmia), or their size was reduced (microphthalmia). The first is linked to the absence of genes such as *chokh/rx3* [52], while the second is linked to the expression of many different genes, such as *sox2* [53], *otx2* [54], *pax6a*, or *pax6b* [55]. Regarding chemical exposure, these two morphological aberrations have been described after treatments with a variety of chemicals, such as phenylthiourea [56], gold nanoparticles [57], di-butyl phthalate [58], and PCBs (Aroclor 1254) [59]. Retinal

defects have been shown in workers exposed to solvents or heavy metal, and defects in photoreceptor cells were described in zebrafish exposed to PBDEs or PCBs [60]. However, to the best of our knowledge, this is the first time the pear-like shape and microphthalmia are described as a malformation caused by these kinds of mixed organic pollutants. We did not observe a correlation of this malformation with any of the other defects that we observed, indicating that a specific mechanism is involved.

One previously described zebrafish mutant, the *cap-g*^{s105} mutant, presents a reduction of retinal cell number and smaller eyes similar to what we observed here [61]. The *cap-g* gene codes for a component of the condensin I complex involved in the regulation of chromosome condensation and segregation during mitosis. The *cap-g*^{s105} mutation of this gene causes increased apoptosis in proliferating retinal stem cells, leading to a small eye phenotype, which could be partially rescued by interfering with the expression of the pro-apoptotic gene *p53*. We thus decided to test the effect of the POP125× mix on zebrafish larvae that had been previously micro-injected with antisense morpholino directed against the *p53* gene. Although injection of the MO^{p53} alone seems to generate some eye deformities on its own, the eye malformation induced by POP125× was significantly reduced in fish injected with MO^{p53} (Figure 3F), similar to what was observed for the *cap-g*^{105} zebrafish mutant [61]. Further support for this mechanism and the genes involved in this striking phenotype will be given in the transcriptome analysis section below.

3.5. Heart Rate Is Severely Affected after 96 h of Exposure, Especially When PFAAs Were Present

Next, we tested the effect of the total POP mix on the heart rate of the zebrafish larvae at 96 hpf. We witnessed a significant, dose-dependent increase in the heart rate upon treatment with the total mixture (Figure 4A). Testing the sub-mixtures at 125× concentration, the most pronounced effects were observed in those treatments where PFAA mix was present (Figure 4B). A weaker, but significant difference was observed as well with the Cl mix, while no significant effect was found using only the Br mix.

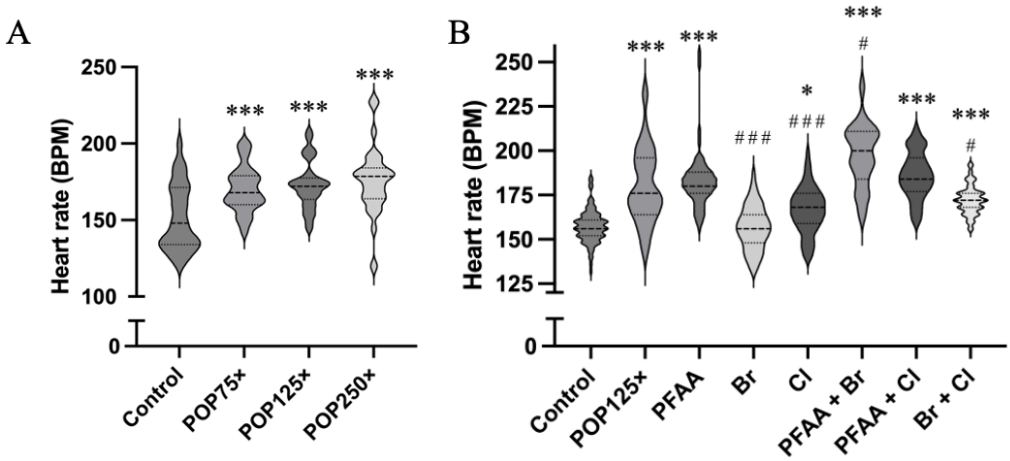


Figure 4. (**A**) Heart rate (BPM, beats per minute) of treated and untreated zebrafish using 3 different concentrations of the total POP mix. (**B**) Heart rate of all the fish exposed to the different treatments and measured at 4 dpf. Kruskal–Wallis test and Dunn's multiple comparison, $n = 30$, $p < 0.05$ (*), $p \leq 0.001$ (***). Asterisks indicate when significant differences were found compared to control, hash (#) sign when differences were found compared to PFAA.

All binary mixtures (PFAA + Br, PFAA + Cl, Br + Cl) significantly increased the heart rate; however, the PFAA mix exerted a dominant effect while the increase caused by Br + Cl was clearly lower compared to those due to any of the treatments where PFAA was present. The highest heart rate was recorded in the fish exposed to the binary mixture PFAA + Br (197 ± 17 BPM vs. Control = 157 ± 9 BPM).

PFOS and PFOA have been shown to increase the heart rate in 72 hpf zebrafish larvae at, respectively, 500 ng/mL and 75 µg/mL [62], indicating that PFOS may be the main

agent here. Chemicals such as perfluorononanoic acid (PFNA) can alter gene expression linked to cardiac development by dysregulating genes such as *amhc*, *nppa*, *nkx2.5*, *edn1* and *tgfb2* [63], but no effect on heart rate was shown. Similarly, there are 12 dioxin-like PCBs (e.g., PCB 118) that have been associated with heart conditions such as hypertension and cardiac defects [64,65]. These effects have been linked to the activation of the aryl hydrocarbon (AhR). More interestingly, even small doses of 1,2,5,6-tetrabromocyclooctane (HBCD) cause arrythmia through dysregulating the function of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA2a) [66]. The latter is encoded by the *atp2a2a* and *atp2a2b* genes, of which only *atp2a2a* was significantly induced after exposure to POP125× (see below). Thus, the role of Atp2a2 in inducing arrythmia is very likely, although the precise mechanism remains unclear. Finally, the cardiotoxic effects of organochlorine pesticides have been clearly described previously [67]. The mechanisms may vary but most of the compounds within the different mixes undoubtedly have the potential to alter the heart rate, leading to cardiac conditions such as arrythmia, hypertension, and other cardiac defects.

3.6. Fish Were Hyperactive and Responded Notably to Changes in Illumination

To test for behavioral effects, indicative of potential neurological defects, we used a standard 10-min light–dark swimming activity protocol to assess the parameters "time spent active", "distance travelled", and "swimming speed" in 10 s intervals. Due to the high number of multiple malformations in the larvae in POP250x, this dose was excluded from the behavioral analyses.

3.6.1. Dark–Light Response

In Figure 5, we illustrate the changes in behavior observed after treatment with the total mix POP125× compared to the control, untreated larvae at 5 dpf. As expected, we observe an increase in all parameters in control larvae during the dark phase, compared to the light phase. We can also observe the initial increase in all parameters at the start of the dark phase, which decreases in time while the larvae acclimate to the new situation. This decrease is even much faster in the POP125×-treated larvae. In contrast, we observe a stronger spike in activity when switching to the light phase, indicating that the larvae do perceive the change in lighting conditions, but rapidly return to a slightly higher activity compared to the dark phase.

The effects recorded during the dark and light phases (excluding the spikes, see below) were different depending on the compounds used (Figure 6). For instance, POP125× was the only compound that increased the swimming speed (SWS) during the dark phase, whereas binary mixtures containing Cl (PFAA + Cl and Br + Cl) decreased the speed significantly compared to controls (Figure 6A). Cl alone decreased the speed significantly only when compared to PFAA, itself slightly, but not significantly, higher than control. Thus, it appears that Cl was mainly responsible for decreasing swimming speed during the dark phase. During the light phase, more striking effects on the swimming speed were seen. Overall, all treatments (except Br) caused a faster swimming speed; however, only those mixtures where PFAA was present caused a significant increase compared to the control group. Fish exposed to the POP125× mix were swimming the fastest, followed by the binary mixtures PFAA + Br and PFAA + Cl, and finally PFAA (Figure 6B). Thus, while only PFAA alone caused a significant increase in swimming speed, addition of Cl or Br in the binary mixtures further enhanced this effect.

Time spent active (TSA) was similarly significantly affected (Figure 6C,D). POP125× caused the highest increase in activity in both the dark and light phases. During the dark phase, the presence of PFAA and PFAA + Cl significantly decreased TSA values, similar to the Cl and Br + Cl groups. During the light phase, a dramatic increase in activity was observed in fish exposed to POP125×, while PFAA, PFAA + Br, and PFAA + Cl caused a significant, but weaker, increase. Br, Cl, and Br + Cl did not affect TSA relative to control, they were thus significantly different from PFAA.

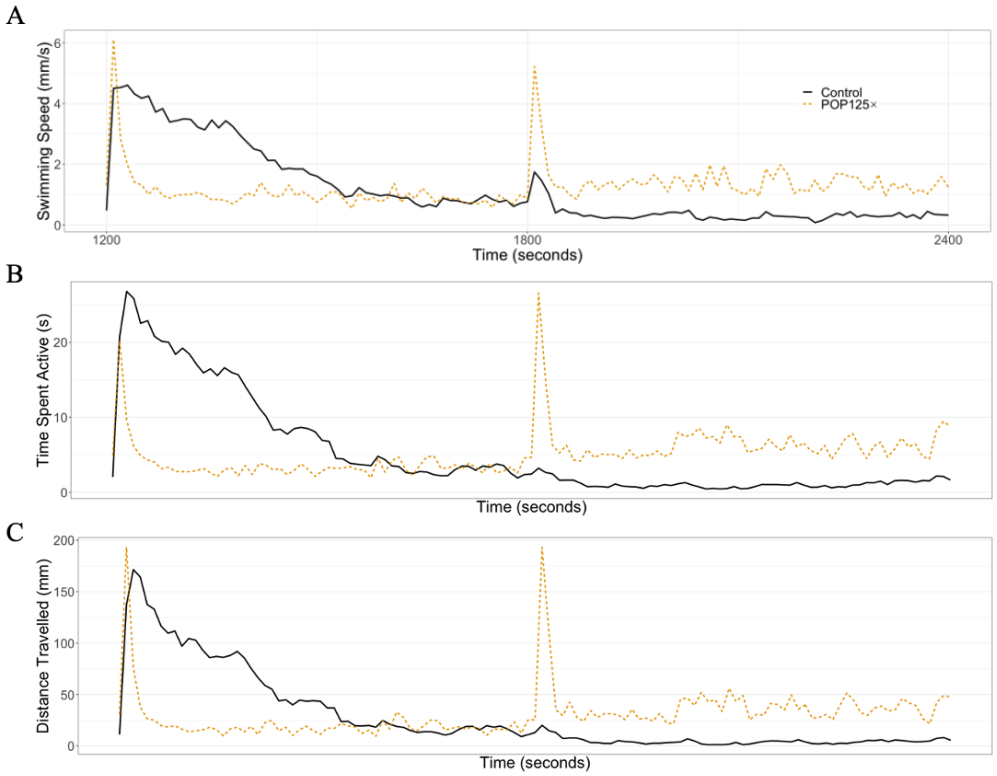


Figure 5. Actogram example of (**A**) swimming speed, (**B**) time spent active, and (**C**) distance travelled during 20 min of the tests, starting with 10 min dark phase followed by 10 min light. For comparison, control larvae are shown alongside POP125×-treated larvae. Two spikes can be appreciated when the fish entered a different phase.

The distance travelled (DT) during the dark phase was significantly higher in fish treated with POP125×, but lower in fish treated with PFAA + Cl and Br + Cl. During the light phase, a large increase in DT was seen upon POP125× treatment, while PFAA, PFAA + Br, and PFAA + Cl, caused weaker, but still significant, increases, similar to what was observed for the TSA (Figure 6E,F).

Behavior is a complex endpoint, hard to analyze, and where many variables could be playing a role and inducing changes. One of the first hypotheses we thought of to explain the altered behavior was related to compounds binding to brain aromatase or Cyp19a1b. This protein (or its isoforms) is present from 24 hpf [68] and several studies have reported changes in swimming behavior triggered by compounds such as fadrozole (a well-known aromatase inhibitor) or other endocrine disrupting chemicals like a PCB mixture (aroclor 1254), PBDE-47, or PFOA in various fish species [69–73]. However, involvement of classical endocrine disruptors was ruled out for environmental effects, either due to the high concentrations used (fadrozole), or to the observation that hormone antagonists did not revert the changes [73]. In our experiments, the most obvious alterations of behavior were caused by the PFAA sub-mix (with PFOA and PFOS at the highest concentrations in the mix). We also observed a significant effect of Br or Cl, also adding to the effect when used in combination with PFAA, although never reaching the extent of the full POP125× mix. Thus, we cannot discard eventual synergistic or additive effects caused by the presence of the other chemicals within the mix. The behavior altering properties of PFAAs have been described before, in various settings. At very low concentrations between 7–700 ng/L, PFOS or PFOA led to decreased activity in 5 dpf larvae when tested alone, but increased activity when both compounds were tested together [72]. Increased activity was also observed for PFOA at 400–4000 ng/mL [44] or at 300 to 2000 ng/mL considering only the dark phase [37], consistent with our findings. These authors also suggested that hyperactivity was linked to alterations in calcium signaling involving the

ryanodine receptor Ryr and affecting muscular contractions. Also, sensitization of the RYR by PCBs (e.g., 28, 138 and 153) can cause a developmental neurotoxicity [74], hence affecting the photomotor response of the larvae.

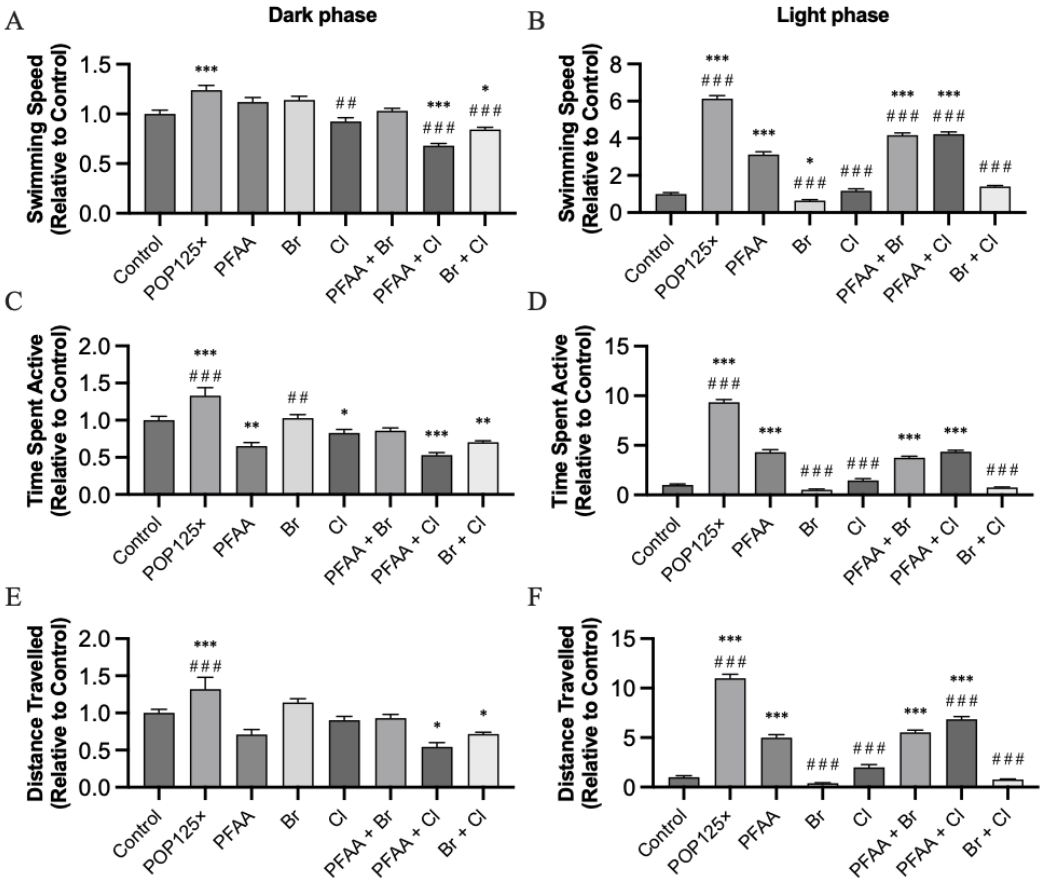


Figure 6. Behavior tests during the dark (**left**) and light (**right**) phases: swimming speed (**A**,**B**), time spent active (**C**,**D**), and distance travelled (**E**,**F**) for all treatments. All data were calculated excluding 50 s in the transition zone between light and dark phases, and the values were normalized relative to the corresponding controls for dark and light phases, $n = 72$, $p < 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***). Asterisks (*) indicate when significant differences were found compared to control, hash (#) sign when differences were found against PFAA.

3.6.2. Startle Response

As mentioned above, we noticed that each time fish were recorded, we could observe a dramatic increase in their activity (spike) at the moment of drastic transition from light to dark or back to light. Thus, we decided to focus on this spike response by analyzing only the 50 s around the transition. For the light–dark transition, we observed an increased startle response for the POP125× mix, which was even higher for the PFAA sub-mix, but somehow attenuated by addition of Br in the PFAA + Br sub-mix and Cl in the PFAA + Cl sub-mix reaching significance only for swimming speed (Figure 7). Interestingly, Br alone had no effect, while Cl alone significantly increased all parameters relative to controls. In the dark–light transition, this increased response was also observed, this time higher for the POP125× mix compared to all other mixtures containing PFAA. Br and Cl alone or in combination had no effect at all compared to control, while only marginally modulating the effect of PFAA in binary mixtures.

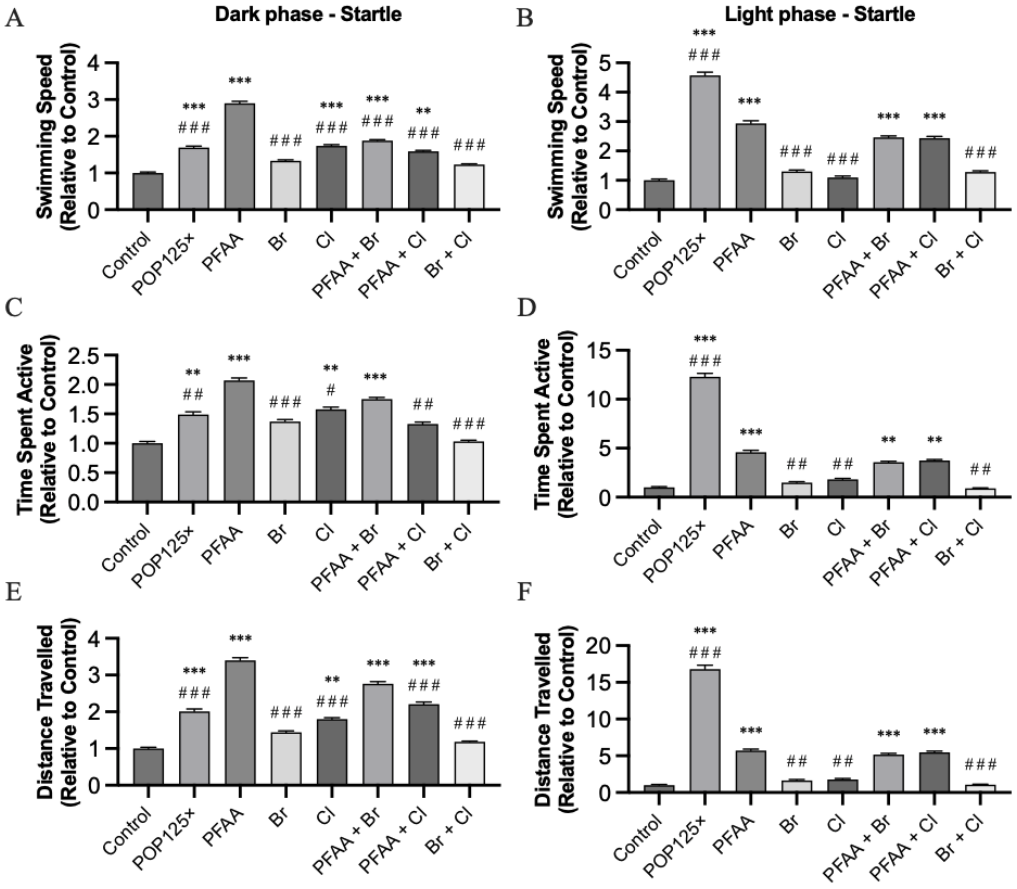


Figure 7. Swimming speed (**A**,**B**), time spent active (**C**,**D**), and distance traveled (**E**,**F**) for all treatments. Left column, parameters during the dark phase, right column, parameters during the light phase. All results were calculated using 50 s during the transition zone between light and dark phases, $n = 72$, $p \leq 0.01$ (**), ≤ 0.001 (***). Asterisks (*) indicate when significant differences were found compared to control, hash sign (#) when differences were found against PFAA.

Taken together, our results indicate that the immediate startle response, presumably corresponding to the larvae reacting to any change in environmental conditions, is amplified by the presence of POPs, especially PFAA. This is consistent with the overall higher activity, as described above; however, the Br and Cl components seem to modulate this startle response more strongly. PBDEs such as BDE-47, -99, -100, and -153 have been shown to alter behavior at concentrations as low as 2.5 µg/mL depending on the congener [46]. According to these authors, the drastic response of the larvae in lighting transition can be explained by alterations in the glutamatergic transmission and changes in electrical coupling in the presence of PBDEs or PCBs.

3.7. Mitochondria Responded Notably to POP Mixture

To test the effect of the POPs on mitochondria, we used the transgenic line *Tg(kdrl-mls:GFP)*, which expresses the green fluorescent protein GFP in the endothelial cells of the vasculature and directs it to the mitochondria via its Mls signal peptide [18]. Note that the expression of the endogenous *kdrl* gene is not significantly affected by POP treatment (see below and Table S2). Exposure of these embryos to POP mixes led to a significantly increased fluorescence with POP125×, while only combined sub-mixes PFAA + Br and Br + Cl caused a significant increase (Figure 8). The higher levels of activity in mitochondria seem to be linked to OCPs and PCBs, which are explained by a POP-induced imbalance in redox, hormone homeostasis, and mitochondrial dysfunction; the mechanisms are discussed in further detail in [75,76].

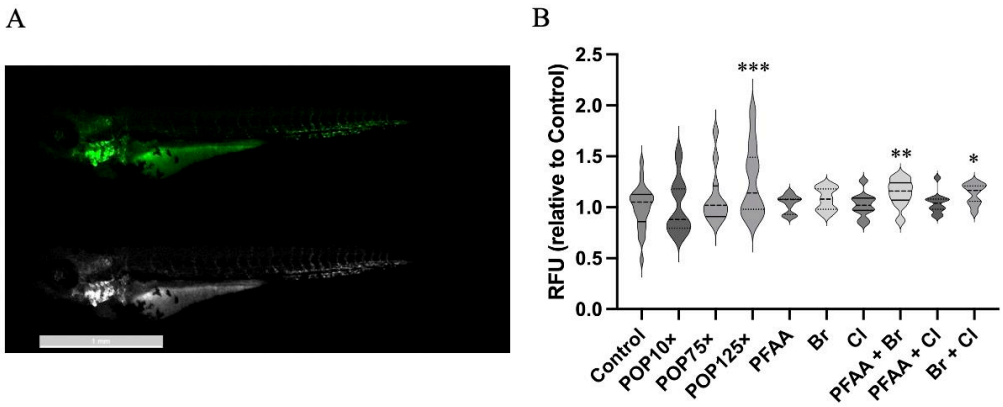


Figure 8. (**A**) Transgenic *Tg(kdrl-mls:GFP)* fish line treated with POP125×. (**B**) Plot with median values of the normalized fluorescence intensity of fish treated with different POP mixes at 96 hpf. There is a clear increasing trend. Kruskal–Wallis test and Dunn's multiple comparison, $n = 21$, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3.8. Gene Expression Is Severely Affected by Exposure to POPs

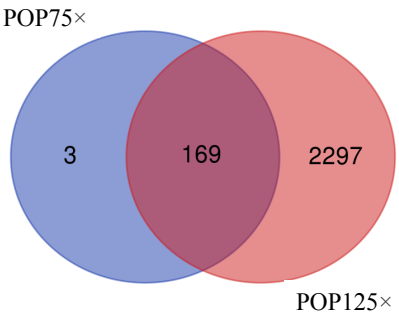
To gain further insight into the molecular mechanisms affected by POP exposure, we compared the whole genome transcriptome of control larvae to that of larvae treated with POP75× or POP125× by RNA-Seq analysis on whole larvae at 5 dpf. The number of differentially expressed genes (DEGs at $p_{adjust} < 0.05$) was 172 and 2466 for, respectively, POP75× and POP125× treatments, with 1531 genes that were upregulated and 935 that were downregulated by POP125×.

