# Physiological and molecular responses to environmental pollutants in Atlantic cod (Gadus morhua)

Characterization of the aryl hydrocarbon receptor signaling pathway and underlying mechanisms of crude oil toxicity

Libe Aranguren Abadía

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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UNIVERSITY OF BERGEN

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Libe Aranguren-Abadía
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# Abstract

Environmental pollutants are harmful substances that can interfere and disrupt numerous physiological processes. Different cellular defence mechanisms are therefore crucial for both detecting pollutants and coordinate the transcription and synthesis of genes and enzymes that are able to metabolize and excrete such compounds from the cells. The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor with an important role in mediating xenobiotic responses to a variety of environmental pollutants in vertebrates, including fish. The marine environment is the ultimate sink for environmental pollutants, and Atlantic cod (*Gadus morhua*) has been used as a bioindicator species to monitor the effects of marine pollution during the last decades. Atlantic cod is also an economically and ecologically important species in the North Atlantic Ocean, whose important spawning grounds are located in areas with proposed offshore oil exploration activities in Norway.

In Paper I, the molecular and functional properties of the Atlantic cod Ahr1a and Ahr2a paralogs were characterized. *In vitro* binding affinity and transactivation studies showed that the cod Ahrs can bind and be activated by several mammalian AHR agonists, but Ahr1a demonstrated the greatest affinity and sensitivity towards 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Tissue-specific expression of *ahr1a*, *ahr2a*, *arnt1* and *arnt2* was assessed in juvenile cod, and *ahr2a* and *arnt1* were the most abundantly expressed genes across the different tissues examined, including liver. The induction of the Ahr signaling pathway was also confirmed *ex vivo* using cod liver slices exposed to different AHR ligands, including 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), 6-formylindolo[3,2-b]carbazole (FICZ) and benzo[a]pyrene (B[a]P), where induction of *cyp1a* expression was observed for all of these compounds. Based on the abundant expression in cod liver, it was suggested that Ahr2a is the most likely protein to be involved in mediating responses to xenobiotic exposure, but a possible role of Ahr1a should not be excluded. Furthermore, the different binding affinities and sensitivities, as well as the tissue-

specific expression profiles of Ahr1 and Ahr2a, indicated that a subfunctional partitioning of the cod Ahrs has occurred.

Paper II focused on further exploring the possible subfunctional partitioning of Ahr1a and Ahr2a during early life stages (ELS) of Atlantic cod. Activation of the Ahr signaling pathway in embryos exposed to B[a]P was demonstrated, and expression and localization of *ahr1a*, *ahr2a* and *cyp1a* were assessed with *in situ* hybridization. Induced expression of *ahr2a* and *cyp1a* was observed in the cardiovascular system and skin, respectively, of B[a]P-exposed cod embryos and larvae. Furthermore, expression of *ahr2a* and *cyp1a* was also evident in the liver of B[a]P-exposed larvae. Our results further supported that a subfunctional partitioning of the *ahr1a* and *ah2a* paralogs has occurred, and that Ahr2a is the major subtype involved in mediating xenobiotic responses during ELS of Atlantic cod. On the other hand, *ahr1a* showed a persistent expression in the eye of cod embryos and larvae independent of B[a]P exposure, suggesting a role of Ahr1a in the development of the eye during early development. An endogenous role of Ahr2a was also indicated by its expression in the jaws and fin nodes of larvae.

In Paper III, a possible photo-enhanced toxicity of crude oil to ELS of Atlantic cod was studied. Several marine fish species, including Atlantic cod, have their spawning and nursery grounds in areas along the coast of Northern Norway, where offshore oil activities have been proposed. The toxicity of crude oil is known to be enhanced by exposure to ultraviolet (UV) radiation present in sunlight. Cod embryos and larvae are transparent and pelagic, and in the possible event of an oil spill during spawning in the spring months, they could experience photo-enhanced toxicity of crude oil. Atlantic cod embryos exposed to crude oil with the presence of UV radiation demonstrated increased mortality at high crude oil doses in comparison to embryos exposed to crude oil alone. Phenotypic outcomes, such as craniofacial malformations and heart deformities were visible in larvae from both experiments at the highest crude oil doses, whereas spinal curvature deformities were predominant in larvae exposed to crude oil with UV radiation. Higher number of differentially expressed

genes (DEGs) and enriched pathways were revealed in co-exposed embryos. Interaction analyses revealed that several of the enriched pathways were affected by the co-treatment with UV radiation, including circadian rhythm, oxidative stress, mitochondrial function, tryptophan metabolism and retinoid metabolism, indicating a photo-enhanced effect of crude oil toxicity on these physiological and cellular processes. Phenotypic traits observed in larvae appear to be manifested in the transcriptome responses affected by the treatments, providing mechanistic insights into crude oil and photo-enhanced crude oil toxicity. Our results suggest that UV radiation increases the toxicity of crude oil in ELS of Atlantic cod.