Interestingly, the huge majority (169/172) of DEGs affected at the lower concentration were also, and more strongly, affected at the higher concentration (Figure 9A, see also Table S2). As an example, the *fbxo32* (involved in muscle morphogenesis and homeostasis, and in larval behavior), *fosb* (transcription factor of the AP1 family), and *cdca7a* (regulation of hematopoietic stem cell differentiation, thymus development) genes were not significantly affected at POP75× (log(fold-change), respectively, 0.64, 0.57, and −0.65, $p_{adj} > 0.05$), but were strongly affected at POP125× concentration (log(fold-change) of, respectively, 2.45, 2.20, and −1.31, $p_{adj} << 0.05$) (Figure 9B). Another such gene is *calcoco1b*, coding for a calcium binding protein acting as a translation elongation factor.

The most highly, and most significantly, regulated genes are mostly upregulated genes. Among the most highly induced genes, we observe *fosl1a*, *fosb*, and *junba* which together form the AP1 transcription factor regulating cell proliferation, differentiation, and stress response [77]. Also, among these upregulated genes are the two paralogs *igfbp1a and igfbp1b*, coding for Igfbp proteins that interact with insulin-like growth factors (IGFs) to stabilize them and modulate their effects on growth and glucose metabolism. The *cyp24a1* gene, coding for a 1,25-dihydroxyvitamin D3 metabolizing enzyme, is significantly upregulated, possibly in relation to the growth retardation observed [78,79]. In contrast, *cyp2aa9* and *cyp2aa8*, coding for xenobiotic metabolizing enzymes [80–82], are among the most downregulated genes, indicating a response to inflammatory status [83]. In addition, many more genes (2297) were differentially expressed in POP125×-treated larvae relative to control, although we did not observe substantial lethality at this stage.

Pathway and gene ontology (Tables S3–S5) analysis points to a dysregulation of the cell cycle, but also of central nervous system development, motor activity, growth, response to stress, and metabolic processes, in particular insulin signaling and glucose metabolism (Tables S3 and S4). Using the list of genes involved in insulin signaling (Table S5) in GENEMANIA, we constructed a network of co-expressed genes encoding proteins with physical interaction (Figure 10). Strikingly, all these genes are significantly upregulated by POP125×, while also distributing in several modules.

A



B

Zfin Name	POP75× log2(fold)	POP75× padj	POP125× log2(fold)	POP125× padj
fosl1a	1.35	3.52×10^{-04}	3.06	3.19×10^{-27}
psme4a	1.43	6.37×10^{-06}	2.81	1.69×10^{-36}
mmp9	1.60	7.46×10^{-06}	2.63	1.32×10^{-22}
pdk2b	1.45	6.74×10^{-06}	2.55	5.67×10^{-28}
calcoco1b	0.96	8.03×10^{-02}	2.53	2.91×10^{-16}
fbxo32	0.64	3.09×10^{-01}	2.45	1.48×10^{-17}
retreg1	1.18	4.88×10^{-03}	2.36	1.56×10^{-15}
igfbp1b	1.27	5.13×10^{-03}	2.34	2.50×10^{-15}
ucp3	1.31	7.46×10^{-06}	2.31	3.19×10^{-27}
slc16a9a	1.15	3.58×10^{-03}	2.27	6.25×10^{-18}
igfbp1a	1.12	3.21×10^{-03}	2.24	3.11×10^{-20}
fosb	0.57	4.75×10^{-01}	2.20	6.94×10^{-14}
lcat	1.19	2.12×10^{-03}	2.06	8.90×10^{-14}
cbx7a	1.16	3.41×10^{-04}	2.05	1.06×10^{-19}
oat	1.44	2.36×10^{-05}	2.05	2.50×10^{-15}
cyp2aa9	-0.74	1.72×10^{-01}	-1.23	1.45×10^{-04}
cdca7a	-0.65	2.40×10^{-01}	-1.31	6.29×10^{-05}
ca4b	-0.91	7.11×10^{-02}	-1.33	1.23×10^{-04}
krtt1c19e	-0.81	4.47×10^{-02}	-1.42	4.95×10^{-07}
cyp2aa8	-1.11	5.06×10^{-03}	-2.18	4.06×10^{-14}

Figure 9. (**A**) Venn diagram comparing the lists of genes affected by POP1 or POP2 treatment. (**B**) Most highly and significantly regulated genes upon treatment with both POP total mix concentrations at 5 dpf. Log(fold change) and significance (adjusted *p*-value) are shown. Upregulated genes are in red, downregulated in green, while genes discussed in the text are highlighted in yellow.

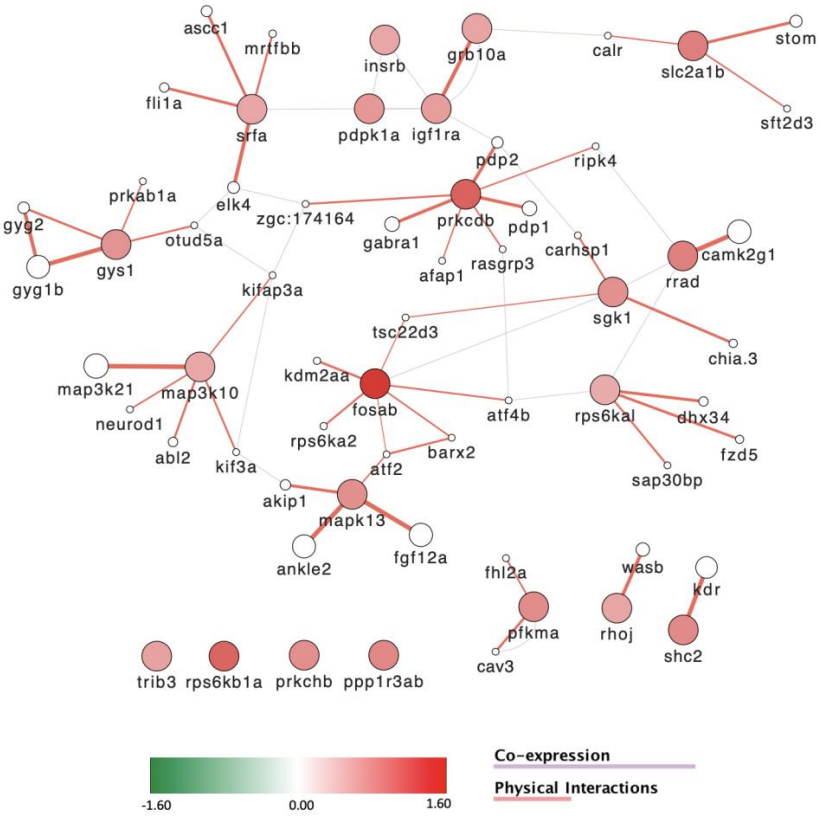


Figure 10. Differentially expressed genes involved in insulin signaling and response. These genes are all upregulated upon treatment with POP125× and distribute into specific co-expression and physically interacting modules.

One of these modules centers around *gys1*, indicating increased glycogen synthesis activity. Other modules involve the insulin receptor (*insrb*), the Glut1 glucose transporter

(*slc2a1b*) [84], a transcriptional regulatory module centered on the serum response factor (*srfa*), or regulatory protein kinases such as the MAPkinases *map3k10*, *map3k21* and *mapk13* genes, the protein kinase C gene *prkcdb* or the serum/glucocorticoid regulated kinase 1 gene (*sgk1*). Together, these observations indicate an increase in metabolism in the POP-treated larvae.

As may be expected from exposure to environmental toxins, oxidoreductase molecular function was identified as significantly affected, with all the genes in the list interestingly downregulated. These genes code for detoxifying enzymes such as alcohol dehydrogenases (*adh5*, *adh8b*), aldehyde dehydrogenases (*aldh16a1*, *aldh9a1a.1*), or cytochromes (*cyb5r3*, *cyp2aa3*, *cyp2r1*, *cyp4v8*, *cyp51*).

Gene enrichment analysis based on the mutant descriptions at zfin (zfin.org; accessed on 6 March 2023)) revealed that mutations in a significant number of the affected genes cause defects in development of the nervous system and the eye, as well as in the mitotic cell cycle (Tables S3 and S4). Based on these results, we used the GENEMANIA database to construct networks for the genes involved in eye and brain development (Figure 11). In both networks, we notice that the genes are all downregulated and that they build a tight network of co-regulated genes. About half of the genes affecting either eye or brain development are common to both networks. When we focused on the genes whose products were shown to physically interact (Figure 11), some similarities and some differences were observed. One common module is formed by the *aurka* (aurora kinase A, a histone serine kinase), the histone deacetylase gene *hdac8*, the polo-like protein serine/threonine kinase gene *plk1*, and the *fbxo5* gene coding for a predicted ubiquitin ligase inhibitor. In the brain, this module is connected through the CDK–cyclin pair Cdk1-Ccnb1 to the gene products of *rpa1* and *smc4*, predicted to be involved, respectively, in DNA repair, replication, and chromosome organization. Two smaller modules are formed by the *birc5a*, *cdca8*, *nono*, and *sfpq* genes, all of which were shown to affect both brain and eye formation when mutated (zfin.org). In the eye, the *cdk1* gene is connected to several members of the condensin I complex, including Smc2, Smc4, Ncapg, Ncaph, and Ncapd2 (Figure 11). This observation is reminiscent of the putative involvement of the *cap-g* gene in the eye defect that we observed. Interestingly, not only mutation of the *cap-g* gene, but also morpholino knockdown of the *capd2* and *caph* genes (coding for two other components of the condensing I complex), led to reduction of retinal cell number and smaller eyes similar to what we observed here [61].

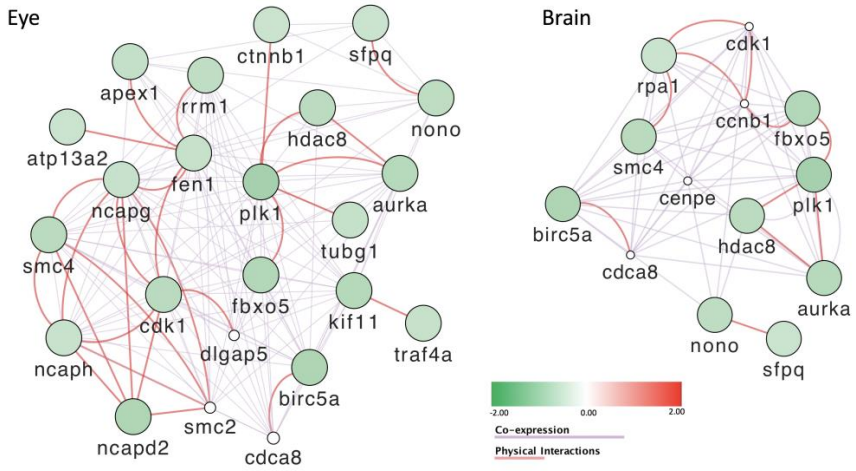


Figure 11. Differentially expressed genes that are involved in brain and eye development. These genes are all downregulated in zebrafish larvae treated with POP125× and form co-expression and physically interacting networks.

Note that the description of the *cap-g* mutant also mentions a behavioral effect, presumably based on oculomotor and optokinetic tests mainly revealing visual impairment [61]; thus, we cannot rule out that the eye defect we observed may impact the larval behavior.

Previous studies involving RNA-Seq analysis of whole larvae exposed to the POP mixture in a similar setting, albeit at lower concentrations, revealed interesting results. The PPAR pathway was shown to be affected [37], similar to our observation that the nuclear receptor genes *pparda* and *ppargc1a* were upregulated (log(fold) 0.89 and 0.64, respectively) along with those for other nuclear receptors such as *rxrab*. Conversely, our data set did not reveal calcium ion transport or signaling to be significantly affect, nor the genes involved in ryanodine receptor signaling such as *ryr1a*, *ryr1b*, *ryr2*, *myl7*, *actc1*, or *tnnc1*.

Another concern with environmental pollution by POPs is their effect on sexual maturation of growing embryos. While we did not perform fertility experiments in our larval development tests, data concerning the effects of sexual hormones on 2–4 dpf larvae are readily available [85,86]. Among the genes most highly upregulated by estradiol (E2) [85], only *vtg1* was moderately and non-significantly downregulated (Table S5), other vitellogenin genes and the aromatase gene *cyp19a1b* were not affected.

In contrast, some of the most regulated genes by 17-ß-testosterone [86] were also significantly affected upon POP125× treatment, such as *insig1*, *col10a1a*, *matn1*, *tmx3a*, *gnb3a*, or *gng13b*. Altogether, these results tend to argue for the presence of an androgen antagonistic activity in the POP mix, as was previously shown in cellular assays [13].

The effect on retina and eye development is particularly interesting. The observed defect phenocopies exactly that previously found in a *capg* mutant and in morphants for *capd2* and *caph* [61], all components of the condensin I complex specifically required for retina and eye formation. Microinjection of *p53* morpholino oligonucleotides at the one–two cell stage was able to rescue the *capg* phenotype [61], but also the eye defect in larvae exposed to POP125×. Whole larvae RNA-Seq revealed that neither the *p53* gene *tp53* expression was affected, nor did the term apoptosis appear in pathway analysis, further supporting the notion that decreasing condensin I action will specifically induce *p53*-dependent apoptosis in the developing retina.

4. Conclusions

This study sheds some new light on the effects of a realistic mixture of POPs on vertebrate physiology, with a focus on early developmental stages. Although informing on the effect on wildlife when present in the environment, our findings are also relevant for pregnant women relative to the health of the unborn child.

As discussed above, we believe that the concentrations tested here (POP75× and POP125×) represent realistic doses that may be reached in the environment, but also in human blood due to the stability and bioaccumulation characteristics of these compounds. Furthermore, we have been using a mixture, and sub-mixtures thereof, that represent the composition of that found in a Scandinavian population. Although not directly transposable to humans, our results obtained on zebrafish larvae allow us to identify potential risks to human fetuses and can inform about the molecular mechanisms that may be involved.

The effects that we observed, and discussed above, at non-lethal concentrations of the POP-mix are growth and developmental retardation as illustrated by the retarded inflation of the swim bladder, increased heart rate, increased metabolism, and mostly increased behavioral activity. Differential expression of genes upon exposure to POP125× was indeed consistent with an increase of metabolism (insulin signaling), but further revealed impacts on musculo-skeletal development and function, brain development, and several signaling pathways. Additional studies will be required in the future to investigate which component of the POP mix is responsible for any specific effect and through which molecular mechanism it acts.

The effects observed here are mainly due to the PFAA sub-mix; this single sub-mix, alone or in combination, caused effects close to those observed with the total POP mixture. This is of high relevance at the present time, as these substances (per- and polyfluoroalkyl acids) are intensely scrutinized as the most harmful chemical group for their deleterious effects on environment and human health. Initiatives and regulations have been introduced very recently by the European Chemicals Agency [87] and the US Environmental Protection

Agency [88] to investigate the origins and effects of these compounds, aiming at reducing or banning their use. Furthermore, the spotlight of public opinion has been drawn to the topic by recent movies such as "Dark waters" [89] inspired by a story in the New York Times [90] and a recent report in The Guardian [91].

Contributions of the Cl and Br sub-mixes were observed, enhancing the effect of the PFAA mix for eye malformations, behavior, mitochondrial effect, and weakly for heart rate. One interesting exception was observed for the startle response during the light to dark transition, where the PFAA sub-mix alone caused a stronger increase compared to the POP mix, which was attenuated upon addition of either the Cl or Br sub-mix in the binary mixtures.

In conclusion, our study highlights the need to study environmental pollution, not only on single compounds, but rather to consider the more realistic situation of exposure to mixtures. One striking result from our studies is that, although some of the compounds within our mixtures are described as endocrine disrupting chemicals, we did not observe significant estrogenic effects. This illustrates the fact that individual compounds in the mix may antagonize the activities of other chemicals. Our results comparing specific sub-mixtures to the total mixture further support this conclusion, with sub-mixes either enhancing or suppressing the effects of another. Finally, we also reveal a novel defect caused by POP contamination on eye development, which we propose to result from inhibition of the condensin I complex.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxics11040357/s1. Figure S1: Network of genes affected by POP125× treatment and involved in eye (A) and brain (B) development. (C) Venn diagram comparing number of genes involved in brain and eye development. Table S1: List of compounds and concentrations in the POP mix and in the PFAA, Br, and CL sub-mixtures. Table S2: List of differentially expressed genes in zebrafish larvae exposed to POP75× and POP125×: gene Ids and names, log2(FoldChange), *p*-value, and adjusted *p*-value. Table S3: Gene ontology analysis of the genes affected by POP125× treatment. Enrichment analysis of up- or downregulated genes in Genotype–Phenotype database, GO-terms, KEGG, Reactome, and Wikipathways databases. Table S4: Gene ontology analysis of the genes affected by POP125× treatment: GSEA analysis using the Genotype–Phenotype database, GO-terms, KEGG, Reactome, and Wikipathways databases. Table S5: List of genes differentially expressed upon POP75× or POP125× treatment, and involved in brain, eye development, insulin signaling, oxido-reductive regulation, estrogen, or androgen regulation.

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Data Availability Statement: Raw and processed sequencing data have been deposited in NCBI's Gene Expression Omnibus [92] and are accessible through GEO Series accession number GSE208019 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE208019; accessed on 8 April 2023).

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Highlights of the research paper “A Realistic Mixture of Persistent Organic Pollutants Affects Zebrafish Development, Behavior, and Specifically Eye Formation by Inhibiting the Condensin I Complex”

Lethality and Growth Reduction

Exposure to a realistic mixture of POPs at high, but realistic concentrations (e.g., 125x human blood concentration) resulted in significant lethality and reduced growth in zebrafish larvae. PFAA mixtures contributed significantly to the observed reduction in whole body length.

Developmental Toxicity

Common developmental defects included edemas and impaired swim bladder inflation, affecting nearly 100% of exposed larvae. These conditions can impede normal larval swimming and overall development.

Eye Malformations

Zebrafish exposed to POP mixtures exhibited notable eye deformities, including microphthalmia (small eyes) and pear-shaped eyes, likely due to inhibition of the condensin I complex.

Cardiac and Behavioral Effects

POP exposure led to a marked increase in heart rate, especially when PFAAs were present. Behavioral tests showed hyperactivity, altered responses to light and dark phases, and an amplified startle response during phase transitions.

Mitochondrial Dysfunction and Gene Expression:

Exposure to the POP mixture increased mitochondrial activity and oxidative stress. Transcriptomic analysis showed that genes related to insulin signaling, stress response, and nervous system development were differentially expressed, indicating widespread effects on metabolic and developmental pathways.

b. Chapter 2 – Research paper “A realistic mixture of ubiquitous persistent organic pollutants affects bone and cartilage development in zebrafish by interaction with nuclear receptor signaling”.

Aim

The objective of this research is to explore further the effects of a realistic mixture of 29 persistent organic pollutants (POPs) on bone and cartilage development in zebrafish. This section specifically aims to investigate how this mixture, which reflects an environmentally relevant complex mixture, influences skeletal formation by affecting nuclear receptor signaling, vitamin D, and retinoic acid pathways. This approach is intended to help understand the mechanisms by which these pollutants may lead to skeletal malformations and to highlight the potential health implications for populations exposed to such mixtures.

PLOS ONE

RESEARCH ARTICLE

A realistic mixture of ubiquitous persistent organic pollutants affects bone and cartilage development in zebrafish by interaction with nuclear receptor signaling

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Abstract

"Persistent organic pollutants (POPs)" have a plethora of deleterious effects on humans and the environment due to their bioaccumulative, persistent, and mimicking properties. Individually, each of these chemicals has been tested and its effects measured, however they are rather found as parts of complex mixtures of which we do not fully grasp the extent of their potential consequences. Here we studied the effects of realistic, environmentally relevant mixtures of 29 POPs on cartilage and bone development using zebrafish as a model species. We observed developmental issues in cartilage, in the form of diverse malformations such as micrognathia, reduced size of the Meckel's and other structures. Also, mineralized bone formation was disrupted, hence impacting the overall development of the larvae at later life stages. Assessment of the transcriptome revealed disruption of nuclear receptor pathways, such as androgen, vitamin D, and retinoic acid, that may explain the mechanisms of action of the compounds within the tested mixtures. In addition, clustering of the compounds using their chemical signatures revealed structural similarities with the model chemicals vitamin D and retinoic acid that can explain the effects and/or enhancing the phenotypes we witnessed. Further mechanistic studies will be required to fully understand this kind of molecular interactions and their repercussions in organisms. Our results contribute to the already existing catalogue of deleterious effects caused by exposure to POPs and help to understand the potential consequences in at risk populations.

Introduction

Persistent Organic Pollutants (POPs) are toxic chemicals that possess characteristics of special concern, they do not degrade easily, hence persisting in the environment for long periods of time. In addition, they bioaccumulate and are transferred through the food chain, exerting their effects at many different levels within the environment and potentially also in humans. POPs are particularly concerning for countries within the European Union where the ageing

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Abbreviations: ACT, Angle between ceratohyals; Br, Brominated compounds; Br+Cl, Dual mixture of brominated and chlorinated compounds; Cl, Chlorinated compounds; DCH, Distance between the frontal end of the ceratohyals and the line connecting the posterior ends of the hyosymplectics; LC, Length of ceratohyals; MPH, Distance covering the entire Meckel's cartilage, palatoquadrate, and the hyosymplectics; MPQ, Distance between the left and right Meckel's cartilage/Palatoquadrates; PFAA, Perfluorinated compounds; PFAA+Br, Dual mixture of perfluorinated and brominated compounds; PFAA +Cl, Dual mixture of perfluorinated and chlorinated compounds; POP125×, Total mixture of 29 perfluorinated, brominated, and chlorinated compounds at 125 times the average concentration found in blood.

population is high and there is a high degree of industrial activity that still relies on the use of POPs for daily life products such as plasticizers, flame retardants in electronic devices, furniture and fire-resistant clothing, and even in our kitchens with the non-stick cookware [1, 2]. Hence, regulations, survey and especially epidemiological studies must be paramount in places where ageing populations are large and increasing [3].

Several studies have found links between exposure to POPs and a plethora of adverse effects [4–10]. Moreover, being widely spread in the environment and some of our food sources (*e.g.*, seafood) [11, 12], POPs are under constant surveillance, however monitoring programs mostly consider each compound individually. A more holistic approach is needed as POPs are rarely found completely alone [13–16]. Investigations of POPs as mixtures present their own set of challenges and their biological and epidemiological implications are complex to understand. Increasingly, researchers in the past couple of years have addressed the problem of POPs as mixtures by investigating their effects using *in vitro*, *in vivo* and *in silico* approaches. Several studies have used constructed mixtures based on levels actually found in the blood of a Scandinavian human population [17]. This particular mixture is made of 29 compounds found at high levels in food, blood, and breast milk. Polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/PCDF) and dioxin-like polychlorinated biphenyls (PCBs) were thus deliberately excluded. Relative concentrations of the compounds were based on estimated daily intake levels from Scandinavian studies. In addition, several sub-mixtures were designed, containing either chlorinated (Cl), brominated (Br), or perfluorinated compounds (PFAA) to be able to assign specific effects to one of these classes [17]. The total POP mixture was shown to antagonize the androgen receptor transactivation and nuclear translocation [18], inhibit the transactivation activity of the aryl hydrocarbon receptor [19], and to enhance the nerve-growth-factor-induced neurite outgrowth in PC12 cells at high concentration [18–21]. It also induces cytotoxicity and some of the sub-mixtures affect the number of cells, nuclear area and mitochondrial membrane potential in human A-498 kidney cells [21]. Recently, zebrafish larvae exposed to this POP mix at realistic concentrations, or sub-mixtures thereof, presented growth retardation, edemas, retarded swim bladder inflation, and hyperactive swimming behavior [22]. Microphthalmia was also observed as a striking malformation, probably due to impaired function of the condensin I complex involved in chromosome segregation during mitosis.

One of the lesser studied issues in environmental risk assessment is whether POPs can cause deleterious effects on bone and cartilage development. Indeed, their diverse effects on general metabolism (e.g. as endocrine disruptors) carry the risk of affecting skeletal development (scoliosis, craniofacial) as well as pathologies such as osteoporosis or osteoarthritis [23]. Some POPs, such as polychlorinated biphenyls (PCBs), pesticides and dioxins, have been found in connective tissue such as cartilage and bone in several species where its uptake can be traced [23–25]. Some studies suggest a direct deleterious effect on the function of chondrocytes after exposure to POPs [26]. It has been hypothesized that these POPs could cause damage to such tissues by disrupting the balance between cartilage formation and degradation, which could lead to its breakdown and consequently to the development of osteoarthritis [27]. Chondrocyte and osteoblast malfunctions may result from several factors such as gene mutations [28, 29], environmental stress causing shifts of the glycolytic pathway [30], age-related effects, and even sex hormone deficiencies [31].

For our study we have chosen the zebrafish larvae as our testing model. This model organism has received extra attention as attempts have been made to reduce the use of animals for experiments. The zebrafish's popularity has increased in the past years due to its many advantages, such as a high degree of similarity in the genome relative to humans, the lower operational costs compared to other models, their capacity to produce often and numerous

offspring [32]. This species has been used to model diseases, genetic conditions, effects of pollutants, and many more across different disciplines. Furthermore, it is a promising model to test environmental chemicals and craniofacial skeletal development [33–35].

Here we looked at the morphological defects, specifically those observed in bone and cartilage of zebrafish larvae after exposure to an environmentally relevant POP mixture. Furthermore, we analyzed the transcriptome to obtain clues about the mechanisms involved, and through cluster analysis, in an attempt to elucidate the potential binding mechanisms, we also compared the compounds using their structural properties with model (ant)agonists.

Results

POP exposure led to craniofacial alterations and severe disruption of the chondrocranium morphology

Previous experiments have tested the total mixture of 29 POPs on zebrafish larvae [17], where a lethal concentration of LC50 = 386-fold the mean human blood concentration (POP386×) was found [22], while exposure to POP75× or POP125× resulted in more than 95% survival at 4 dpf. On the other hand, POP125× did affect behavior, heart rate, and eye development [22] and can be considered as a realistic scenario in highly exposed populations (*e.g.* sea food, environmental/natural disaster). Thus, the POP125× mix (S1 Table) was chosen to expose WT zebrafish larvae during the first 4 days for studying the effects on skeletal development.

At 5dpf, the larvae were stained with alcian blue (AB) to reveal the cranial cartilage elements. Morphometric measures were performed on these treated larvae, revealing a significant decrease in the angle between ceratohyals (ACT) and in the distance between the left and right Meckel's cartilage/palatoquadrates (MPQ) (Fig 1A and 1B). In contrast, longitudinal measures such as the length of the ceratohyals (LC) or the distance between the frontal end of the ceratohyals and the line connecting the posterior ends of the hyosymplectics (DCH) were unchanged (Fig 1C and 1D), while the combined distance covering the entire Meckel's cartilage, palatoquadrate, and the hyosymplectics (MPH) was significantly decreased (Fig 1E). Thus, globally the length of the head cartilage seems unaffected by POP125× treatment, while the medial region of the skeleton appears to be narrower. This is also illustrated by the inward curving of the palatoquadrate, leading to an extremely narrow angle in its connection to the hyosymplectics (arrow in Fig 1F). This deformity was observed in around 50–60% of the treated animals.

To assess which one of the components of the POP mix was responsible for the observed defects, different sub-mixes (PFAA, Br, Cl) at equivalent 125× concentrations were tested, as well as their dual combinations (PFAA+Br, PFAA+Cl, Br+Cl) in independent experiments (Fig 1). A significant decrease in the angle between ceratohyals (ACT) was observed only for PFAA+Br and PFAA+Cl, similar to the distance between Meckel's cartilage and palatoquadrate (MPQ), with PFAA+Cl being the most effective ($p < 0.001$). The length from the frontal part of the ceratohyals to the back of the hyosymplectic (DCH) was unaffected by any of the treatments. The length of the ceratohyal (LC) was altered in groups such as PFAA and PFAA +Cl, but not PFAA+Br, while the distance covering hyosymplectic, palatoquadrate and Meckel's cartilage (MPH) was decreased by all treatments containing PFAA within their formulation. We also evaluated the differences between PFAA alone and the other treatments. Only three observed parameters had significant differences: the width of the angle (ACT) where POP125×, PFAA+Br and PFAA+Cl had smaller values than PFAA, and in both MPH and MPQ where the measured length was larger in Br than PFAA.

To further assess the occurrence of craniofacial deformities, each fish was observed, then assessed, and catalogued. Three categories were established based on the severity of the skull

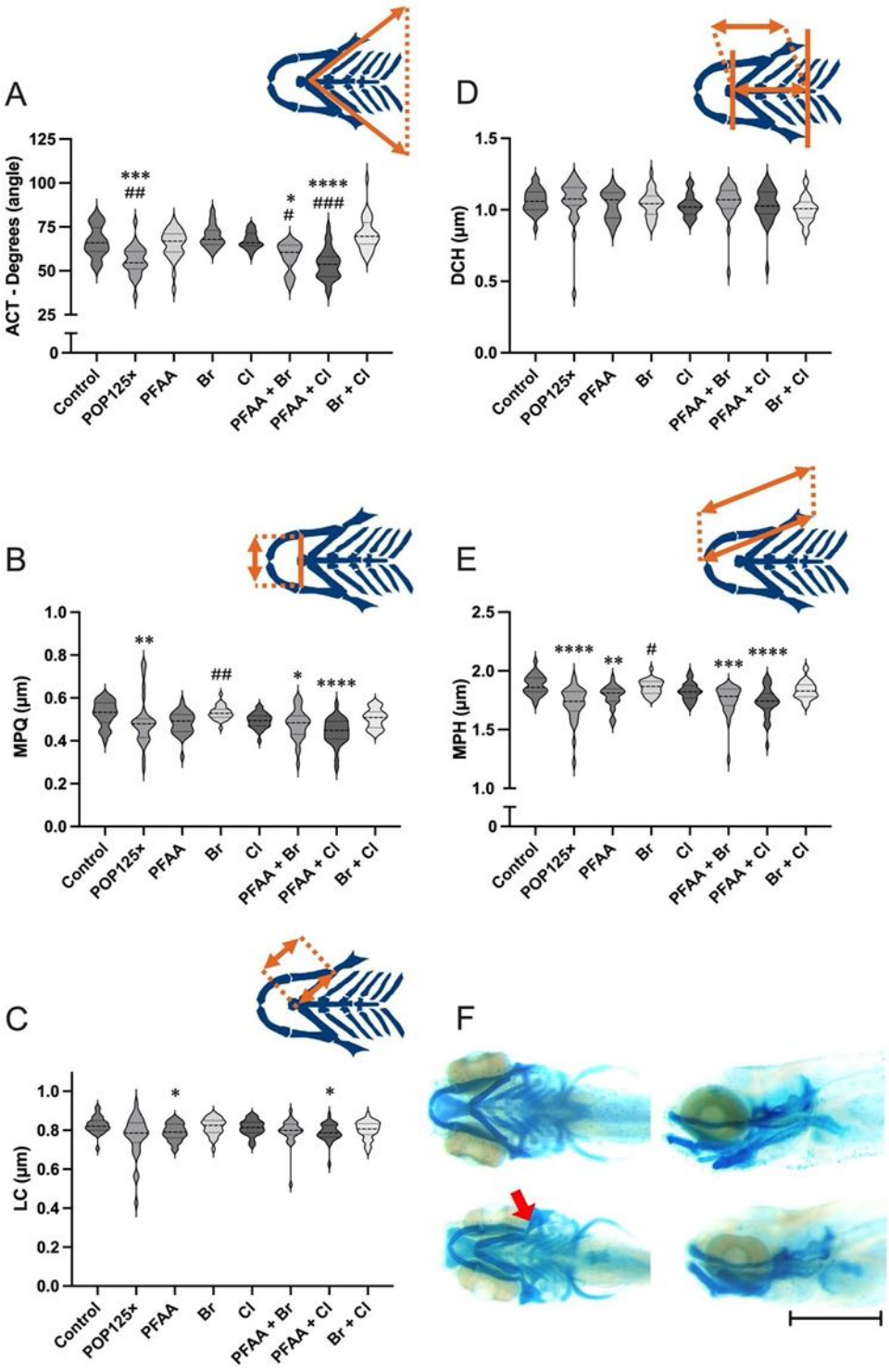

Fig 1. Morphometric analysis of the chondrocranium in 5dpf zebrafish larvae exposed to POP mixtures. A) Aperture of the angle between ceratohyals (ACT), B) Distance between the left and right Meckel's cartilages/ palatoquadrates (MPQ), C) Length of the Ceratohyal (LC), D) Distance between the frontal end of the ceratohyals and the line connecting the posterior ends of the hyosymplectics (DCH); E) Combined distance covering the entire Meckel's cartilage, palatoquadrate, and the hyosymplectics (MPH); F) Alcian blue staining of controls (upper pictures) and larvae exposed to POP125× (Bottom pictures), bar represents 1 mm. Kruskal-Wallis with Dunn's multiple comparison test. $p < 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***), < 0.0001 (****). Asterisks (*) when differences were found compared to Control, pound sign (#) when differences were found compared to PFAA.

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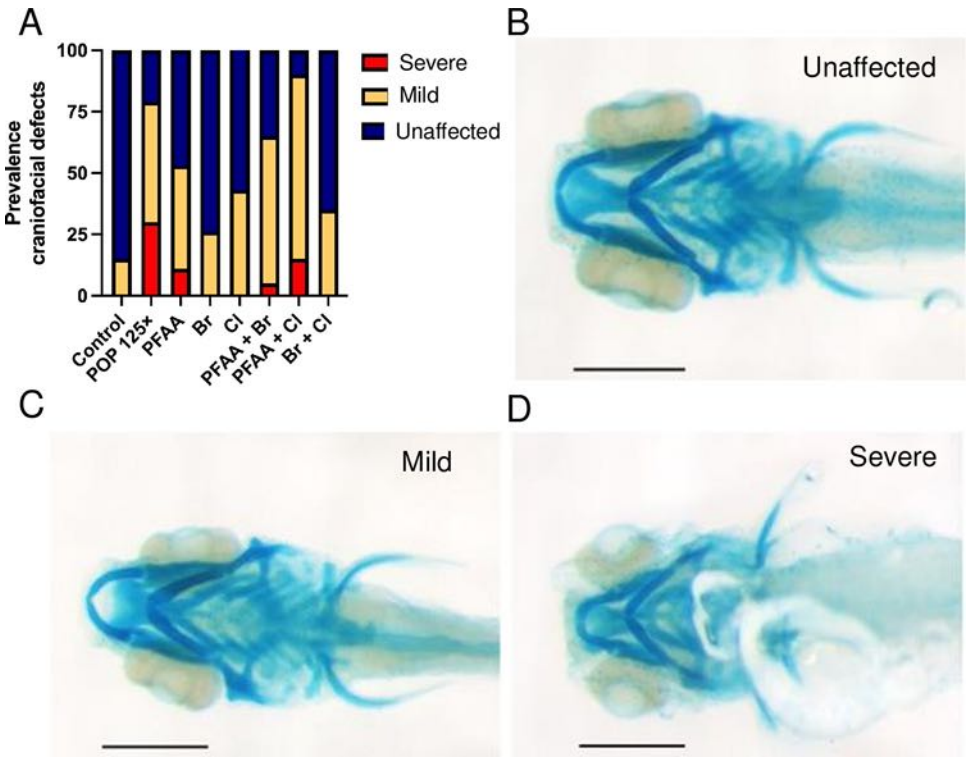

Fig 2. Prevalence of craniofacial defects and examples of such defects. (A) Percentage of fish presenting craniofacial defects, (B-D) Examples of phenotypes used to catalogue fish in each category: (B) normal, (C) mild, (D) severe phenotype. The scale bar represents 200 μm.

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defects. For this analysis, the shape of the different structures composing the head was qualitatively assessed, then assigned to one of the three categories, namely "Unaffected", "Mild", and "Severe" (Fig 2 and S2 Table). In the "Unaffected" category, only Br and Br+Cl revealed no significant differences, while all other treatments resulted in significant decreases compared to control. This translated in a slight, but significant increase in "Mild" phenotypes for the Cl treatment, in contrast to the very significant increases by those formulations where PFAA was present, that is POP125×, PFAA, PFAA+Br, PFAA+Cl. "Severe" phenotypes were only found in the PFAA-containing groups, but only POP125× reached significance due to a high variability. Finally, comparing all exposure groups containing PFAA vs PFAA alone, the POP125× and the PFAA+Cl treatments showed a significantly lower incidence of the "normal" category, while PFAA+Br caused a significant increase in the 'mild' category.

Mineralized bone formation is compromised following 4-day continuous exposure

To assess the effect of POPs on bone mineralization, we performed alizarin red staining on 10 dpf fixed larvae after treatments, as at this stage most cranial bone elements are already mineralizing [36, 37]. After the different treatments from day 0 to day 4, the larvae were grown until 10dpf before staining them using a traditional staining protocol (euthanize then stain). Unfortunately, no survivors were obtained at this stage from the larvae treated with POP125×, PFAA and PFAA+Cl. The area of the opercle was measured, as it is the most prominent structure at this stage (Fig 3). In surviving individuals treated with PFAA+Br, the area of the opercle was significantly smaller compared to controls and those subjected to other treatments. The

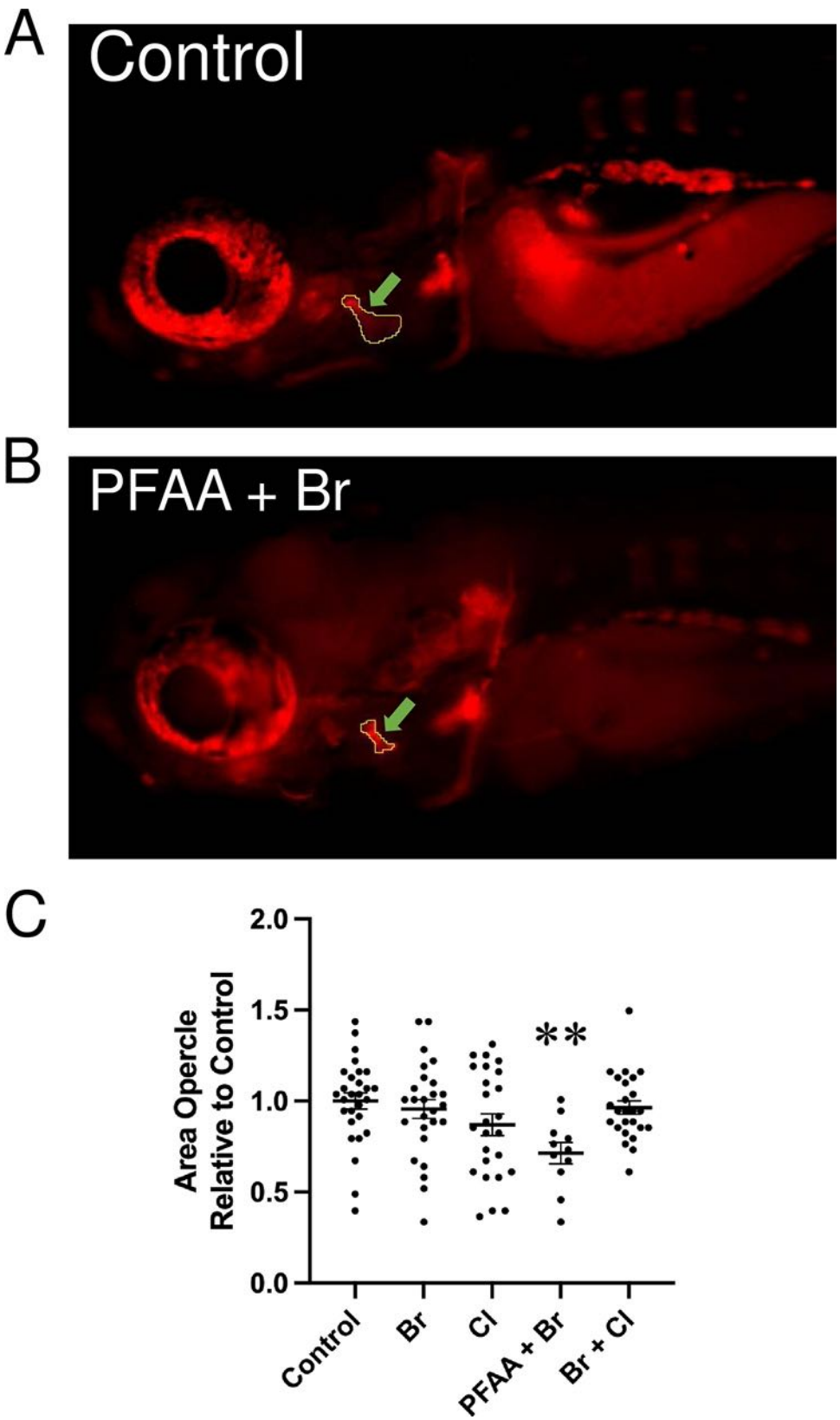

Fig 3. Area of the opercle in fish stained with alizarin red at 10 dpf. (A, B) Live alizarin red stained fluorescent image of a control larva (A) and a larva treated with PFAA+Br (B) (lateral view, anterior to the left). Arrows point at

the area of the opercle outlined in the images. The scale bar represents 500 μm. (C) Opercle area measured in control larvae and larvae upon treatment with the indicated mixture ($n \geq 11$). Ordinary one-way ANOVA and Dunnett's multiple comparison test were performed. Significant difference relative to control is indicated: p-value<0.05 (*), p value $\leq$ 0.005 (**).

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mixtures Br+Cl and Br alone did not have any remarkable effect. On the contrary, Cl treated fish had slightly smaller area sizes, but not enough to be considered as significantly different from controls.

Following the assessment of bone mineralization, in a second experiment, the transgenic line *Tg(col10a1a:col10a1a-GFP)* was used, which contains the coding sequence for the fluorescent protein GFP inserted into the coding region of the endogenous *col10a1a* gene, thereby causing a fusion protein *col10a1a-GFP* mRNA to be expressed under the control of the endogenous *col10a1a* promoter [38]. The expressed fusion protein is secreted from the cells and then binds to mineralized or un-mineralized (total) bone matrix in the living larvae. To detect specifically mineralized bone structures in the same individuals, the larvae were stained live with alizarin red for red fluorescence detection. The green fluorescence revealed a significant decrease in the total bone matrix only upon treatment with Br and PFAA+Br (Fig 4A). Surprisingly, no significant difference was observed in the live staining of mineralized bone between all the treatments, possibly due to the longer manipulation time compared to the direct fixation used above (Fig 4B). The obtained ratio between total and mineralized bone matrix, only PFAA+Br revealed a significant decrease in the opercle area (Fig 4C). Further, this analysis indicates that mainly the PFAA+Br mixture causes a significant decrease in mineralized bone formation, with a major effect on deposition of the unmineralized bone matrix.

Modulation of expression of genes related to skeletal development

Differentially expressed genes (DEGs) upon POP75× and POP125× treatment were previously analyzed by whole larvae, whole transcriptome RNA-Seq [22](the data are accessible through GEO Series accession number GSE208019 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE208019)). In light of the defects in skeletal development reported here, the list of DEGs was reanalyzed according to their known expression pattern, as available from zfin-org. Interestingly, this analysis revealed a significant enrichment in down-regulated genes that are expressed in the pharyngeal arch 3–7 skeleton and the splanchnocranium (visceral head skeleton), while only GSEA identified the pectoral fin bud (S3 Table). In addition, the functional enrichment analysis of DEGs previously carried out was reconsidered (S3 Table). In molecular functions, many membrane receptors appeared to be affected in their expression and signaling, while one class of nuclear, ligand-regulated receptors was striking. Perturbations of the vitamin D and retinoic acid pathways were shown to affect skeletal development and may lead to malformations of the cranium [39–47]. Indeed, the genes coding for vitamin D3 receptor (*vdra)*, retinoic acid receptor (*rargb*), and peroxisome proliferator-activated receptor (*pparda*) were all significantly upregulated ((log2(fold-change) of 1.90, 1.95, and 1.82, respectively) as well as that for their common heterodimerization partner Rxr (*rxrab*: log2(fold-change) = 1.79). Using Cytoscape, a network of these zebrafish genes (S3 Table) was constructed, based mainly on their shared protein domains (Fig 5), while a similar analysis based on their human homologs revealed a dense network of genes linked by known physical, genetic and signaling pathway interactions of the encoded proteins. It was observed that the *rxrab*, *vdra*, *vdrb*, *rargb* and *pparda* genes are upregulated in a dose-dependent manner upon exposure to POP75× and POP125×. In that context, it is also interesting to mention that the gene *cyp26c1*, coding for an enzyme involved in retinoic acid degradation and regulating bone mineralization [39–47] is

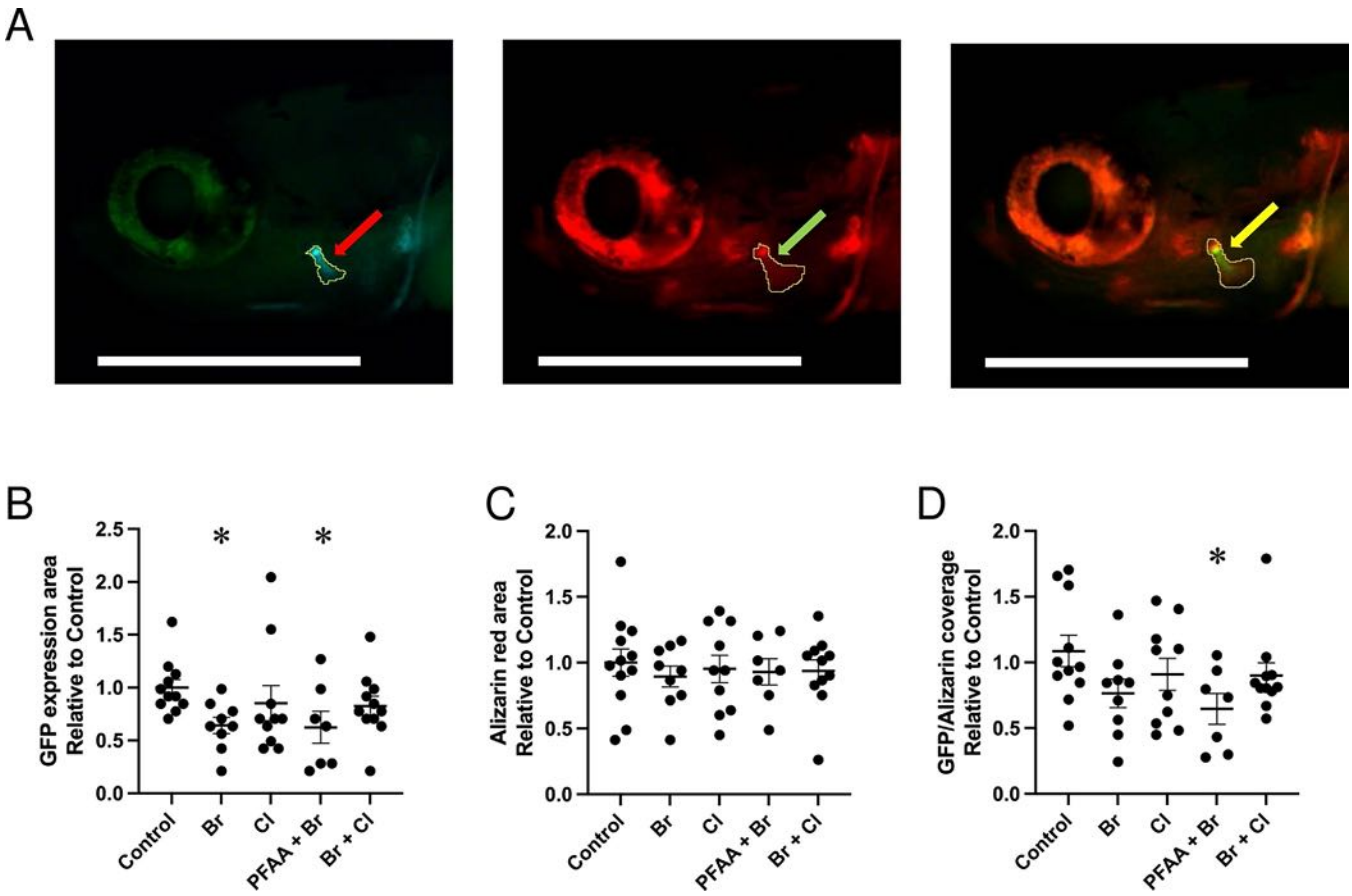

Fig 4. GFP staining in *Tg(col10a1a:col10a1a-GFP)* and alizarin red staining of the opercle in 10 dpf zebrafish. (A) Example of an individual control larva illustrating the green fluorescence of the *col10a1a-GFP* fusion protein (left), the red fluorescence of the live alizarin red staining (middle) and an overlay of both (right). The outline of the fluorescent areas is shown (red, green, and yellow arrows). Lateral view, anterior to the left, the scale bar represents 500 μm; (B) Plot of the opercle area revealed by the green fluorescent *col10a1a-GFP* fusion protein; (C) Plot of the opercle area stained with alizarin red D) Plot of the ratio between the green fluorescent and red fluorescent opercle area measured in control larvae and larvae upon treatment with the indicated mixture ($n \geq 7$). Ordinary one-way ANOVA and Dunnett's multiple comparison test were performed. Significant difference relative to control is indicated: p value < 0.05 (*).

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upregulated upon POP treatment (log2(fold-change) = 0.8; p-value = 0.006) (S4 Table). The *cyp2r1* gene, involved in vitamin D biosynthesis, is downregulated, while the gene for vitamin D degrading enzymes *cyp24a1* is significantly upregulated [48].

Another interesting molecular function and Reactome pathway was that for collagen biosynthesis and modifying enzymes, which appeared to be preferentially downregulated, pointing to defects in the extracellular matrix affecting cartilage and bone formation. These genes aggregate into two connected networks linked by shared protein domains and coexpression in zebrafish, while their human homologs form a dense network of downregulated genes whose encoded proteins are linked through physical, co-expression, co-localization, and genetic interactions (Fig 6). Interestingly, all these genes are downregulated in a dose-dependent manner upon exposure to POP75× and POP125× treatment. The different collagen genes form a specific cluster of related genes, while another cluster is formed by genes coding for enzymes involved in collagen maturation. The *plod3* gene codes for a procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 enzyme whose human homolog causes craniofacial deformities and bone fragility when mutated [49]. Similarly, the *p3h1*, whose human ortholog is involved in *osteogenesis imperfecta* [50] and *p3h3* genes code for prolyl-3-hydroxylases, while the *p4ha1a* and the

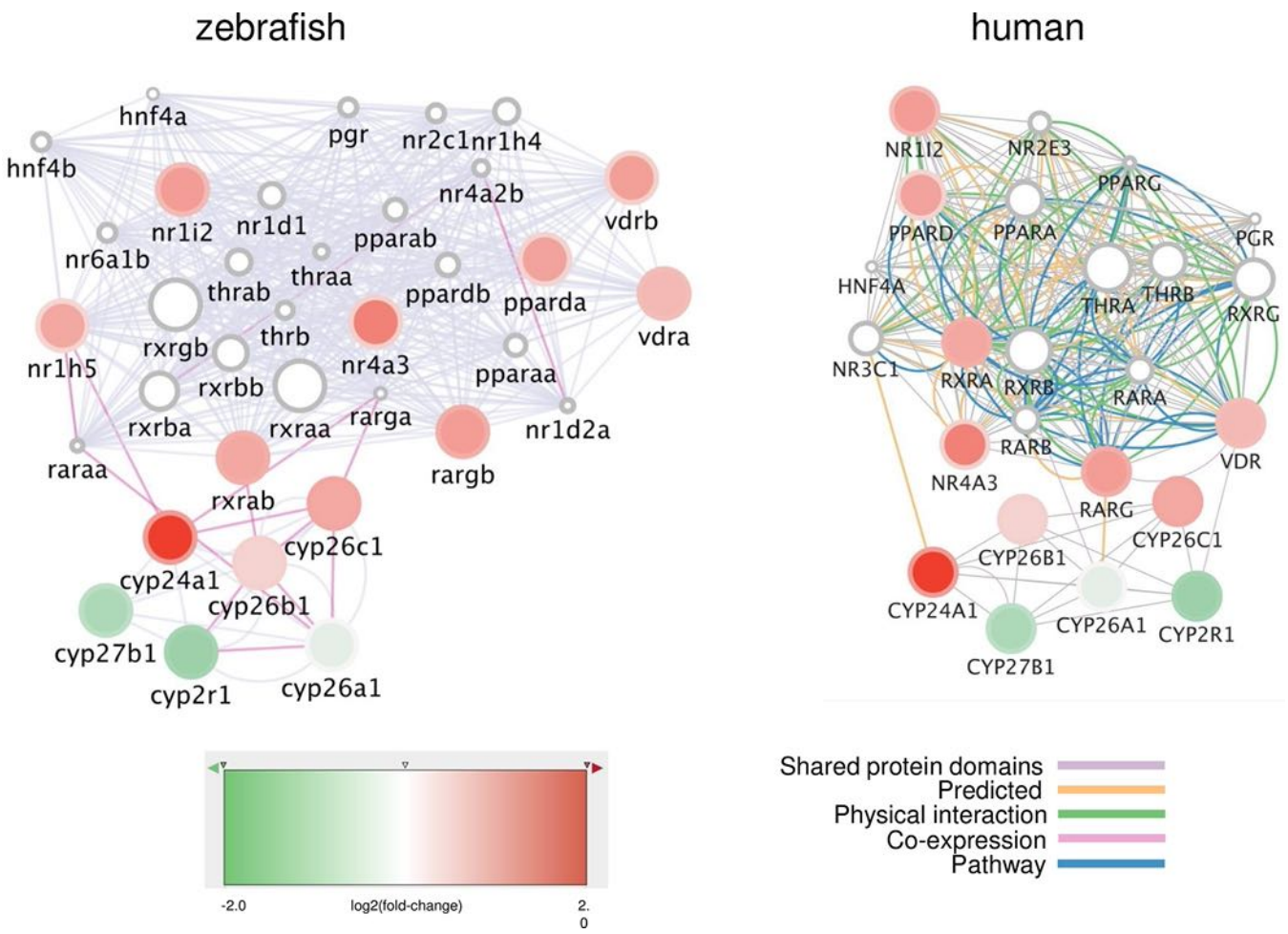

Fig 5. Networks of genes coding for nuclear receptors differentially expressed upon exposure to the total POP mixture. Networks were generated on Cytomine using GENEMANIA annotations. The nodes represent genes, the fill colors indicate the log2(fold-change) upon POP125× exposure, while the rim circle color gives the log2(fold-induction) due to POP75× exposure. All genes are upregulated in a dose-dependent way. The edges (connecting lines) represent links between nodes, based on "Shared protein domains" in the zebrafish network (left), or on specific interactions and pathways as indicated for the human network (right).

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p4ha2 genes code for prolyl-4-hydroxylases whose mutation in mouse causes impaired ECM, chondrodysplasia, and kyphosis [51].

Chemical similarities between POPs and retinoids or vitamin D

In an attempt to connect chemical structures of pollutants to specific pathways they may affect, the structural similarity of the molecules within the POP mix with some 'model' compounds known to act on specific pathways was evaluated. We decided to focus on the signaling that we found to be affected by the POPs and who are known to affect skeletal development, the vitamin D and the retinoic acid pathways. Agonists for the Vdr (calcitriol) or Rar (retinoic acid) were used as reference compounds and their chemical similarities were computed to all compounds in the POP mix. In addition, 4 triazole fungicides with a potential to be endocrine disruptors and posing serious concerns were chosen to complete the analysis: flusilazole, triadimenol, diniconazole and hexaconazole [52–59]. Hierarchical agglomerative nesting clustering was performed using the chemical fingerprints (toxprints) of all these compounds (Fig 7).

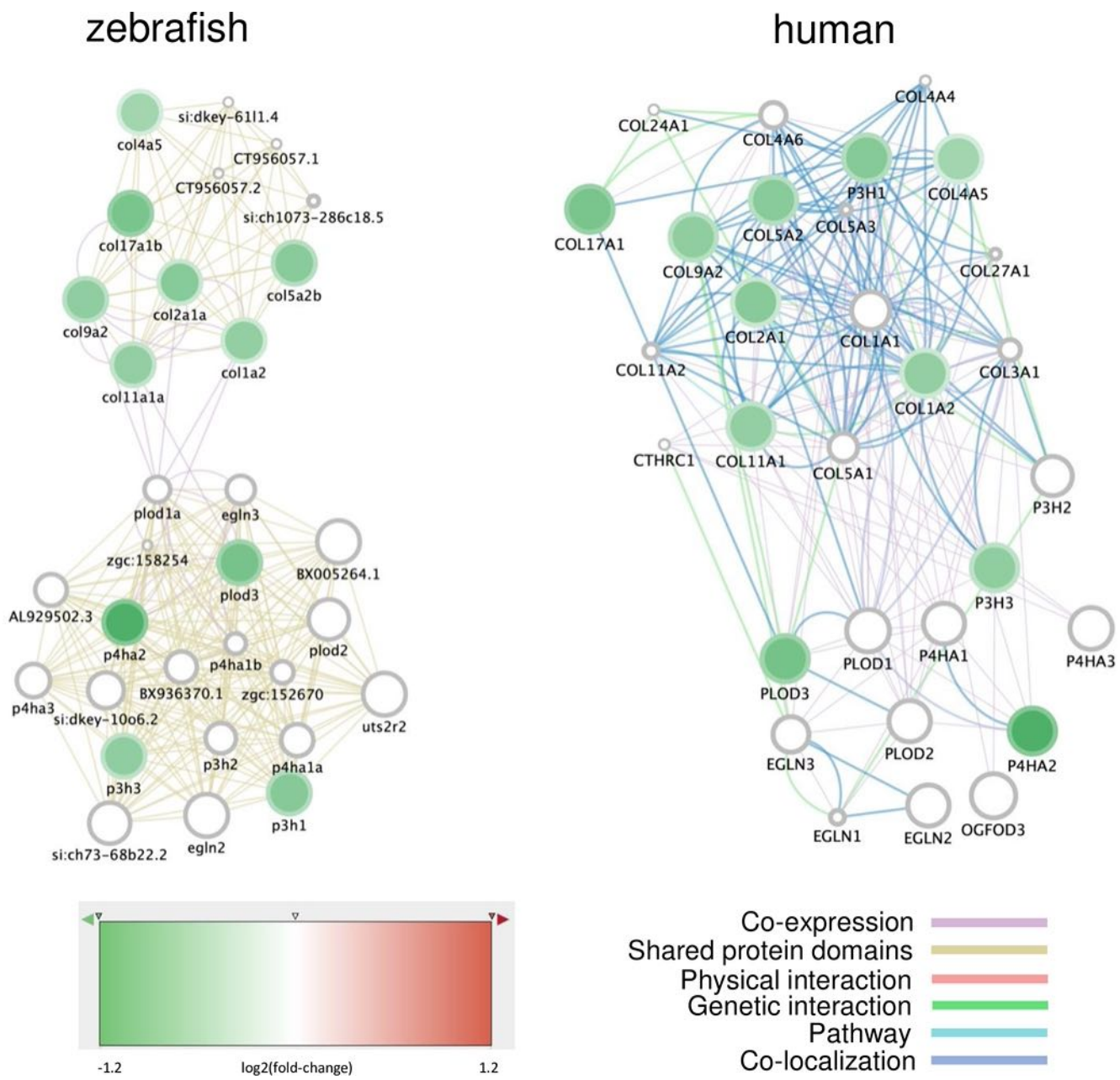

Fig 6. Networks of genes involved in collagen synthesis and differentially expressed upon exposure to the total POP mixture. Networks were generated on Cytomine using GENEMANIA annotations. The nodes represent genes, the fill colors indicate the log2(fold-change) upon POP125× exposure, while the rim circle color gives the log2(fold-induction) due to POP75× exposure. All genes are down-regulated in a dose-dependent way. The edges (connecting lines) represent links between nodes, based on "Shared protein domains" and "Co-expression" in the zebrafish network (left), or on specific interactions and pathways as indicated for the human network (right).

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As expected, closely related compounds such as polychlorinated biphenyls (PCBs), brominated flame retardants (PBDEs), and perfluoroalkyl acids (PFAAs) clustered together, indicating that the applied method is successful in grouping chemicals (Fig 7). Interestingly, the compounds arranged in two main clusters, with an agglomerative coefficient of 0.9568738. The first cluster (orange box) is headed by calcitriol, with the nearest relatives dieldrin and

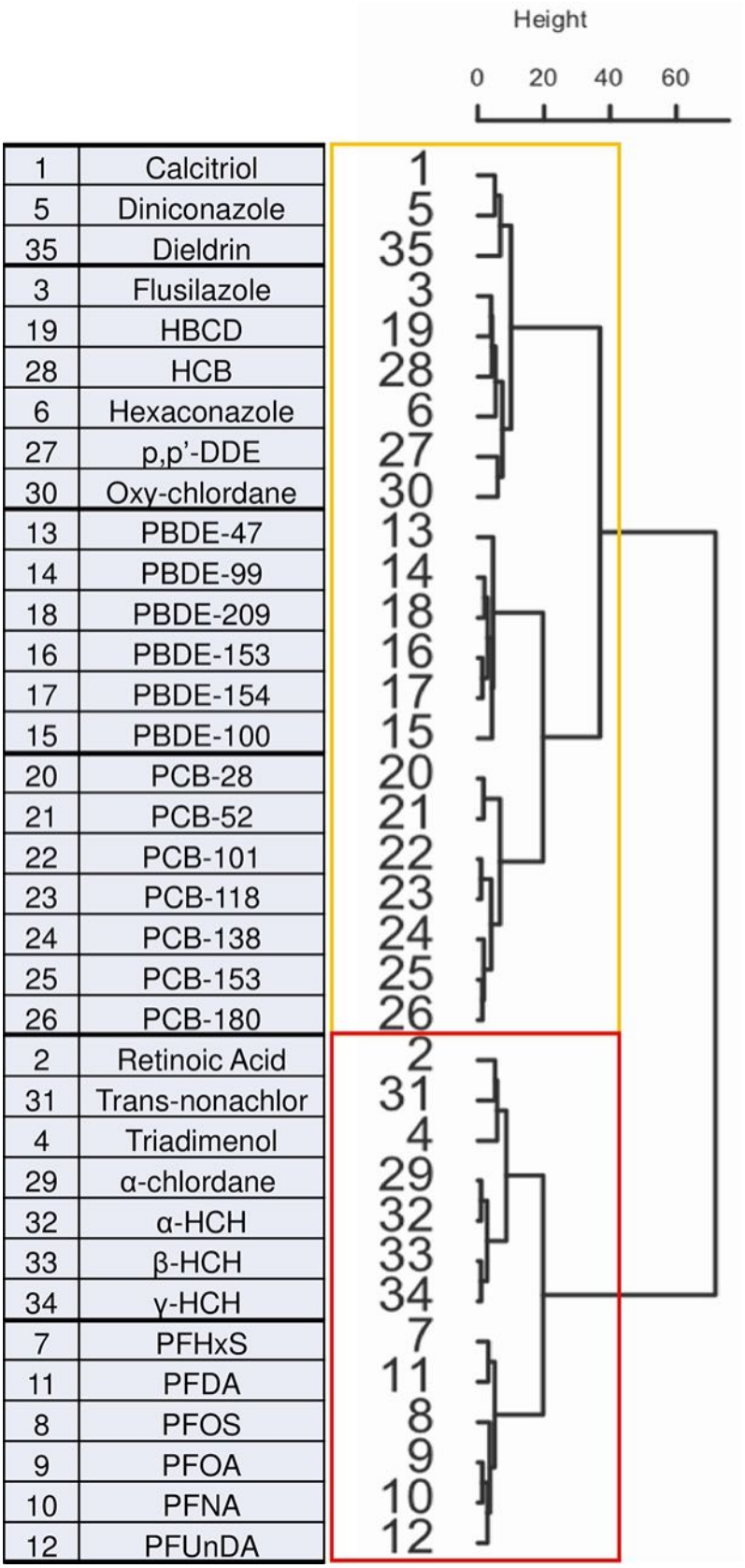
Height
0
20
40
60
1 Calcitriol
5 Diniconazole
35 Dieldrin
3 Flusilazole
19 HBCD
28 HCB
6 Hexaconazole
27 p,p'-DDE
30 Oxy-chlordane
13 PBDE-47
14 PBDE-99
18 PBDE-209
16 PBDE-153
17 PBDE-154
15 PBDE-100
20 PCB-28
21 PCB-52
22 PCB-101
23 PCB-118
24 PCB-138
25 PCB-153
26 PCB-180
2 Retinoic Acid
31 Trans-nonachlor
4 Triadimenol
29 α-chlordane
32 α-HCH
33 β-HCH
34 γ-HCH
7 PFHxS
11 PFDA
8 PFOS
9 PFOA
10 PFNA
12 PFUnDA

Fig 7. Molecular fingerprints organized in hierarchical agglomerative nesting clusters. Dendrogram revealing the chemical fingerprint similarity relations between reference compounds and the components of the POP mixture (29 compounds).

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diniconazole, followed by HBCD, HCB, *p*,*p*′-DDE, flusilazole hexaconazole, and oxy-chlordane, while the remaining PCBs and PBDEs sub-clusters were connected more distantly.

The second clearly distinct cluster (red box) grouped chemicals more similar to all-trans-retinoic acid (ATRA), with trans-nonachlor and triadimenol as the closest relatives followed by the organochlorinated pesticides α-chlordane and HCH, and finally the PFAA cluster.

Taken together, this clustering experiment shows that it is possible to classify compounds based on their chemical fingerprints, while also giving an indication on their possible target pathway through which they may exert their effects. In the pollutant list considered here, some compounds are more likely to act through binding to the Vdr, while others would preferentially act on the retinoic acid pathway.

Discussion

A lot of research effort during the last decades has backed up the affirmation that environmental pollutants, and specifically POPs are a real threat to both humans and the environment. Most of the studies on environmental toxicity use standardized protocols to evaluate lethal, morphological, or reproductive effects, while human health studies focus on carcinogenicity, genotoxicity, or adverse effects on specific organs such as liver, brain, or reproductive organs as also investigated by the pharmacological industry during the drug discovery process [60].

This work focused on the potential of POPs to induce defects in the skeleton of developing vertebrates, using the zebrafish larva as a convenient and sensitive model system. Moreover, the fact that POPs are nearly exclusively found as mixtures [13–16] was considered, thus limiting the interpretation of the biological relevance for health and environment of studies, though insightful, focusing on single compounds. Therefore, a mixture of POPs was tested based on the concentrations found in the blood of a Scandinavian population [17] and sub-mixtures thereof. Skeletal deformities in the chondrocranium were observed as well as decreased bone mineralization in 5dpf larvae exposed to the POP. The observed malformations complement the effects on growth, heartbeat, behavior, and eye development that were previously reported [22].

This is the first time such an extensive evaluation of skeletal effects has been conducted using a realistic mixture of POPs that we are aware of. On the one hand, the effects on morphology of the head cartilage elements, potentially leading to craniofacial deformities in the adult, were investigated, and bone mineralization was assessed using the growing opercle as one of the most prominent bones to be observable. To some extent, altering the normal function and viability of chondrocytes could lead to alteration of the osteoblast activity and the subsequent formation of bone [26], however the opercle is an intramembranous bone which does not depend on a preformed cartilage matrix to develop. Nevertheless, many regulators and signaling pathways are common to both processes, thus some of the mechanisms involved in deficiencies may be shared.

Some of the compounds within the mixture have been tested previously as single compounds. PCBs such as PCB 28, PCB153, and the flame retardant metabolite (6-OH-BDE-47) were found to cause incomplete fusion of the ethmoid plate and reduced size of the jaw and branchial cartilages in zebrafish [61, 62]. *In vitro* experiments using the murine chondrogenic ATDC-5 cell line and human T/C-28a2 immortalized chondrocytes that were exposed to non-dioxin-like PCBs such as PCB 101, PCB 153 and PCB 180 showed that these compounds induce chondrocyte apoptosis after exposure [27]. In humans, PCB exposure might be linked

to rheumatoid arthritis and osteroarthritis [63], while a recent overview [26] reported possible associations of PCB and PFAA contaminations with increased incidence of osteoarthritis in human cohorts.

Osteotoxicity has been witnessed across species following exposure to one or several of the chemicals within the mixtures used here. For instance, bone density in polar bears has been negatively correlated to the presence of POPs such as *p*,*p*′-DDE, HCH and PBDE-153 [64]. In humans, exposure to organochlorine compounds has also produced changes in bone mineral density [65, 66]. Some of these effects can even be witnessed across generations and through maternal exposure. The offspring of female goats orally exposed to PCB 153 had alterations in the bone composition following exposure [67]. Perfluoroalkyl substances such as Perfluorooctanoic acid (PFOA), Perfluorooctane sulfonate (PFOS), Perfluorononanoic acid (PFNA) and Perfluorohexane sulfonate (PFHxS) have been found in human bones and, since they tend to accumulate, they have the potential to affect bone turnover, hence altering bone geometry and mineral density. Uptake of PFAS by osteoclasts was seen in *in vitro* experiments, and PFNA was present within human bones [68–70]. The presence of PFOA [71] or other PFAAs [68, 72–74] in human blood serum has been associated with lower bone density, changes in cell differentiation, and bone weakness through several stages in life. Some PFAAs, such as PFOA, have been shown to bind peroxisome proliferator-activated receptors (PPAR) receptors, whose deregulation can lead to metabolic disorders and contribute to bone defects [3, 68, 73, 74].

Mild to severe effects on cartilage development were observed in 5dpf larvae caused by the different treatments, with the total mix POP125× being the most deleterious. Sub-mixes Br, Cl, and Br+Cl, were in general not causing very significant defects, only PFAAs alone caused a significant increase in the "mild" phenotype. The dual mixtures PFAA+Br and PFAA+Cl caused significantly higher percentages of malformations, close to or even exceeding those observed with the total mix. Thus, it appears that the significant malformations caused by the total POP125× mix results from largely cumulative actions of each of the sub-mixes, with a stronger effect caused by PFAAs.

A similar picture emerges concerning bone mineralization of the opercle in 10dpf larvae, although here the POP125× mix, PFAA, and PFAA+Cl could not be assessed due to their lethality at this stage. However, a decrease in mineralization upon PFAA+Br treatment was observed. Moreover, a decrease in the total bone matrix (as assessed by staining by the transgenic fusion protein Col10a1a:GFP) was observed, caused by the Br and the PFAA+Br mixtures, as well as a decrease in the ratio between total bone matrix/mineralized bone matrix caused by PFAA+Br (Fig 4D). The discrepancy between the two alizarin red staining experiments, one performed directly on fixed WT larvae (Fig 3), the other by live staining of living larvae, is probably due to the different timing and protocol of the observation and/or slight differences in the development of the WT and the transgenic line. Taken together, these observations indicate that bone formation is primarily decreased by interfering with the capacity of osteoblasts to deposit the bone extracellular matrix. This conclusion is supported by the transcriptomic analysis of 5dpf larvae treated with POP75× or POP125× mixtures which clearly identify a dose-dependent decrease of collagens and collagen maturation enzyme genes upon exposure (Fig 5).

Many hormones, such as parathyroid hormone (PTH) [39], 17-α-ethinylestradiol (EE_2) and 17-β-estradiol (E_2) [75, 76], as well as environmental pollutants such as 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) [77] have been shown to induce malformations such as bent palatoquadrate cartilages, shorter ceratohyal cartilages, changes in the angle of the Meckel's cartilage and even some missing structures, while benzo (*a*) pyrene (BaP) was shown to affect the expression of several skeletal genes (*sox9a*, *spp1*, *col1a1*) [78]. However, the POP125× mixture used here does not contain, on purpose, any dioxin-like compound (PCBs),

nor BaP [17]. Previous studies showed by RNA-Seq analysis that this mixture does not present estrogen agonistic or antagonistic properties as none of the classical target genes for the estrogenic pathway were found to be affected [22]. Similarly, PTH-like compounds would affect calcium homeostasis for which no relevant gene was detected. In contrast, down-regulation of target genes for the androgen receptor was observed, while anti-androgenic properties of the POP-mix were previously shown in a cell-based assay [18]. Androgens are known to be required for osteoblast differentiation and bone growth [79], it is thus possible that interference with androgen signaling is causing some of the observed effects. However, this transcriptomic analysis revealed in addition a dose-dependent increase in the expression of the *vdra*, *vdrb*, the *rarga*, *rargb*, the *rxrab*, and the *ppard* genes. Ppar receptors are mainly involved in lipid metabolism, however effects of Pparb/d on skeletal health have been shown [80], and PFOA was revealed as an agonist of the PPARa receptor, hence possibly directly affecting bone homeostasis [71]. Transcriptomic analysis further revealed that regulation of genes coding for enzymes involved in vitamin D and retinoic acid metabolism support an increase of these signaling pathways, which are well known for their involvement in skeletal formation. Vitamin D is well known for its role in preserving bone integrity in humans [81], a *vdra* deficient zebrafish presented delayed vertebral ossification, while treatment with exogenous vitamin D was shown to cause cranial skeleton deformities in developing zebrafish larvae [39]. Retinoic acid similarly has well described effects on bone formation and homeostasis in humans [82], while its effects in zebrafish have been well studied in individuals deficient in the RA degrading enzyme Cyp26b1, causing increased RA levels and severe developmental and craniofacial deformities [40, 42, 44]. Interestingly, comparison of the chemical fingerprints with the *bona fide* ligands revealed a clear separation into compounds more related to vitamin D and those more similar to retinoic acid. Taken together, these considerations support a hypothesis that disruption of one, or several of these pathways may, at least in part, be involved in the skeletal defects caused by the POP mix.

This study also illustrates the extent to which using specific transgenic lines, coupled to live staining techniques that are available for performing studies in zebrafish larvae can help to identify developmental toxicity rapidly and easily, as well as mechanisms of action and potential molecular targets of individual compounds and mixtures. A decrease in mineralized bone formation, as observed here, may be due to inhibition of osteoblast proliferation, differentiation, inhibition of ECM deposition, or the final step of mineralization (Fig 8) [36, 83, 84]. Here, the transgenic line *Tg(col10a1a:col10a1a-GFP)* was used to reveal total (i. e. unmineralized and mineralized) bone ECM, combined with live staining of the mineralized ECM [38]. This experiment revealed that the POP125× more strongly affected deposition of the unmineralized ECM than the subsequent mineralization. This is consistent with the observed decrease in expression of the collagen and collagen maturation enzyme genes. Furthermore, manually checking the RNA-Seq results from treated larvae [22] did not reveal any changes in expression of genes involved in osteoblast differentiation, such as *sox9a*, *runx2b*, *sp7*, or *spp1* [85]. Similarly, genes involved in inflammation or oxidative stress (*sod*, *gsh*) were not significantly affected. In conclusion, our results strongly suggest that mainly the deposition of collagenous bone ECM is affected by the POP125× mixture tested here in 5 dpf zebrafish larvae (Fig 8).

Molecular fingerprints of a compound are based on substructure keys that are used to search for structure similarity, representing different aspects of a molecule [86]. This similarity is then used to propose toxicity alerts based on these chemical features, common scaffolds, and varied ring, bond, and atom types. This kind of approaches have been used for drug discovery and virtual screening. Here, structural similarity was applied to identify the chemicals most likely to bind to and exert effects on receptors such as retinoic acid, vitamin D, and *pparda*, as suggested from our transcriptomic analysis. The chemical fingerprints approach used here,

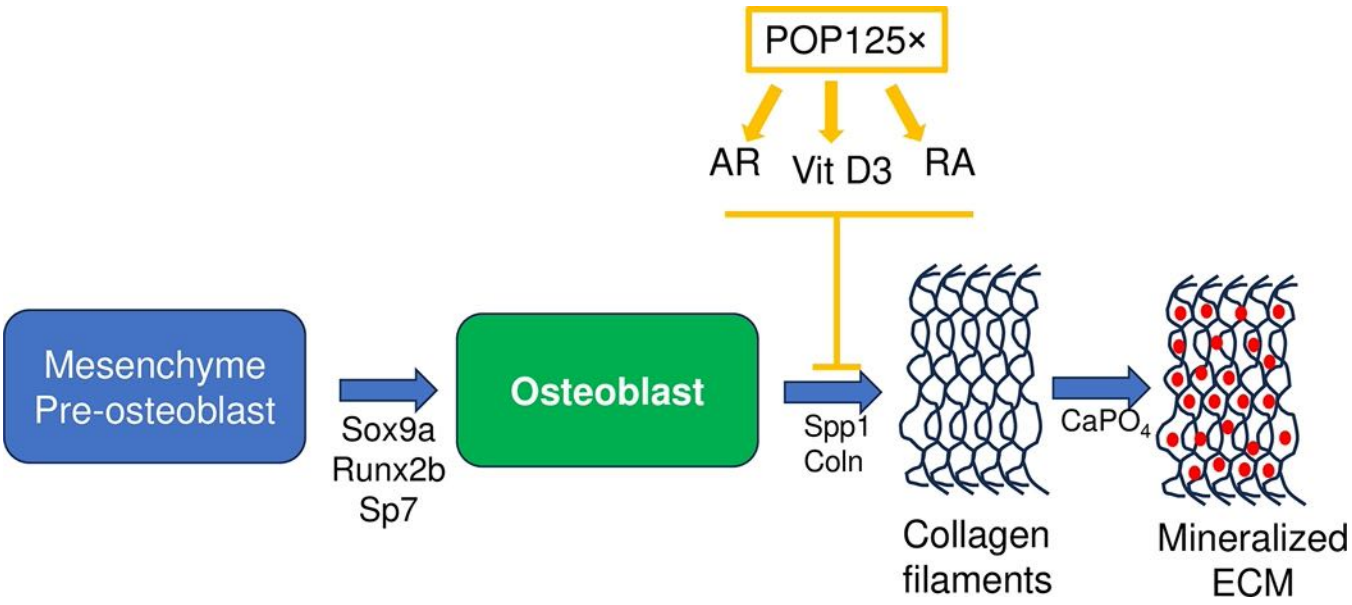

Fig 8. Schematic diagram of the effects of POP125× on skeletal development. Represented is the bone-forming osteoblast cell, originating from pre-osteoblastic, mesenchymal cells through expression of regulatory genes such as *runx2b* or *sp7*. They secrete the collagenous (Coln) bone ECM, which itself is subsequently mineralized through deposition of $CaPO_4$ cristals. POP125× inhibits mineralized bone formation primarily by interfering with collagen secretion, acting on the vitamin D (vit D3), the retinoic acid (RA), and/or the androgen receptor (AR) pathways.

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and other similar ones also based on structural similarity of the compounds [87], may be useful to suggest potential mechanisms of action of a compound, and it could aid in focusing on specific components of a mixture to reduce the experimental testing load. Thus, using chemoinformatic data has major potential implications in reducing specifically animal testing, while dealing with the tremendous task of evaluating risks caused by mixtures. Further work is needed to confirm and improve such a strategy, also including other kinds of properties (*e.g.*, toxicity, environmental levels, bioavailability), however it does have potential implications for regulatory agencies, both dealing both with environmental and human health regulations, that are moving towards non-testing studies, as laid out for example in the New Approach Methodologies [88] for human risk assessment.

Taken together, we propose that a realistic mixture of POPs, as derived from the blood of a Scandinavian population, causes skeletal malformations and decreased bone formation, most likely through affecting the vitamin D and retinoic acid signaling pathways. Our study helps to understand the potential effects caused by exposure to a realistic mixture of POPs, an exposure scenario that could be reached in particular populations (or subgroups within a population) of humans or wildlife, especially those living by the ocean and relying heavily on fish as a food source [89–92].

Materials and methods

Zebrafish husbandry and ethical considerations

Adult wild-type zebrafish (WT) of the AB strain and *Tg(col10a1a:col10a1a-GFP)* [38] were obtained from breeding facilities at the GIGA-Institute, Liege, Belgium. Fish maintenance, breeding conditions, and egg production were described [93, 94] and are in accordance with internationally accepted standards. Animal care and all experimentation were conducted in

compliance with Belgian and European laws (Authorization: LA1610002 Ethical commission protocol ULg19-2134 and ULg19-2135).

Chemicals, persistent organic pollutant mixtures and exposure tests

Dimethyl sulfoxide (DMSO, >99.9%, CAS number 67-68-5) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The stock solutions for total POP mixture and six sub-mixtures were designed and prepared by the Norwegian University of Life Sciences, Oslo, Norway [17] as indicated in S1 Table. Briefly, the stock solution of the total POP mixture was designed to represent a mixture of 29 compounds at 1,000,000-fold the mean concentrations found in the blood of a Scandinavian population, while the sub-mixtures consisted of the same concentrations of either one single class of these compounds (PFAA, Br, Cl) or of two combined (PFAA+Br, PFAA+Cl, Br+Cl) classes. The stock solutions were diluted in DMSO to obtain a final concentration of 125-fold the mean concentrations found in the blood of a Scandinavian population (125×) [22]. Exposure tests were performed in 6 well-plates, with 25 fertilized eggs per well in 4 mL of E3 medium supplemented or not with the test compounds. DMSO concentrations were 0.1%. For each experiment, 150 fertilized eggs were selected, 50 as controls and 100 for the specific treatment, to ensure a sufficient number of treated individuals for the tests. Each treatment was repeated at least three times in independent experiments. To keep stable chemical concentrations, we used a static-renewal approach where at least 90% of the media was refreshed every 24 h. Exposure started between 0 to 6 h post fertilization (hpf), the larvae were treated for 96 h before being transferred to fresh E3 medium without compounds for further growth for one or 6 days.

Craniofacial morphometrics

Cartilage integrity was assessed in 10 wildtype zebrafish larvae per treatment that were staged, euthanized with an overdose of tricaine (400 mg/L) (MS-222, Ethyl 3-aminobenzoate methane sulfonate; Merck, Overijse), fixed in PFA 4% overnight (ON) at 4˚C and stained with alcian blue (Sigma-Aldrich/Merck, Overijse, Belgium) solution (EtOH 80%/Mg 20mM, 0.02% Alcian blue) at 5 days post fertilization (dpf) (120hpf) [95]. Pictures were taken using an Olympus (Antwerp, Belgium) stereomicroscope and camera SZX10 (4x magnification) and Cell B software. Head cartilage and bone skeletons were analyzed using methods previously described [39].

Regarding bone integrity, for the first type of exposure tests we used AB (wildtype) zebrafish. At 10 dpf, 10 zebrafish larvae per treatment were staged, euthanized with an overdose of tricaine (MS-222) and stained ON with 0.05% alizarin red (A5533, CAS 130-22-3, Merck, Overijse, Belgium). On the next morning, fish were rinsed three times with E3 media and observed with an Olympus stereomicroscope and camera SZX10 (4x magnification) and Cell B software. For the second type of experiments, heterozygote parents of the transgenic *Tg (col10a1a:col10a1a-GFP)* line were outcrossed with AB wildtype zebrafish. At 10 dpf, 10 heterozygote transgenic zebrafish larvae per treatment were staged and live stained with alizarin red (dissolved in E3 media at a concentration of 0.1% alizarin plus 500 μL 1M HEPES). Fish was incubated for at least 2 hours, then euthanized with an overdose of tricaine, and mounted in methylcellulose and observed with an epifluorescence stereomicroscope Leica M165 FC (Leica Microsystems, Diegem, Belgium). Pictures were taken, then transferred and analyzed with FIJI (ImageJ2, v. 2.3.0/1.53f).

RNA extraction and RNAseq

RNA was extracted from pools of 65 larvae at 5 dpf using the RNA mini extraction kit (Qiagen, Hilden, Germany). Details of RNA extraction protocol are described in [22]. Then, the

integrity of total RNA extracts was assessed with BioAnalyzer analysis and provided RIN (RNA integrity number) scores for each sample (Agilent, Santa Clara, CA, USA). cDNA libraries were generated from 100 to 500 ng of extracted total RNA using the Illumina Truseq mRNA stranded kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. cDNA libraries were then sequenced on a NovaSeq sequencing system, in 1 ×100 bp (single end). Approximatively 20–25 M reads were sequenced per sample. The sequencing reads were processed using Nf-core rnaseq pipeline 3.0 with default parameters and using the zebrafish reference genome (GRCz11) and the annotation set from Ensembl release 103 (www.ensembl.org; accessed 1 May 2020). The analysis for differential gene expression was performed using the DESeq2 pipeline. Pathway and biological function enrichment analysis was performed using the WEB-based "Gene SeT AnaLysis Toolkit" (http://www.webgestalt.org; accessed on 10 November 2022) based on the integrated GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), Panther, and WikiPathways databases (all accessed on 10 November 2022 via http://www.webgestalt.org). An additional database was constructed using the Gene-mutant/Phenotype database from zfin (zfin.org; accessed on 6 March 2023). The cut-off values were set for the false discovery rate (FDR) to "adjusted p-value < 0.05 and the fold change > 1.5.

Chemoinformatics–Cluster analysis of toxprints

We obtained canonical SMILES for each of the 29 compounds within the POP mixture, as well as for Vdr and Rar agonists calcitriol or retinoic acid, respectively, and additional fungicides [96]. Chemical fingerprints (Toxprints) were obtained using Chemotyper [97] (S1 Table), then applying unsupervised classification, to perform a Hierarchical Agglomerative Nesting Clustering with the "Ward's method" [98] using R version 4.2.1 (2022-06-23) — "Funny-Looking Kid" [99], the library "*cluster*" and the function "*agnes*" within that library [100]. The positive controls and list of the analyzed compounds and their fingerprints are in the S1 Table.

Data and statistical analysis

Generated data were transferred to Prism 9.0.0 (v86), then every data set was tested for normality (*e.g.*, D'Agostino & Pearson test) and equal variances (Bartlett's test). Thus, parametric, or non-parametric tests were performed, each case is indicated in their respective figure. Confidence was assigned at alpha = 95% and a p-value of ≤ 0.05 was considered as significant.

Supporting information

S1 Table. List of compounds, SMILES, IUPAC name, concentration and chemical fingerprints within the POP mix and chemicals used for the cluster analysis.
(XLSX)

S2 Table. Prevalence of craniofacial defects in 5 dpf zebrafish larvae. Average in percentage and standard deviation values in percentage of the prevalence of micrognathia in zebrafish larvae at 5dpf. Mixed-Effect model (Treatment and Treatment*Phenotype), uncorrected Fisher's LSD test. p values: * <0.05; ** < 0.01; *** <0.001, **** < 0.0001. Asterisk (*) when differences were found against Control, pound sign (#) when differences were found against PFAA alone.
(DOCX)

S3 Table. List of genes differentially expressed upon exposure to POP75× or 125×. The list is focused on genes for nuclear receptors (Fig 5), genes involved in collagen synthesis (Fig 6), genes for transcription factors, and genes whose expression was found in zebrafish pharyngeal arches at any stage of zebrafish development. The columns give the zebrafish gene name, log

(fold-change) at POP75×, adjusted p-value, log(fold-change) at POP125×, adjusted p-value, and corresponding human gene name.
(XLSX)

S4 Table. Gene ontology analysis of differentially expressed genes upon POP125× exposure, focused on processes linked to skeletal development. The table first lists the GSEA analysis using the expression database in zebrafish (highlighted are the genes with decreased expression in the indicated organs = negative enrichment scores), followed by a separate over-representation analysis (ORA) of up (UP)- or down (DOWN)-regulated genes, and finally ORA analysis against the Reactome and GO-molecular fuction (MF) databases.
(XLSX)

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Software: Gustavo Guerrero-Limón.

Supervision: Jérémie Zappia.

Validation: Marc Muller.

Writing – original draft: Gustavo Guerrero-Limón.

Writing – review & editing: Marc Muller.

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Highlights of the research paper “A realistic mixture of ubiquitous persistent organic pollutants affects bone and cartilage development in zebrafish by interaction with nuclear receptor signaling”

Craniofacial and Cartilage Development

Exposure to a realistic mixture of 29 POPs caused significant craniofacial deformities in zebrafish, including a narrowing of the skeletal midline and malformations in structures like the Meckel’s cartilage and ceratohyal cartilage.

Bone Mineralization

Larvae exposed to specific POP mixtures, especially those containing PFAA and brominated compounds, showed reduced mineralization of cranial bones, indicating impaired osteoblast activity and decreased bone matrix formation.

Nuclear Receptor Signaling Disruption

Transcriptomic analysis revealed altered expression of nuclear receptor genes, especially those involved in vitamin D and retinoic acid pathways, suggesting these pathways as primary targets in the observed skeletal defects.

c. Chapter 3 – Research paper "Exploring Estrogen antagonism using CRISPR/Cas9 to generate specific mutants for each of the receptors."

Aim

The objective of this section is to investigate the effects of estrogen receptor antagonism in zebrafish by generating specific mutants for each estrogen receptor (Esr1, Esr2b, and Gper1) using the CRISPR/Cas9 gene editing tool. The study aims at understanding how disrupting each receptor impacts zebrafish development, behavior, bone development, heart rate, and other physiological endpoints. This approach allows for a more precise examination of the roles of individual estrogen receptors in response to environmental endocrine disruptors, and specifically to antagonistic compounds.

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Exploring estrogen antagonism using CRISPR/Cas9 to generate specific mutants for each of the receptors

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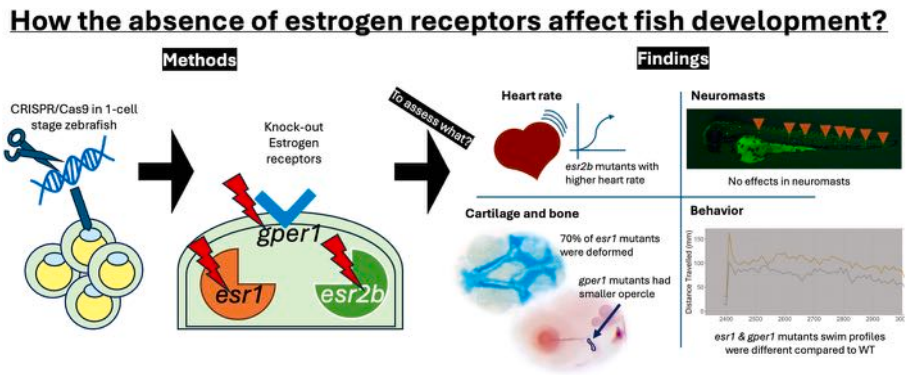
HIGHLIGHTS

- Genetic tools to study endocrine antagonism.
- Individual estrogen receptors invalidated in zebrafish.
- Receptor-specific estrogen antagonism.
- Effects on growth, behavior, bone development, and heart beat.

GRAPHICAL ABSTRACT



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ABSTRACT

Endocrine disruptors are chemicals that have been in the spotlight for some time now. Their modulating action on endocrine signaling pathways made them a particularly interesting topic of research within the field of ecotoxicology. Traditionally, endocrine disrupting properties are studied using exposure to suspected chemicals. In recent years, a major breakthrough in biology has been the advent of targeted gene editing tools to directly assess the function of specific genes. Among these, the CRISPR/Cas9 method has accelerated progress across many disciplines in biology. This versatile tool allows to address antagonism differently, by directly inactivating the receptors targeted by endocrine disruptors. Here, we used the CRISPR/Cas9 method to knock out the different estrogen receptors in zebrafish and we assessed the potential effects this generates during development. We used a panel of biological tests generally used in zebrafish larvae to investigate exposure to compounds deemed as endocrine disrupting chemicals. We demonstrate that the absence of individual functional estrogen receptors (Esr1, Esr2b, or Gper1) does affect behavior, heart rate and overall development. Each mutant line was viable and could be grown to adulthood, the larvae tended to be morphologically grossly normal. A substantial fraction (70%) of the *esr1* mutants presented severe craniofacial deformations, while the remaining 30% of *esr1* mutants also had changes in behavior. *esr2b* mutants had significantly increased heart rate and significant impacts on craniofacial morphometrics. Finally, mutation of *gper1* affected behavior, decreased standard length, and decreased bone mineralization as assessed in the opercle. Although the exact molecular mechanisms underlying these effects will require further investigations in the future, we added a new concept and new tools to

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explore and better understand the actions of the large group of endocrine disrupting chemicals found in our environment.

1. Introduction

In the past decade, one of the major breakthroughs within the field of biology is undoubtedly the discovery, description, and application of the CRISPR/Cas9 system to perform gene editing. Although other gene editing tools already existed, such as chemical mutagenesis coupled to "targeting induced local lesions in genomes" (TILLING) (Wienholds et al., 2003), "transcription activator-like effector nucleases" (TALENs) (Bedell et al., 2012) or morpholino antisense RNAs (Nasevicius and Ekker, 2000), the advent of the "clustered, regulatory interspaced, short palindromic repeats" CRISPR/Cas9 (Gagnon et al., 2014) system has greatly improved speed and accessibility of such tools (Housden et al., 2017).

In the field of ecotoxicology, gene editing tools have been used to generate transgenic or mutant lines to study the underlying molecular mechanisms of chemical compounds and their toxic effects (Zhao et al., 2023). Some examples across species have been useful to determine how certain compounds could be more toxic in the presence or absence of specific genes/receptors. This is the case of the *Daphnia magna* CYP360A8 mutant, where the absence of a functional cytochrome P450 (CYP) clan 3 gene enhanced the observed toxic effects from exposure to paraquat in comparison to their wildtype counterparts (Religia et al., 2021). Similarly, generation of a mutant in the aryl-hydrocarbon receptor gene *arh2* in zebrafish has revealed its involvement in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced developmental defects (Garcia et al., 2018).

The zebrafish has been used as a model organism due to its many perks. It is a model organism sharing around 84% of gene orthologues known to be related to human diseases. They are cost-effective, easy to handle, they have high reproductive rates and a rather easy to manipulate genome (Segner, 2009; Howe et al., 2013). Historically, fish have been used as sentinel species to indirectly assess the health of the environment and to model effects on humans. Their use has yielded good, reliable, and precise data, while they are cost-effective approaches compared to their mammalian counterparts.

Estrogen receptors (ERs) are targets for many chemical compounds, they trigger responses such as activation or inhibition of gene expression or phosphorylation of signaling molecules. Four genes code for ERs in zebrafish: three corresponding to the classical nuclear receptors acting on transcription of their target genes depending on the presence of their ligand (Menuet et al., 2002), which are *esr1* (ortholog to mammalian ERα) and *esr2a* and *esr2b* (closer to mammalian ERβ). Moreover, the *gper1* gene encodes a membrane receptor acting on the MAPKinase pathway (Liu et al., 2009; Pang and Thomas, 2010). Exposure of zebrafish larvae to estrogens leads to feminization of their gonads, with males being sterile (Van den Belt et al., 2002; Fenske et al., 2005). Transcriptomic analysis of whole larvae mRNA has revealed regulation of target genes such as *vtg1* et *cyp19a1b* at these early stages (Hao et al., 2013; Schiller et al., 2013b) upon β-estradiol exposure. Estrogens affect cartilage, variations in their concentration may lead to cartilage disruptions when zebrafish is exposed during early development (He et al., 2018). Besides their involvement in female sexual maturation, little is known about the function of individual ERs. Their expression pattern during development is known in the liver, brain, lateral line, pituitary, and other tissues (Bardet et al., 2002; Lassiter et al., 2002; Menuet et al., 2002; Froehlicher et al., 2009; Liu et al., 2009; Pang and Thomas, 2009; Hao et al., 2013). Recently, effects of estrogen exposure on early development of zebrafish larvae have been shown (Soares et al., 2009; Schiller et al., 2013a). Gper1 has been shown to be involved in brain development (Shi et al., 2013), liver growth (Chaturantabut et al., 2019), and oocyte maturation (Pang and Thomas, 2009), while Esr1 and Esr2b seem to be required for lateral line development (Froehlicher et al., 2009; Gamba et al., 2010).

In our study, we focused on the developmental effects caused by antagonizing estrogenic signaling, either by antagonistic chemical compounds, or using a novel approach by knocking out genes expressing three of the four known estrogen receptors by means of CRISPR/Cas9. We knocked out the *esr1*, *esr2b* and *gper* genes in zebrafish and performed a panel of biological tests on zebrafish larvae to determine the effects such a selective antagonism may cause.

2. Materials and methods

2.1. Zebrafish husbandry and ethical considerations

Fish maintenance, breeding conditions, and egg production were described in detail (Westerfield, 2007; Lammer et al., 2009) and are in accordance with internationally accepted standards. Adult wild-type zebrafish of the AB strain were obtained from breeding facilities at the GIGA-Institute, Liege, Belgium. Animal care and all experimentation were conducted in compliance with Belgian and European laws (Authorization: LA1610002 Ethical commission protocol uLg19-2134 and Ulg19-2135).

2.2. Generation of mutant zebrafish lines, DNA extraction and genotyping

To generate mutant lines for *esr1*, *esr2b* and *gper1* genes, we used the CRISPR/Cas9 method (Hwang et al., 2013; Doudna and Charpentier, 2014). Guide RNA (gRNA) sequences were introduced into the Alt-R™ Cas9 system from Integrated DNA Technologies (IDT, Leuven, Belgium). Their sequences were, for *esr1* (exon 2): 5′-TGCTTCAGTGGGATACTACC-3'; for *esr2b* (exon 2): 5′-GGGCAGTGCAGAGAGTGAGG-3'; and for *gper1* (exon 3): 5′-GGAGGTTGACCACCAAAATG-3'. The solutions were made following protocols from the manufacturer (IDT, Leuven, Belgium). The Alt-R crRNA (gRNA for the gene of interest) and tracrRNA were resuspended in Nuclease-Free IDTE Buffer to reach a final concentration of 100 μM each (Raman et al., 2023). Prior to injection, we also added to the final mix 0.5 μL tracer dye (0.5 mg/mL, rhodamine dextran (RD), Molecular Probes, Carlsbad, California USA). Microinjection was carried out into one- and two-cell stage zebrafish embryos using a pneumatic PicoPump PV 820 assembled on an Olympus dissecting microscope. Every injected droplet was calibrated to contain around 2 nL. After 24 h, the DNA from single embryos was extracted and the genomic region surrounding the target sequences for the genes of interest was amplified using PCR. Primer sequences used for PCR were, for *esr1*: forward 5′-GAGTACCCGAACCCCACAC-3′, reverse 5′-TGGACGAGGAATCCAGGTAG-3'; *esr2b*: forward 5′-GATTACTCTCCACCGCATGG-3′, reverse 5′-TGAACGTGTTTCGCTGTAGG-3'; *gper1*: forward 5′-AGTTTTACATCATCGGCCTGTT-3′, reverse 5′-CACCAGAATAAGATCTGCCACA-3'.

2.3. Search of mutants and streamlining genotyping – DNA extraction, qPCR, and sequencing

DNA was isolated from whole larvae or from finclips of adults at various stages of development in 50 mM NaOH by heating at 95 °C water bath for 20 min. The solution was cooled down on ice for 10 min and the DNA extraction was stopped by adding Tris-HCl 1 M, pH = 8.0, 1/5th the volume of NaOH and spun down using a desktop centrifuge for 2 min to recover the supernatant. The extracted DNA was stored at 4 °C until further processing. Genomic fragments covering the targeted region were obtained by PCR using the above primers. The mutants were

identified using Heteroduplex Mobility shift Analysis (HMA) by polyacrylamide gel electrophoresis, selected DNAs were further processed for Sanger sequencing to identify the exact position and extent of the mutation (Raman et al., 2024).

Homozygous mutants were obtained by crossing heterozygous parents carrying the desired mutation and then further tested in each of the assessed endpoints. After each experiment, genotyping of each individual was performed in either 5 days post fertilization (dpf) or 10 dpf fish. qPCR was performed to identify and sort the mutants into 96 well-plates, one fish in each well to be easily located for further analysis. DNA extraction was performed as described above by adapting the volume to fit in the 96-well-plates. Only homozygous WT or mutants were then assigned to their location for phenotype determination.

2.4. Alizarin red (AR) staining

Larvae were euthanized at 10 dpf with an overdose of MS-222 (400 mg/L) (Ethyl 3-aminobenzoate methane sulfonate; Merck, Overijse, Belgium), fixed in PFA 4% overnight at 4 °C and thereafter rinsed three times with PBST for 10 min each time. They were then bleached using H_2O_2 3%/KOH 0.5% during 30 min, then washed thrice for 10 min with 25% glycerol/0.1% KOH to remove the bleaching solution and subsequently stained with alizarin red-S (Merck, Overijse, Belgium) at 0.05% in the dark for 30 min. The pictures were obtained on a dissecting microscope (Olympus and camera SZX10 using cell B software), from at least 96 individuals in three or more independent experiments for each condition.

2.5. Alcian blue (AB) staining

Larvae were euthanized at 5 dpf with an overdose of MS-222 (400 mg/L) (Ethyl 3-aminobenzoate methane sulfonate; Merck, Overijse, Belgium), fixed in PFA 4% for 16 h at 4 °C and thereafter rinsed three times with PSBT for 10 min each time. The larvae were then stained with 1 ml of alcian blue (0.04% Alcian blue/10 mM $MgCl_2$/80% EtOH pH 7.5) for 16 h on low agitation. The pictures were obtained on a dissecting microscope (Olympus and camera SZX10 using cell B software), coming from at least 96 individuals in three or more independent experiments for each condition.

2.6. Estrogen receptor antagonist exposure tests

We have selected three antagonist compounds, one for each of the estrogen receptors. The chosen chemicals were MPP, PHTPP and G15 antagonists for ERα, ERβ and GPER1, respectively (https://www.tocris.com/products/). Stock solutions were prepared in Dimethyl sulfoxide (DMSO, >99.9%, CAS number 67-68-5; Merck kGaA, Darmstadt, Germany). Every exposure test was performed at least three times independently using three final concentrations (10, 100 and 1000 nM) of each compound. 6-well plates were used and in each well 25 fertilized eggs were placed in E3 medium mixed with one of the exposure treatments. The DMSO concentration was corrected to achieve a total concentration of 0.1%, including in the control groups. To keep stable chemical concentrations, we used a static-renewal approach where at least 90% of the media was refreshed every 24 h. Exposure started between 0 and 6 h post fertilization (hpf); the larvae were treated for at least 96 h.

2.7. Morphological observations, heart rate, and behavior

A set of morphological features was recorded, including presence of edemas, inflation of the swim bladder, eye malformations, etc. Pictures of treated and untreated larvae at different stages were taken. All observations were made with a stereomicroscope Leica M165 FC (Leica Microsystems©, Leica, Wetzlar, Germany). Standard length was estimated in fish at 5- and 10- dpf using the FIJI line tool for measurement (ImageJ2, v. 2.3.0/1.53f).

Heartbeats were counted manually using an inverted Nikon Eclipse TS100 microscope and a hand counter for 15 s. To obtain the beats per minute (BPM), measurements were multiplied by 4. The heart rate was estimated on 96 hpf larvae that were acclimated to the lighting conditions for no less than 5 min prior to counting; the larvae were not immobilized by anesthetics or other means. Each larva was observed sequentially at least three times. Ten larvae were observed per mutation and each experiment was performed at least in triplicate.

Neuromasts were counted in each fish at 4 dpf. The neuromasts of the posterior lateral line (PLL) were stained using Di-Asp (4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide) (Di-Asp; Sigma D3418, USA) (Harris et al., 2003). Live staining was achieved by incubating larvae in 5 mM Di-Asp for 5 min and then rinsing three times with normal E3 rearing medium. Stained larvae were euthanized with an overdose of MS-222 (400 mg/L), then mounted in Petri dishes with 3% methylcellulose, placed on their lateral side, and visualized under fluorescent light on a stereomicroscope Leica M165 FC (Leica Microsystems©, Leica, Wetzlar, Germany). 24 larvae were used for each mutant and each experiment was performed at least in triplicate.

Behavioral tests were conducted on zebrafish larvae at 120–125 hpf as previously described (Guerrero-Limón et al., 2023); every test was performed between 10:00 and 13:00 to maintain a constant position in the circadian cycle. Special care was taken during the entire rising period to avoid interference of environmental factors. Exposed larvae were shielded from loud noises, changes in the temperature of the incubator (27–28 °C) and the raising medium (~26° at the time of testing), changing light conditions and activities in the room by putting them in a specific enclosure harboring its own, constant dark/light regime. Prior to each behavioral test, the zebrafish larvae were inspected under a stereomicroscope to select and transfer to the testing plates only individuals devoid of any malformation that might interfere with mobility outcome (e.g., yolk sac or pericardial oedemas, spinal aberrations, aberrations in pigmentation, and/or loss of equilibrium, etc.). The larvae were placed individually in a well of a 96-well plate and observed using a ViewPoint® Zebrabox system and its tracking software (ViewPoint Life Sciences, Lyon, France). The light level was set to 20% on the ViewPoint software (7.45 klux, TES 1337 light meter), while infrared light (850 nm) was used to track larval activity. We applied a light–dark cycle that lasted for a total of 1 h and consisted of 20 min of light, allowing for the larvae to acclimate to the situation and discarded from the analysis, followed by 10 min of darkness, 10 min of light, 10 min of darkness and 10 min of light. The video and tracking software were used to screen larval locomotion behavior in10 s intervals; the distance travelled (DT) and the time spent active (TSA) were determined and, from these parameters, the mean swimming speed when active (SS) was calculated by dividing the cumulated distance travelled by the total time spent active. For each condition, at least 3 batches of 24 individuals each were observed in independent experiments, taking care that always one control (WT or untreated) batch was present on the same 96-well plate.

2.8. Data and statistical analysis

Morphological data were transferred to Prism 9.0.0 (v86) (Graphpad, San Diego, CA, USA). Each data set was tested for normality (e.g., using a visual cue (QQ plot), D'Agostino–Pearson omnibus normality and Shapiro–Wilk tests) and equal variances (Bartlett's test). Therefore, parametric or non-parametric tests were performed depending on the case, as indicated in each figure.

Raw behavioral data sets consisted of tables holding the positions of

each larva in each video frame (30 FPS). This table was first trimmed to eliminate very short, oscillating, and likely artefactual movements, and then aggregated into 10-s periods for further analysis. These data were transferred to R version 4.0.2 to analyze motility during the dark and light phases. To assess behavior, we used linear mixed effect (LME) models within the "nlme" package (Pinheiro and Bates, 2000). Three dependent variables were used, either the "mean time spent active" (seconds), the "mean distance travelled" (mm), or the "mean swimming speed" (calculated as the mean distance travelled/mean time spent active) within each 10 s period, with "compound" and "time" as the categorical and continuous independent variables, and "batch" as a random effect. The "Anova" command within the "car" library (Fox and Weisberg, 2019) was used to extract the results for the main effects whereas the "lsmeans" command (Lenth et al., 2022) 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 was used for the model. We performed our analyses using the 600 s (10 min) of every alternating phase (dark-light). Confidence was assigned at $\alpha = 95\%$ and a p-value of ≤ 0.05 was considered as significant, $p \leq 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***), ≤ 0.0001 (****).

3. Results

3.1. Generation of estrogen receptor mutants

To elucidate the role of the estrogen receptors in development, we generated mutants for each of the *esr1*, *esr2b*, and *gper1* genes using the CRISPR/Cas9 method. We focused our studies on three specific lines: line ulg078 disrupting the *esr1* coding region with a 43-nucleotide deletion in exon 2, -line ulg079 disrupting the *esr2b* coding region with a 31-nucleotide deletion in exon 2, -and ulg080 disrupting the *gper1* coding region with a 22-nucleotide deletion in exon 3. Heterozygous mutant populations were established for each line, the mutations and the resulting disruption of the coding sequence was thoroughly checked in homozygous mutant offspring before further characterization. Detailed description of the mutants can be found on http://zfin.org.

3.2. Streamlining genotyping for large number of samples was successful using qPCR

Genotyping individual fish can be time and resource-consuming using traditional means such as the Heteroduplex Mobility Assay, where a polyacrylamide gel is used to tell apart individuals that have a mutation from those being wildtype. Here we streamlined a process where we would identify mutants, heterozygotes and wildtype fish using the differences in the melting curves of the fragment amplified from the genomic target region for each individual. Briefly, depending on the type of mutation, melting curves would appear at a lower or a higher temperature, if the mutation is large enough, the peaks are easily discriminated both in heterozygotes and homozygotes (Fig. S1).

3.3. $esr1^{-/-}$ mutants present malformations in craniofacial cartilage

Initial analysis of larval growth at 5 dpf did not reveal any obvious morphological malformations, nor did we observe any significant differences in standard length between WT and their mutant siblings for any of the estrogen receptors (Fig. 1A).

Skeletal development in zebrafish starts with the formation of cranial cartilage that can be easily observed at 4 dpf by alcian blue staining. We thus compared WT larvae to homozygous mutants for each of the three receptor genes *esr1*, *esr2b*, and *gper1*.

Mutation of the *esr2b* or *gper*1 genes did not cause any defects in craniofacial development. In contrast, mutation of the *esr1* gene led to 70% of the $esr1^{-/-}$ mutant fish presenting malformations in the cranial cartilage compared to WT (Fig. 1B), ranging from mild (31%), severe (29%) to very severe (10%) (respectively Fig. 1C and D) and consisted in significant reduction or total absence of structures such as Meckel's cartilage, ceratohyals, palatoquadrates, and ceratobranchials. The ethmoid plate, although present, was clearly deformed and/or reduced

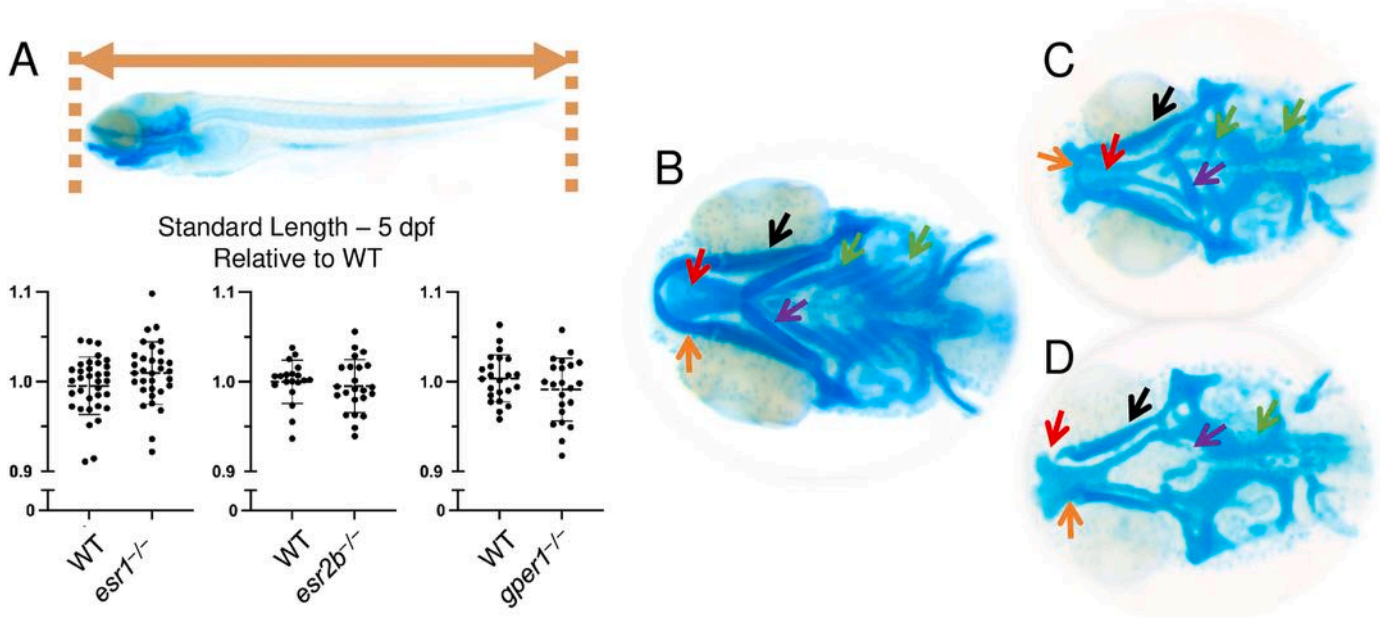

Fig. 1. Phenotypes found in *esr1* mutants. A) Lateral view of a WT sibling, illustrating the measure of the standard length (orange arrow) and graphs representing the measures of standard lengths (relative to the mean of the WT larvae) of each mutant compared to their WT siblings; B) Dorsal view of the stained head cartilage of a 5 dpf WT larva; C) Observed chondrocranium malformations in *esr1* mutant fish: total absence of ceratobranchial 1–5 (green arrows), reduced Meckel's cartilage (orange arrow), thinner palatoquadrate (black arrow) and deformed ceratohyals (violet arrow; D) another example of observed malformations in 5 dpf zebrafish larvae: heavily reduced or total absence of Meckel's cartilage (orange arrow), deformed ethmoid plate (red arrow), thinner palatoquadrate (black arrow), heavily deformed ceratohyal (violet arrow) and absence of ceratobranchials 1–5 (green arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in size.

To obtain a more precise and quantified analysis of craniofacial features, morphometric analysis was performed on individual images (Aceto et al., 2015; Guerrero-Limón et al., 2023). The assessed parameters were "Angle between ceratohyals" (ACT), "Distance between the frontal end of the ceratohyals and the line connecting the posterior ends of the hyosymplectics" (DCH), "Length of ceratohyals" (LC), "Distance covering the entire Meckel's, palatoquadrate, and hyosymplectics" (MPH), and the "Distance between the left and right Meckel's cartilages/Palatoquadrates" (MPQ) (see also Fig. 2).

These measures could not be performed on the severely affected *esr1*$^{-/-}$ mutant fish, therefore we only analyzed the 30 % (10 individuals) that were less affected, which were indeed not significantly different from their wildtype counterparts (Fig. 2). Although all assessed parameters were slightly different in *esr2b*$^{-/-}$ mutants compared to their WT siblings, only LC was significantly lower (Fig. 2A). Analysis of the *gper1*$^{-/-}$ mutants revealed that the DCH and LC were significantly different from their wildtype siblings (Fig. 2A and B), all other parameters were unchanged.

3.4. The transmembrane receptor Gper1 plays a role in overall growth and bone mineralization

To assess bone formation in the estrogen receptor mutants, we decided to measure the size of the opercle in 10 dpf larvae. Measuring the standard length of the 10 dpf larvae revealed that all mutants were slightly smaller compared to their wildtype siblings, however only the *gper1*$^{-/-}$ mutants were significantly smaller than WT (Fig. 3A). In addition, after performing alizarin red staining for the calcified tissues, we measured the area of the mineralized opercle in each larva. This measure revealed that only the *gper1*$^{-/-}$ mutant displayed a significantly smaller opercle area compared to its WT siblings (Fig. 3B–D).

3.5. Swimming behavior is differently affected by mutation of the estrogen receptors

Next, we tested the swimming behavior at 5 dpf of the different mutants in comparison to their WT siblings. Tracking the fish movements could provide important insights that could be linked to the effects caused by the studied factor, positional data is collected in a semi high-throughput fashion, then actograms of 10 s intervals are plotted to find changes in patterns (Fig. 4 and Fig. S2). We used the previously described alternating 10 min dark/light cycling protocol to measure the "swimming speed when active", the "time spent active", and the "distance travelled" respectively during the dark and the light phases (Guerrero-Limón et al., 2023). As expected, the larvae were significantly more active during the dark phases (Fig. 4).

The *esr1*$^{-/-}$ mutants showed a significantly reduced time active and distance covered in both the dark and the light phase, while swimming speed was significantly reduced only during the light phase. No change in swimming behavior was observed for the *esr2b*$^{-/-}$ mutants (Fig. 5B). In contrast, the *gper1*$^{-/-}$ mutants were significantly less active and covered a significantly smaller distance only during the light phase (Fig. 5F and I, respectively).

3.6. The heart rate was significantly increased in *esr2b*$^{-/-}$ mutants

The heart rate was measured in mutants and their WT siblings at 4 dpf. Both *esr1*$^{-/-}$ and *gper1*$^{-/-}$ mutants presented a slightly lower heart rate, but only *esr2b*$^{-/-}$ zebrafish mutants revealed a significantly increased heart rate compared to their WT siblings (Fig. S3).

3.7. Neuromasts were unaffected by mutations

Previously, a defect in neuromast development was reported upon morpholino knock-down of the *esr2b* gene in zebrafish. Using Di-Asp staining, we thus compared neuromast development in each of the estrogen receptor mutant lines relative to their WT siblings. No significant difference was observed in the number of neuromasts for any of these mutants (Fig. S4).

3.8. Exposure to chemical estrogen receptor antagonists affects zebrafish larvae behavior and heart rate

To gain further insight into the specific effects caused by inhibition of each of the estrogen receptors, chemicals with known antagonistic properties on specific human estrogen receptors were tested in zebrafish to determine whether they would mimic the effects on some of the endpoints observed in mutants.

First, the ERα -specific antagonist MPP was shown to have no effect on zebrafish development at any of the concentrations tested (10–1000 nM) and, importantly, had no effect on cranial cartilage development in 4 dpf larvae. In contrast, several effects on swimming behavior were observed upon treatment with ER antagonists. The ERα-specific antagonist MPP caused a significant increase in distance travelled at 100 nM, and in swimming speed at 100 and 1000 nM in the dark phase, while we found a surprising inverted "U"-shaped pattern in the light phase, with only the 10 nM dose causing a significant increase in all measures compared to untreated or higher doses (Fig. 6A). A similar inverted "U"-shaped pattern was observed using the inhibitor of the ERβ (PHTPP) in the light phase, where the values for all parameters were the highest at the 100 nM dose (Fig. 6B). In the dark phase, the same inhibitor caused a significant decrease in all parameters at the lowest, 10 nM dose, followed by an increase at 100 nM, and a dramatic decrease at the 1000 nM dose (Fig. 6B).

Finally, the inhibitor of GPER1 (G15) had significant effects only at the highest concentration, with an increase of the Swimming Speed in the light phase and a decrease on the Time Spent Active in the dark phase (Fig. 6C).

The exposure tests using estrogen receptor antagonists revealed a decreased heart rate at 1000 nM PHTPP relative to control, while the GPER1 antagonist G15 increased the heart rate at all concentrations tested (Fig. 7).

4. Discussion

4.1. Disrupting estrogen signaling in zebrafish

Estrogen receptors play important roles within the bodily functions of an organism, substantial effort has been put into elucidating the function of these receptors in fish using different approaches. Total antagonism was previously tested using aromatase inhibitors such as fadrozole that blocks the synthesis of endogenous estrogens and thus would affect all receptors. The effects observed were impairment of gonad histology and severe pathological changes (Luzio et al., 2016), gonadal sex differentiation (Fenske and Segner, 2004), altered gene expression in brain and ovaries (Villeneuve et al., 2009), developmental toxicity, delayed hatching and decreased heart rate (Santos et al., 2014), while reduced uptake of vitellogenin into developing oocytes (Ankley et al., 2002) and inhibition of both brain and ovarian aromatase activity was observed in fathead minnow (*Pimephales promelas*) (Villeneuve et al., 2006). However, this approach is limited when investigating the effects on early development, as it would not affect the maternal hormone stock provided by the mother within the egg. Known anti-estrogenic compounds, such as tamoxifen or ICI 182,780 are also difficult to use, due to their complex actions on the different receptors (e.g. partial agonism of tamoxifen, or agonism of ICI 182,780 on GPER1). Therefore, we decided to investigate the function of each receptor separately by knocking out each one separately to assess their effects individually. Individual receptors have been previously targeted using morpholino antisense RNAs to specifically block transcription, or by targeted mutation of the individual genes. The focus of these studies was

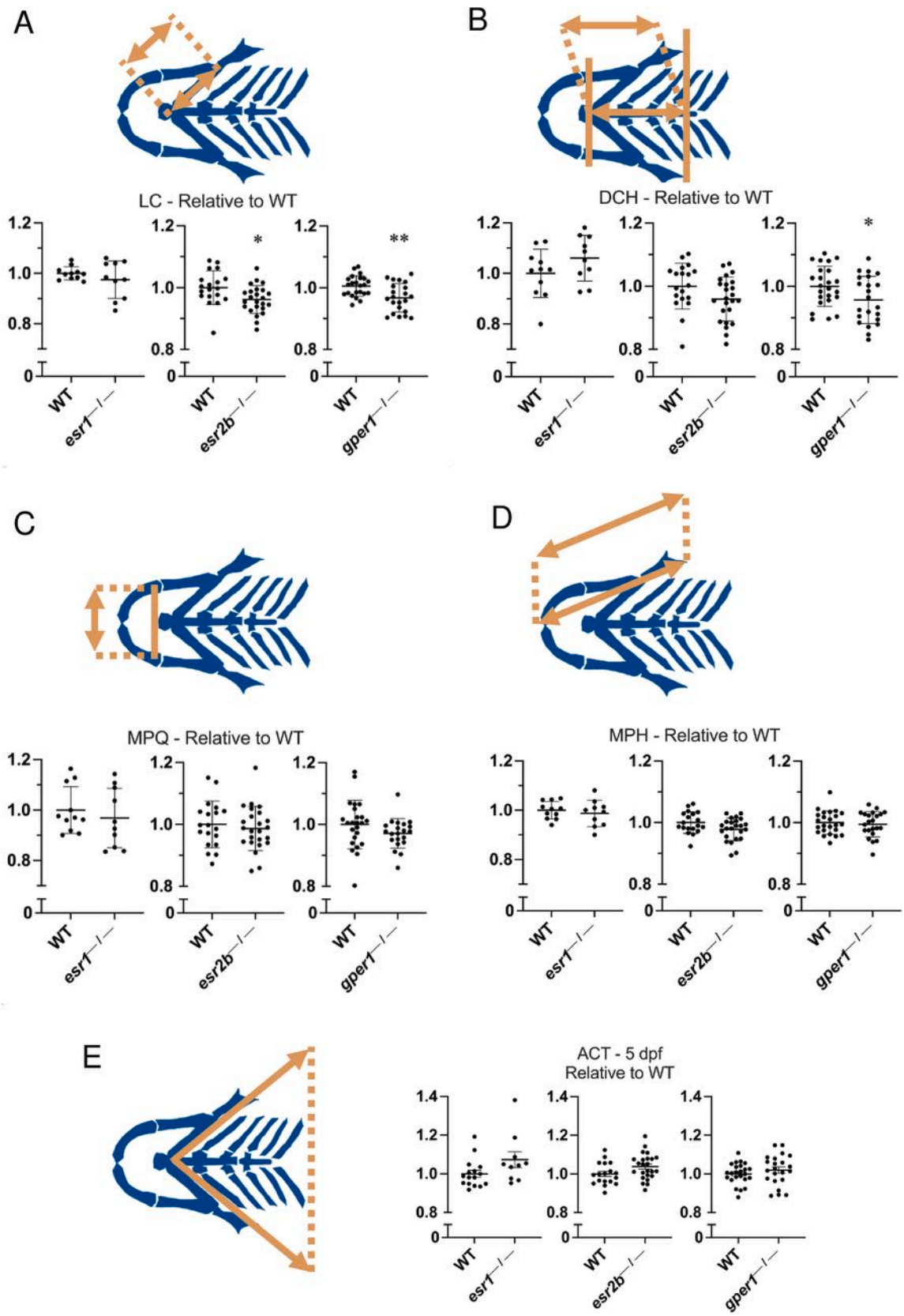

Fig. 2. Morphometrics of *esr1*$^{-/-}$, *esr2b*$^{-/-}$, and *gper1*$^{-/-}$ mutants compared to WT. Illustrations above each plot, or on the left for figure E, are a representation of how the different measurements were performed. A) Length of ceratohyals (LC); B) Distance between the frontal end of the ceratohyals and the line connecting the posterior ends of the hyosymplectics (DCH); C) Distance between the left and right Meckel's cartilage/Palatoquadrates (MPQ); D) Distance covering the entire Meckel's cartilage, palatoquadrate, and the hyosymplectics (MPH); E) Angle between ceratohyals (ACT). An Unpaired Mann-Whitney *t*-test was performed on 24 fish per group (either Mutants or WT) per assessed parameter. Asterisks (*) indicate when significant differences were found for a mutant strain compared to its WT siblings, $p < 0.05$ (*), $p < 0.01$ (**). All values were normalized relative to the mean value of the corresponding WT siblings for each mutant strain.

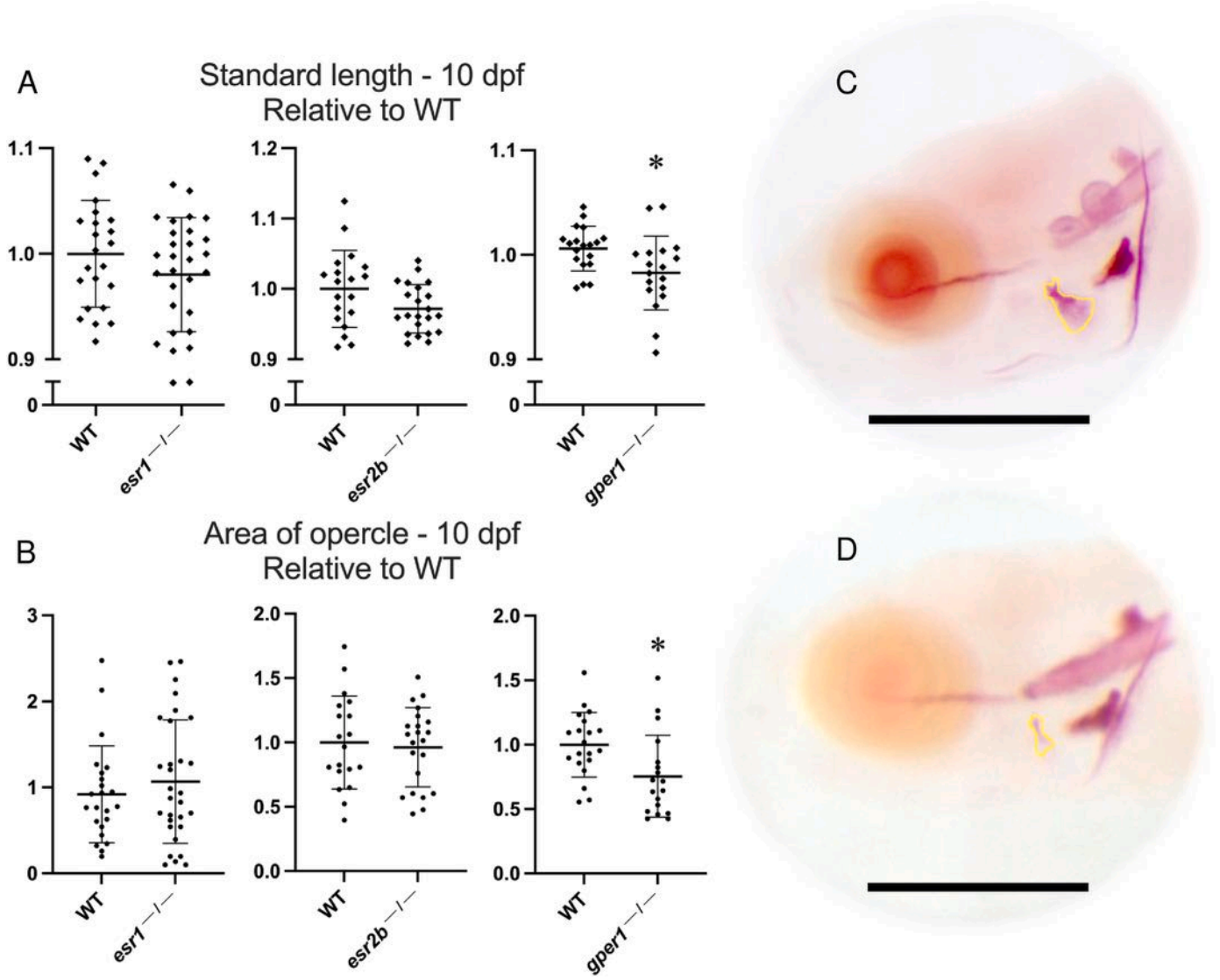

Fig. 3. A) Standard length of mutant larvae at 10 dpf relative to their WT siblings; B) opercle area measured for all mutant larvae relative to their WT siblings; C) Example of alizarin red stained WT larva and D) a *gper1*$^{-/-}$ mutant, the opercle area measured using FIJI is outlined in green, the size bar represents 500 μm. Unpaired Mann-Whitney *t*-test. Asterisks (*) indicate when significant differences were found for each mutant strain compared to its WT siblings, $p < 0.05$ (*). All values were normalized relative to the mean value of the corresponding WT siblings for each mutant strain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

on sexual maturation, sex reversal and fertility or function of reproductive organs in older animals (about 3 months old) (Pang and Thomas, 2010; Lopez-Munoz et al., 2015; Lu et al., 2017; Chen et al., 2018, 2019; Bertotto et al., 2019; Wu et al., 2021). Impairment of the antiviral response in adults was observed in *esr2b* mutants (Lopez-Munoz et al., 2015), while defects in early development were observed in the lateral line in *esr2b* and in *esr1* morphants (Froehlicher et al., 2009; Gamba et al., 2010) and dopaminergic signaling was affected in *esr1* morphants (Bertotto et al., 2019). Growth retardation and brain defects were detected in *gper1* morphants (Shi et al., 2013), as well as impaired liver growth (Chaturantabut et al., 2019) and an increased embryonic heart rate (Romano et al., 2017).

4.2. Generation of mutants and streamlined genotyping

The generation of mutant zebrafish lines for any gene is greatly facilitated by using the CRISPR/Cas9 technology, which often results in obtaining several deficient lines, however working with mutant zebrafish can still be painstakingly labor-intensive. As a rule of thumb, the larger the number of individuals, the more reliable the statistical tests and their results will be. In situations like those described here, the absence of a clearly observable phenotype at early stages means that each larva obtained from heterozygous parents has to be individually genotyped before analyzing the results of a test. Here we used a previously reported method (D'Agostino et al., 2016) that allowed us to screen a large number of samples at once. Using the cost-effective and rapid screening technique of Melting Curve Analysis, which requires a real-time PCR instrument now widely available, we were able to run several batches of zebrafish larvae in skeletal staining and behavioral tests. We therefore chose, after initial selection and validation by sequencing, the mutant lines with the largest deletions/insertions to increase the difference in size relative to WT of the amplified genomic fragment, and to simplify the detection of mutant fish. Thanks to this, the fish were easily and efficiently genotyped so that we were able to obtain results faster than using more traditional methods such as Heteroduplex Mobility Shift assays or sequencing. Thus, we decided to evaluate different developmental endpoints in the different estrogen receptor mutants that we obtained. Interestingly, none of the mutants

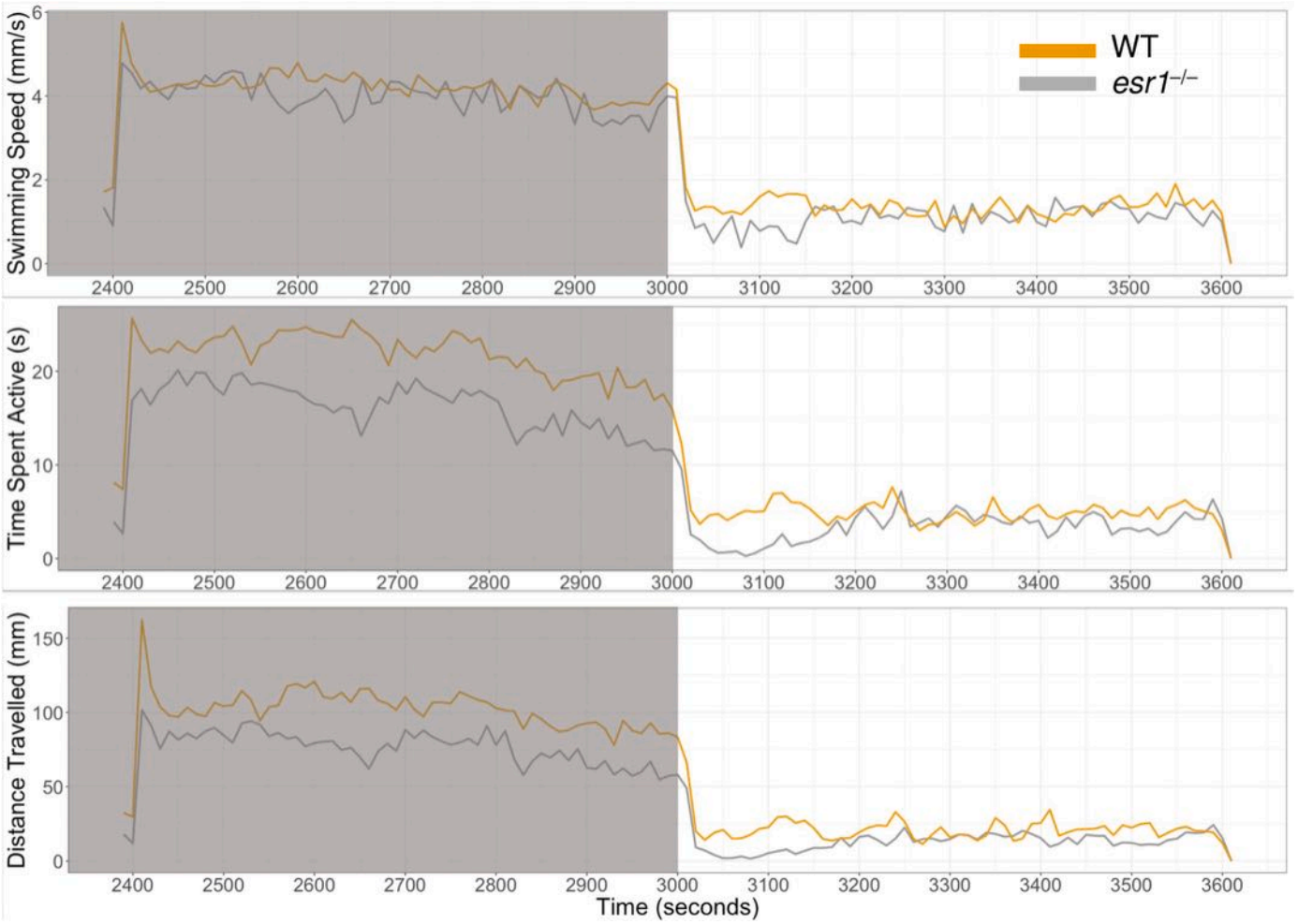

Fig. 4. Actograms of the recorded parameters in 5 dpf zebrafish larvae of WT and *esr1*$^{-/-}$ fish. The graphs represent the mean Swimming Speed when active, Time Spent Active, or Distance Travelled of 72 individuals observed in10 s intervals during 20 min, with a switch from dark to light that occurred at Time 3000 s.

displayed an increased mortality or extensive larval deformities, except 70% of the population of *esr1* mutants, consistent with the previously published results where a grossly normal development was reported. How far this resistance to lethality is due to mutual compensation of the different homologous receptors remains to be seen, however we also did observe specific developmental defects in the mutants for individual receptors. All the results of this study are summarized in Table 1.

4.3. Neuromasts

The neuromasts are a sensory organ that is part of the lateral line system in teleost fish and is composed of sensory hair cells surrounded by supporting cells (Nicolson, 2005). It has been described previously that neuromast development and function can be disrupted upon exposure to some toxicants such as heavy metals (Hernandez et al., 2006), organophosphate flame retardants such as TCPP (Xia et al., 2021), bisphenol A (Lam et al., 2011), various pharmaceuticals (Ton and Parng, 2005) and even complex matrices such as stormwater runoff (Young et al., 2018).

Although these treatments do not specifically affect estrogen signaling, it was also shown that knock-down of the *esr2b* gene using antisense morpholino oligonucleotides reduced the number of neuromasts at 72 hpf in the posterior lateral line in the morphants (Froehlicher et al., 2009), while knock-down of *esr1* caused a delay in the migration of the posterior lateral line primordium between 35 and 41 hpf (Gamba et al., 2010). Here, none of the mutants in estrogen receptor genes were affected in the number or positioning of the neuromasts at 96 hpf. Discrepancies between phenotypes observed in morphants and in mutants targeting the same gene have been previously observed (Schulte-Merker and Stainier, 2014). Two opposing explanations have since been formulated: on one hand, morpholino antisense oligonucleotides are able to target mRNAs with a similar, but not identical sequence, leading to off-target inhibition of a different gene, which would not be phenocopied by the mutant (Joris et al., 2017); on the other hand, deficient mRNA produced in a mutant is able to trigger a compensatory mechanism, not induced by antisense morpholinos, leading to higher or ectopic expression of a closely related gene (Rossi et al., 2015; El-Brolosy et al., 2019). The existence of a family of estrogen receptors makes the second explanation likely, however a compensation would not be complete as we still observe some significant phenotypes in the mutants. Further experiments will be required to determine the precise role of ERs in neuromast development (if any).

4.4. Effects on skeletal development

Estrogen receptors have important roles in bone and cartilage development, for instance, chondrogenesis may be disrupted in the presence of certain estrogenic compounds such as 17β-estradiol (E2). The latter is a pleiotropic factor for chondrocytes and osteocytes, it plays an important role in regulating the proliferation, differentiation, and the synthesis of the cartilage and bone extracellular matrix (Fushimi et al., 2009; Lara-Castillo, 2021). Its presence in high concentrations causes

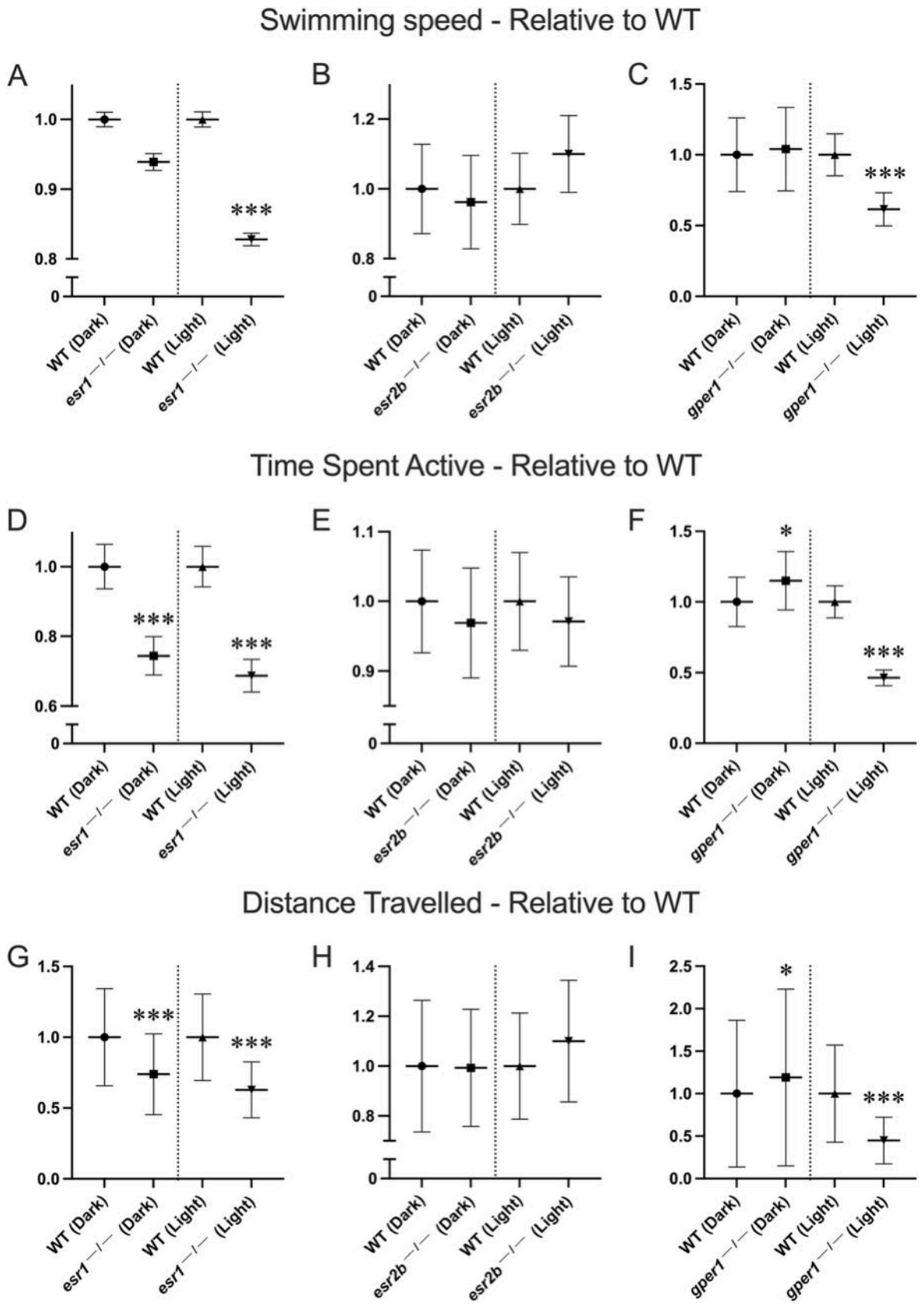

Fig. 5. Relative mean values of the three assessed parameters in each mutant strain during the motility test: Swimming speed (A–C), Time Spent Active (D–F) and Distance travelled (G–I). Asterisks (*) indicate when significant differences were found compared to WT of each mutant strain, $p < 0.05$ (*), <0.001 (***). All values were normalized relative to the mean value of the corresponding WT for each mutant strain.

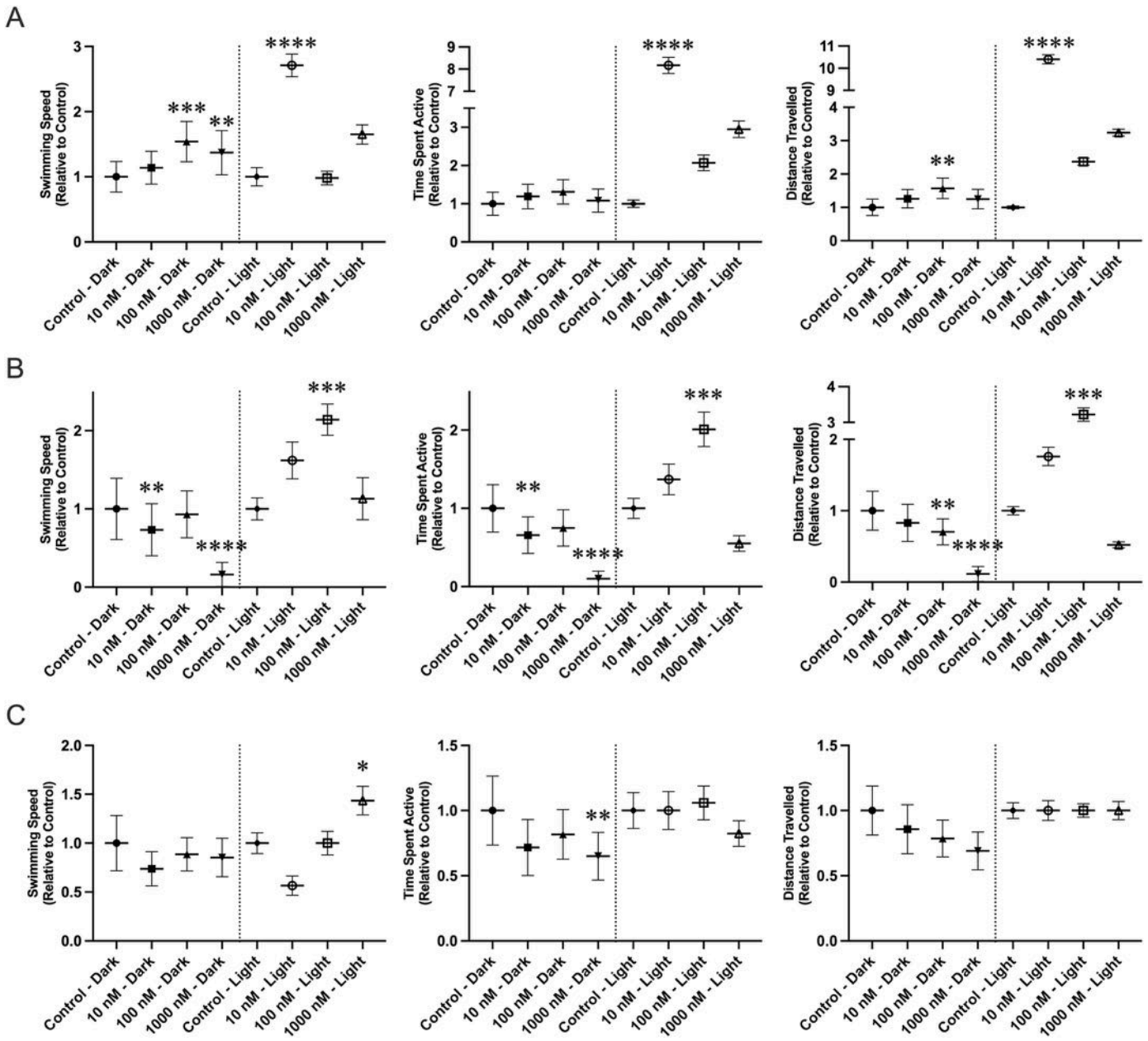

Fig. 6. Zebrafish larvae behavioral parameters recorded following 96 hpf chronic exposure to (by row) A) MPP (ERα antagonist); B) PHTPP (antagonist of ERβ) C) G15 (antagonist of GPER1). Asterisks (*) indicate when significant differences were found compared to control, $p < 0.05$ (*), <0.01(**), <0.001 (***), <0.0001 (****). All values were normalized relative to their respective control group.

craniofacial abnormalities in zebrafish larvae at ages 6 dpf to 40 dpf (Zare Mirakabad et al., 2019; Stewart et al., 2023), indicating that estrogenic signaling is involved in normal cartilage development. The underlying mechanism has been studied by whole transcriptome sequencing (He et al., 2018), revealing that genes coding for ECM proteins, such as collagens, were downregulated, while MAPK, TGF-β, and cell cycle signaling was upregulated following exposure to 17β-estradiol. Fish in those experiments had craniofacial deformities at 7 dpf. Other endocrine disruptors, such as glyphosate, were shown to affect craniofacial skeleton, bone, and behavior (Staal et al., 2018; Díaz-Martín et al., 2021).

We observed a dramatic effect of *esr1* mutation on craniofacial development, while significant effects were also observed in *gper1* and *esr2b* mutants by morphometric analyses at 5 dpf. These results clearly indicate that ERs are involved in regulation of the fine-tuned morphogenetic processes leading to an intact chondrocranium during these early stages, and that Esr1 is the main player. Interestingly, at later stages, we observe that only mutation of the *gper1* gene decreases mineralization of the opercle, an intramembranous bone that forms without a previous cartilage matrix. Taken together, these results indicate that different ERs play distinct roles in regulating the various pathways required for building an intact skeleton in zebrafish larvae.

4.5. Behavior of mutants

Previous studies revealed that exposure to E2 or 17α-ethinylestradiol (EE2) resulted in decreased "time spent active" and "total distance moved" in a dark-light behavioral test similar to the one used here (Fraser et al., 2017) or in longer durations of inactivity, exposing themselves in the center areas (Nasri et al., 2021). Here, we observed a decrease of activity in both phases in the *esr1* mutants, while the *gper1* mutant was less active in the light phase, but slightly more active in the

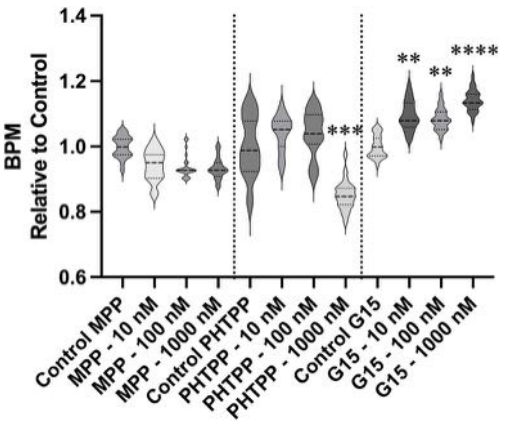

Fig. 7. Heart rate of zebrafish larvae measured at 4 dpf. Asterisks (*) indicate when significant differences were found compared to control, $p < 0.01$(**), <0.001 (***), <0.0001 (****). All values were normalized relative to control of each treatment.

Table 1
Recapitulation of the effects observed in ER mutant larvae and upon exposure to specific antagonists. The arrows indicate an up-(↑) or down-(↓)-regulation of the considered endpoint, (/) indicates no observed effect, n.d. means "not determined". The concentrations of antagonists resulting in a maximal effect are also given.

	esr1$^{-/-}$	*esr2b*$^{-/-}$	*gper1*$^{-/-}$	MPP	PHTPP	G15
Cartilage	↓↓↓↓	↓	↓↓	/	/	/
Bone	/	/	↓↓	n.d.	n.d.	n.d.
Behavior D	↓↓	/	↑	↑	↓↓	↓
Behavior L	↓↓↓	/	↓↓	↑↑↑ (10 nM)	↑↑ (100 nM)	↑
Heart rate	/	↑	/	/	↓	↑↑↑

dark phase (Table 1). Behavior is a complex endpoint that may respond to many factors; however, brain development may be an important one. EE2 was shown to decrease neuron proliferation and expression of tyrosine hydroxylase, a marker for dopaminergic neurons (Nasri et al., 2021), while *esr1* morphants have decreased dopamine levels and metabolism (Bertotto et al., 2019) and *gper1* morphants display decreased proliferation of brain cells and impaired development of peripheral neurons (Shi et al., 2013).

4.6. Heart rate in mutants

Previous studies showed that acute exposure for 1 h of zebrafish larvae to E2 led to an increased heart rate at 48 hpf, an effect that was attributed to the Gper1 receptor because ICI 182,780 (an antagonist of nuclear receptors but agonist for Gper1) was able to mimic this effect, acting most probably by increasing circulating levels of thyroid hormone T3 (Romano et al., 2017). The authors also show that larvae derived from a homozygous *gper1*$^{-/-}$ mother present lower maternal E2 levels, leading to decreased heart rates. The experiments reported here, using two heterozygous parents to generate *gper1* mutants, reveal that, at presumably normal levels of endogenous E2 in the larvae and presumably also some maternally deposited receptor, the absence of a functional *gper1* gene caused a trend to decreased heart rate, but did not lead to a significant effect.

4.7. Effects following antagonist exposure tests

In an attempt to correlate the results obtained with specific ER mutants with those observed using specific inhibitors, we chose to test three estrogen antagonists based on their alleged specificity against the human receptors. As the specificity and the affinities of these compounds for the zebrafish receptors are very controversial (Menuet et al., 2002; Notch and Mayer, 2011; Gorelick et al., 2014), we decided to use up to 1000 nM concentrations for these tests. Consistent with these previous observations, we did not find any correlation between the effects observed in the specific mutants and the supposed specificity of the antagonists. Taken together, our results show (confirm) that these antagonists against mammalian ERs are not effective in zebrafish, however we did observe different developmental defects caused by these chemicals that are presumably not endocrine mediated. Model endocrine modulators such as estradiol has, for instance, toxic effects that are not ER mediated, this was demonstrated using MCF7 breast cancer cell lines where high doses (above 10^{-6} M) of estradiol produced cytotoxic effects, but low doses (10^{-12} and 10^{-11} M) promoted cell proliferation (Welshons et al., 2003). Indeed, although orthologs are present across species, binding enzymes tend to react differently to the same compounds. That is the case of the aromatase inhibitor fadrozole, different fish species are indeed more or less sensitive (Doering et al., 2019).

An evaluation of selectivity for each specific receptor would need to use first cellular systems, as used for agonistic studies (Cosnefroy et al., 2012) where a zebrafish liver cell line was used to generate cells harboring only one of each of the receptors to test various chemicals for their ability to induce reporter gene transcription in this setting.

Another aspect that became apparent during these antagonist tests was the non-monotonic effects that we observed in the behavioral tests: e.g. the significantly increased movement parameters at 10 nM MPP or 100 nM PHTPP during the light phase were not observed at lower or higher concentrations (Fig. 6A and B). Such non-monotonic dose-response releationships have been previously described for EDC compounds (Vandenberg et al., 2012). For instance, using Bisphenol A (BPA) at environmentally relevant concentrations of 0.1 μM (low dose) BPA in male zebrafish would exert higher effects on the time spent active in comparison to a higher dose of 1 μM BPA (Weber et al., 2015). Mechanisms such as receptor selectivity, receptor down regulation, desensitization, and competition have been described to explain non-monotonic dose-responses. Using mutants deficient in potential compensatory genes could provide important insights into the processes involved.

5. Conclusions

Estrogen receptors play an important role in the overall development of organisms. Best known for their action in sexual maturation, our results emphasize their importance in early developmental stages. We present a reliable and cost-effective method, using qPCR, to tell apart mutants from WT fish, thus speeding up the findings and enabling medium throughput experiments, thus increasing statistical power of the results and their interpretation in a wider perspective. We have observed how the absence or deficiency of each one of the estrogen receptors (either nuclear or transmembrane) has consequences that can be measured using several biological tests. Although none of the individual ER mutations was lethal, we observed several effects at various frequencies and severity, however the underlying mechanisms remain at present unclear. Finally, we find that additional efforts are required to define receptor-specific antagonists for studying the effects of individual zebrafish estrogen receptors.

CRediT authorship contribution statement

Gustavo Guerrero-Limón: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Marc Muller:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Data curation, Writing – Review & Editing, Visualization, Supervision, Funding acquisition, Project administration.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143100.

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Highlights of the research paper "Exploring estrogen antagonism using CRISPR/Cas9 to generate specific mutants for each of the receptors"

Craniofacial Development

The *esr1* mutants showed significant craniofacial deformities, including malformations in structures such as Meckel's cartilage and ceratohyals. This highlights *esr1* as critical in craniofacial cartilage formation.

Bone Mineralization and Growth

gper1 mutants exhibited reduced bone mineralization in the opercle and shorter standard length, indicating a role of GPER1 in bone development and growth regulation.

Behavioral Changes

Both *esr1* and *gper1* mutants showed altered swimming behavior, with decreased activity during light phases, suggesting a role for these estrogen receptors in regulating normal behavioral patterns.

Heart Rate Variations

esr2b mutants exhibited a significantly increased heart rate, linking this kind of mutation to cardiac function regulation in zebrafish development.

14. General discussion

For decades, researchers have been successfully demonstrating the adverse effects of POPs through experiments establishing links between exposure and development of conditions. The overwhelming evidence is clear and rather than demonstrating their toxic effects, researchers nowadays are more interested in finding the underlying mechanisms to understand how exposure to certain chemicals could cause undesirable effects.

In its broadest context, this work contributes to understanding the consequences of exposure to environmental pollution (with a particular interest in EDCs) using the zebrafish larvae as a model. The rapid development of this species allows to study effects on early embryogenesis up to later stages when many organs and physiological processes are already in place. In a span of a couple of days important and reliable results that may be extrapolated to other species can be obtained. This model provides insight into effects on the vertebrate fauna in the environment, as well as on human health due to the large conservation of biological processes between the two species.

We applied a panel of biological tests to find the disruptive developmental effects in zebrafish following exposure to a realistic and ubiquitous POPs mixture. The toxic effects produced by the Total POP Mixture (TPM) at the tested concentrations are clear. An important aspect is the fact that we could observe a remarkable dose-response relationship, in other words, there was a clear trend: as the concentration of TPM increased, the observed effects became more pronounced. This is especially true concerning the prevalence of typical toxic endpoints such as edemas, eye malformation, heart rate, mitochondrial toxicity. Given the complexity of the TPM representing all major contaminants found in human blood samples, we then tested several sub-mixtures of chemically related compounds that were available at the same concentrations. Trying to elucidate the most toxic sub-mixture, all our evidence points to all the mixtures were PFAA were present (e.g., TPM, PFAA, PFAA+Br, PFAA+Cl). In brief, the overall

severity of effects in a realistic POP mixture can be classified in decreasing order as PFAA < Total Mix < Cl = Br = Control. Obviously, this has to be brought into perspective by considering the relative concentrations of the different compounds (and compound class) that are present in the TPM. For instance, the PFOS and PFOA (being the first and third most abundant in our tested mixture, respectively) have been linked to alterations in heart rate, induction of mitochondrial toxicity, changes in morphometrics, alteration of behavior (locomotor), changes in gene expression, induction of ocular toxicity, non-inflated swim bladder, alterations in the metabolism of lipids, to mention some of the outcomes found in the literature[85,246–255]. Thus, it is reasonable to think that the major culprit for the observed effects is the PFAA cocktail, and more specifically the PFOS and PFOA. Other components, most strongly the chlorinated compounds (Cl), affected the assessed endpoints to some extent, either alone or in combination with the PFAA, but were generally less active than the PFAA.

Assessing mixture effects is challenging, especially regarding EDCs as they might act as ligands to many receptors[256–258]. There is the potential interaction of chemicals within a mixture. For instance, Bisphenol A (BPA) is a well-known endocrine disruptor that alone acts as agonist of the estrogen receptor like estradiol, other structurally similar molecules such as Bisphenol F (BPF) and Bisphenol S (BPS) behave similarly by interacting as agonist of ERs and as androgen antagonists. When HeLa9903 cells were tested using a mixture of BPs (BPA, BPF and BPS, 0.33 µM of each compound within this mixture) findings revealed a higher endocrine effect of the mixture in comparison to each compound tested alone[259]. In our study, we observed that effects may be partially due to a "mixture effect" as some of the analyzed parameters seemed either enhanced or decreased.

Most of the assessed endpoints were screened at 4 or 5 dpf, only a few required observations at 10 dpf (*i.e.*, bone mineralization and survival post-exposure). The observed lethality at 10 dpf led to missing parameters in these cases, and clearly indicates that the accumulated toxic effects lead to lethality between 6 to 10 dpf

at these concentrations. Future studies performed at lower, non-lethal concentrations, will be required to complete the studies.

One of the most striking, novel developmental effects we observed was the malformation of the eyes. This malformation is unusual, as it is different from the eye absence (anophthalmia) or small eye (microphthalmia) that was previously described in mutants or toxin-treated animals[260]. Notably, disruption of thyroid hormone signaling was shown to affect eye development and visual function[261,262], however we did not observe any significant change in expression in any of the typical target genes for this pathway in our RNA-Seq data, such as the thyroperoxydase (*tpo*), thyroid hormone receptors (*thraa*, *thrab*, *thrb*), or the deiodinases (*dio1*, *dio2*, *dio3a*, *dio3b*). The only occurrence of the pear-shaped microphthalmia we found was described in a mutant for the *cap-g* gene, mimicked by knock-down of the *capd2* and *caph* genes, all components of the condensin I complex[263]. This complex is an ancient protein complex conserved from prokaryotes to eukaryotes[264], which has been studied in several organisms as it plays a crucial role in the formation and segregation of structurally stable mitotic chromosomes into daughter nuclei, in other words, it plays an important role in orchestrating the folding of the chromosome during mitosis. It is located in the cytoplasm during the interphase and loaded onto the chromosomes after nuclear breakdown at the end of the prophase[265]. Interestingly, RNA-Seq revealed that exposure to the TPM caused a decrease in the expression of many components of this complex. We thus consider as highly likely that this newly observed developmental effect is mainly linked to the disruption of the condensin I complex. Further proof would be given by testing inhibitors of the condensin I complex, such as Michellamine B or NSC260594, which were proposed by computer-assisted docking and then tested in *E. coli*[266], or by targeting the PARP-1-XRCC1 complex that directly interacts with condensin I for modulating its DNA single-strand break repair function[267].

The RNA-Seq results also allowed us to investigate possible endocrine disruptive properties of the TPM. Indeed, it is extremely difficult to assign a specific effect

caused by a complex mixture to one specific endocrine pathway. We therefore looked at modulation of the entire transcriptome to identify pathways that were affected by the exposure. Although we did not find evidence of estrogenic activity, other NRs and endocrine receptors were affected. For instance, we found clear signs of disruption in pathways involving the Androgen Receptor, but also vitamin D3 receptor (VDR), Retinoic Acid Receptor, Insulin receptor, peroxisome proliferator-activated receptor, etc.

One of the affected receptors was the VDR. Studies show that knocking down VDR (*vdrb*), via morpholino, causes a downregulation of the pre-optic markers (*pax8* and *pax2a*), thus indicating impairment of optic induction; this causes a cascading effect where zebrafish embryos produce fewer sensory hair cells creating conditions where locomotor functions are impaired[268]. Another example comes from disturbing (either through agonist or antagonist (e.g., diethylaminobenzaldehyde) chemicals) the Retinoic Acid (RA) signaling pathway, which could lead to changes in behavior and morphological changes such as fin development. Morphants of *hoxb5b* phenocopied the lack of RA signaling induced by BMS-189456 treatment[269]. Also, knocking down FABP3 (Fatty acid-binding protein 3) caused significant effects on cardiac development through dysregulation of RA signaling[270]. Thus, blocking a signal either through chemicals or applying genetic tools could yield similar insights.

The many disrupted pathways might result from the combination of the different compounds present in the TPM. For instance, brominated flame retardants have been linked to disrupt the vitamin D pathway by making cells less prepared to activate and transport vitamin D and the signaling as found in MCF-7 cells exposed to hexabromocyclododecane, hexabromobenzene and pentabromotoluene[271]. PFOS is an example of a chemical inducing hepatic steatosis (fatty liver) in zebrafish, caused by the increased expression of RARs and other genes associated to fatty acid oxidation[249]. Insulin signaling can be disrupted if the *pik3r1*, *pfkfb3* and *ptb1* genes are altered, as was shown using natural mixtures of POPs as described in Nourizadeh-Lillabadi and colleagues[272]. These authors tested a natural mixture consisting of HCB, chlordanes, DDTs,

PCBs, BDEs, Hexachlorodisilane and HCHs as found in the liver of Burbot from sampled fish coming from Lake Losna and Lake Mjøsa.

Further experiments using the mutant lines generated during this work and the TPM and sub-mixtures could advance our understanding of the underlying mechanisms of action. Furthermore, this approach could be applied using other mutants lacking for instance vitamin D, Retinoic Acid, and other receptors and assessing the same endpoints analyzed here.

As abovementioned, most interesting is the fact that while we observed endocrine activity, we did not observe any estrogenic effects, although some of the compounds of the TPM have been reported to present such capabilities. This is in line with previous results obtained using reporter cell assays to test estrogenic activities of the TPM (M. Amber, personal communication, August 2019). We hypothesize that some of the compounds within the formulation may affect estrogen signaling in opposite directions, thus in the end annihilating each other. It has been demonstrated that in certain mixture scenarios, compounds would produce unexpected results due to an antagonist-agonist interaction where the effects of a compound might be diminished in the presence of another. For instance, AhR signaling can be antagonized by PCB-118 and PCB-138 in mixtures (within our TPM) but can also have agonistic effects depending on the specific environmental context and receptor interactions[273]. It should also be noted that EDCs can be considered as tricky chemicals that might not follow the adage stating that "*the dose makes the poison*", due to the hormesis effect phenomenon. Hormesis was defined by Edward J. Calabrese as "*a biphasic dose-response phenomenon characterized by a low-dose stimulation and a high-dose inhibition*"[274]. For instance, compounds such as *p,p'*-DDE can act as antagonist of the glucocorticoid receptor by reducing its transcriptional activity in the presence of cortisol[239]. However, it has been suggested that *p,p'*-DDE could behave differently and may act as a glucocorticoid agonist if specific concentrations and contexts are given, and especially in the presence of other compounds[239]. Also, perfluorodecanoic acid (PFDA) and perfluorooctane

sulfonic acid (PFOS) can act as agonists or antagonists for various receptors, including glucocorticoid and androgen receptors. Their effects can vary based on concentration and environmental factors[275].

Additionally, is it worth mentioning that GPER1 is understudied and a good candidate to study estrogenic disruption from a non-genomic perspective and using mixtures. In Gogola et al., researchers exposed granulosa cells in human ovary to a mixture of HCB, *p,p'*-DDE, PCB-153, PFOA, and PFOS at different concentrations, where they found that these compounds exert their endocrine effects via the GPER1[143]. This transmembrane receptor is also the target of other EDCs such as genistein, BPA, DDT metabolites, PCBs, Kepone, Atrazine, PBCE, 4-tert-octylphenol, as was reviewed in Périan and Vanacker[276]. In Table 6, a compilation is given of the different compounds and their potential target-organs found in a literature search.

Table 6. List of compounds, their class and organs or tissues where they have been found to exert their effects, as reported in zebrafish studies. This is a list of the compounds that were tested during this work and are part of the POP Total Mixture and/or sub-mixtures. Our original mixture contains 29 different substances that have a different range of available information. The literature search revealed that data gaps on target organs, receptors, etc. is common. However, examples of other aquatic and mammalian species can be sometimes found.

Class	Chemical	Found or acting at sublethal doses on
PFAA	PFHxS	Ovaries, liver, brain [277]
PFAA	PFOS	Gills, organs, roes, pelvic fin, muscle, and brain[278]
PFAA	PFOA	Gills, organs, roes, pelvic fin, muscle, and brain[278]
PFAA	PFNA	No data
PFAA	PFDA	No data
PFAA	PFUnDA	No data
BR	PBDE-47	Swimming bladder, blood vessels, liver, muscle, eggs [279–282]
BR	PBDE-99	Blood vessels, thyroid hormone system, liver, intestine, brain[283,284]
BR	PBDE-100	No data
BR	PBDE-153	Liver[281]
BR	PBDE-154	No data
BR	PBDE-209	Thyroid hormone system, Intestine, eyes[285–288]
BR	HBCD	Heart, thyroid hormone system, liver[289–292]
Cl	PCB-28	Thyroid hormone system[293]
Cl	PCB-52	No data
Cl	PCB-101	No data
Cl	PCB-118	Immune system *(Binary mixture with PCB-81)[294]

Cl	PCB-138	No data
Cl	PCB-153	Physiological processes linked to circadian rhythm[295]
Cl	PCB-180	No data
Cl	p,p'-DDE	Gonads[296]
Cl	HCB	Gonads *(Binary mixture with pentachlorophenol)[297]
Cl	α-chlordane	No data
Cl	Oxy-chlordane	No data
Cl	Trans-nonachlor	No data
Cl	α-HCH	No data
Cl	β-HCH	Neurons[298]
Cl	γ-HCH	Neurons[299]
Cl	Dieldrin	Intestine (gut microbiome), central nervous system, heart[300–303]

Our experimental design was based on the presumed EDC properties of the compounds within the POP mixture. Thus, the original idea was to first assess the general developmental effects of the mixture, and then in a second step to investigate the role of estrogenic signaling in these effects. Therefore, we generated mutant zebrafish lines with ER loss-of-function and we tested specific estrogen receptor inhibitors.

We successfully generated stable zebrafish mutant lines for three estrogen receptors—Esr1, Esr2b, and Gper1—using the CRISPR/Cas9 method. These mutants provided significant insights into zebrafish development in the absence of each individual estrogen receptor, the creation of such mutants is invaluable for understanding the mechanisms underlying various conditions and phenotypes.

Initially, we aimed to create four mutant lines, including Esr2a. However, two main challenges prevented the successful generation of the Esr2a mutant: 1) promising lines faced lethality issues; homozygous mutants carrying two copies of a mutated gene are unviable at times. Given that other groups have successfully generated viable Esr2a mutants[210], lethality seems less likely. On the other hand, *esr2a* expression is present from early stages of development in zebrafish[202,304,305] and is mainly reported on zfin as "whole organism" (see:

https://zfin.org/ZDB-GENE-030116-2#phenotype), thus we did not expect really specific effects and we therefore did not insist.

The phenotypes exhibited by the ER mutant lines are distinct from those observed in the chemical exposures to TPM and sub-mixtures, further supporting the previous conclusion from RNA-Seq analysis that these compounds are not affecting estrogen signaling in zebrafish. Further research in other species is needed, as differences in ligand preferences could be influencing these effects as well.

Finally, testing the compounds described as inhibitors specific for the different mammalian ERs resulted effects that did not correlate with those observed in the corresponding mutants. The most likely reason lies in interspecies differences in the binding affinities of the different ERs[306]. Further studies would have to include *in vitro* and *in vivo* determination of binding affinities, and the search for other, more specific compounds.

Our results are, in a way, a first approach to understand how complex persistent organic pollutant mixtures could impact human health and environment. Zebrafish has been successfully applied to model human conditions. For instance, the "gridlock" mutant mirrors the aortic coarctation condition in humans[307], making it a good model for studying cardiovascular diseases. Also, zebrafish has been used to model metabolic disorders such as diabetes. The mutation of *vHnf1* results in underdevelopment of the pancreas and development of kidney cysts closely mimicking the human condition of maturity-onset diabetes of the young (MODY)[i] type 5[308]. Like these, many examples for the equivalency of zebrafish conditions resembling those in humans exist.

Zebrafish can serve as a model for toxicological studies relevant to human health, but with certain limitations. Zebrafish offer advantages such as their

[i] - *"Maturity-onset diabetes of the young (MODY) is a clinically heterogeneous group of disorders characterized by nonketotic diabetes mellitus, an autosomal dominant mode of inheritance, an onset usually before the age of 25 years and frequently in childhood or adolescence, and a primary defect in the function of the beta cells of the pancreas"* [312].

small size, rapid development, and genetic similarity to humans, which make them suitable for high-throughput toxicology screening. Their genetic homology with humans allows zebrafish to exhibit similar toxicological responses, especially in cardio-, neuro-, hepato-, and nephrotoxicity. Zebrafish have conserved genes, such as the cytochrome P450 (CYP) family, that play a role in drug metabolism and mimic many human metabolic pathways. Additionally, certain drugs known to be cardiotoxic, neurotoxic, or hepatotoxic in humans have shown similar toxic responses in zebrafish embryos and larvae. However, differences remain, primarily due to zebrafish-specific anatomical and physiological traits. For example, zebrafish embryos are surrounded by a chorion, affecting compound absorption differently than in humans. Furthermore, zebrafish regenerate organs and have a simpler liver structure, which can affect the direct translatability of some toxicological outcomes. While zebrafish studies provide significant insights into toxicity mechanisms, confirming results in mammalian models remains essential for a complete understanding (reviewed in Bauer, Mally & Liedtke[309] and Bailone *et al*.[310]). Zebrafish and mammalian toxicity assays can be comparable. For instance, LC50 values at 96 hpf correlated strongly with rat inhalation toxicity, indicating that zebrafish can serve as a proxy for relative toxicity levels found in mammalian models[311].

We anticipate that the information contained within this research work might be relevant to progress the assessment of endocrine disruptors and advance the understanding regarding complex and ubiquitous mixtures. Therefore, our findings can serve as a first screening that would give preliminary hints of the adversity, biological plausible links and exposure scenarios. Although as a species and under certain scenarios where zebrafish is more sensitive to certain chemicals than mammalian models and direct dosing equivalency is challenging, zebrafish remains a vital tool to assess the environmental health, and some adjustments might be needed to make the results comparable to mammalian models[311].

15. General conclusions, prospects and final remarks

The history of POPs serves as a cautionary tale, highlighting the need for ongoing research to comprehend the complexities of mixture toxicity and to mitigate the long-term consequences of human exposure to these persistent pollutants in our quest for progress.

In summary:

- Zebrafish is a powerful model that can be used to understand many different conditions. Its application through different disciplines and its popularity is notorious given its advantages.
- All assessed endpoints in this study were responsive (to a certain extent) to the compounds following exposure. Some were more sensitive than others and the effects were clearly dose-dependent.
- The POP mixture and sub-mixtures behaved differently than expected from a simple addition of individual effects, enhancing or decreasing the observed deleterious effects. In some cases, this could be attributed to a "mixture effect". Proving with a high degree of certainty whether a mixture effect is antagonist, synergistic or additive is complex. However, our results clearly show that some sort of interaction is taking place in the mixtures of all these compounds.
- Our transcriptomic analysis revealed that the total POP mix did not affect estrogenic signaling, however several other nuclear receptor and endocrine pathways were disturbed, such as the androgen receptor, *pparda* and *ppargc1a*, *rxrab*, *vdra*, *rargb*, and *insrb*.
- The transcriptomic analysis also provided insights on the phenotypes observed, giving hopes for developing a biomarker of effect for mixture exposures. One example is the evidence of a novel eye defect linked to the disruption of the Condensin I complex that could be explored further while studying the deleterious effects of POPs and developmental issues.
- Estrogen receptors are important for early developmental stages. They play a role in different processes.

As part of the prospects, one interesting avenue to explore would be to expose mutant zebrafish lines deficient in specific pathways to the POP mixture used here. This will allow us to better understand where and how either genomic or non-genomic responses are linked to the observed effects. Also, the physicochemical interactions of these compounds must be further studied by means of *in silico* modelling. An idea would be to test their binding properties, potencies and how fast they can arrive to a receptor, in a way to model the potential competition for binding sites of the different compounds. Finding links between this kind of compounds, using their physicochemical, molecular structure and binding properties could help developing more robust models that would reduce the need for animal testing.

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