# List of Publications

**Paper I:**

**Aranguren-Abadía, L.,** Lille-Langøy, R., Madsen, A. K., Karchner, S. I., Franks, D. G., Yadetie, F., Hahn, M.E, Goksøyr, A., & Karlsen, O.A (2020): "Molecular and Functional Properties of the Atlantic Cod (*Gadus morhua*) Aryl Hydrocarbon Receptors Ahr1a and Ahr2a". Environmental Science & Technology, 54(2), 1033–1044. https://doi.org/10.1021/acs.est.9b05312.

**Paper II:**

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**Paper III:**

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# Abbreviations

| | |
|---|---|
| aa | Amino acid |
| AHH | Aryl hydrocarbon hydroxylase |
| AHR | Aryl hydrocarbon receptor |
| AHRR | Aryl hydrocarbon repressor |
| AIP | AHR-interacting protein |
| ARE | Antioxidant response elements |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| ATP | Adenosine triphosphate |
| B[a]P | Benzo[a]pyrene |
| bHLH | Basic helix-loop-helix |
| BMAL | Brain and muscle ARNT-like |
| BNF | Beta-naphthoflavone |
| CAR | Constitutive androstane receptor |
| CLOCK | Clock circadian regulator |
| COX2 | Cyclooxygenase 2 |
| cPLA2 | Cytosolic enzyme phospholipase A2 |
| CYP | Cytochrome P450 |
| DDT | Dichlorodiphenyl-trichloroethane |
| DEG | Differentially expressed gene |
| Dpf | Days post fertilization |
| Dph | Days post hatching |
| EC | Excitation-contraction |
| EC50 | Half maximal effective concentration |
| ELS | Early life stages |
| $E_{max}$ | Efficacy |
| ER | Estrogen receptor |
| FICZ | 6-formylindolo[3,2-b]carbazole |
| GST | Glutathione-S-transferase |
| HAHs | Halogenated aromatic hydrocarbons |
| HIFα | Hypoxia-inducible factor alpha |
| HSP90 | Heat shock protein 90 |
| ICZ | Indolo-(3,2,-b)-carbazole |
| IPAS | Inhibitory PAS domain |
| ISCs | Iron sulfur clusters |
| I3C | Indole-3-carbinol |
| Ikr | Cardiac delayed rectifier potassium |
| LTCC | L-type Ca2+ channels |
| Mhc | Major histocompability complex |
| mPTP | Mitochondrial permeability transition pore |
| NCC | Norwegian coastal cod |
| NEAC | Northeast Arctic cod |
| NFKB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLS | Nuclear localization sequence |
| NR | Nuclear receptor |
| NRF2 | nuclear factor erythroid 2-related factor 2 |
| PAH | Polycyclic aromatic hydrocarbon |
| PAS | Per-Arnt-Sim |
| PCB | Polychlorinated biphenyl |
| PCB126 | 3,3',4,4',5-pentachlorobiphenyl |
| PCLS | Precision-cut liver slices |
| PCR | Polymerase chain reaction |
| PER | Periodic |

| | |
|---|---|
| PKCα | Protein Kinase C |
| POP | Persistent organic pollutant |
| PXR | Pregnane X receptor |
| P23 | protein 23 |
| qPCR | Quantitative PCR |
| ROS | Reactive oxygen species |
| RYR | Ryanodine receptor |
| SERCA | Calcium ATPase 2 |
| SIM | Single-minded |
| SS | Spineless |
| SR | Sarcoplasmic reticulum |
| TCDD | 2,3,7,8-tetrachlorodibenzo-*p*-dioxin |
| TGO | Tango |
| UV | Ultraviolet light |
| UVA | Ultraviolet light A |
| UVB | Ultraviolet light B |
| VDR | Vitamin D receptor |
| XRE | Xenobiotic response elements |
| 3-MC | 3-methylcholanthrene |
| ΣPAH | Sum of PAHs |

# Contents

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# 1. Introduction

## 1.1 Environmental pollution in the Anthropocene

The significant impact of human activities on nature and wildlife has made the Anthropocene a proposed geological era (Waters et al., 2016). Climate change, overfishing, deforestation, loss of biodiversity, and different types of environmental pollution are among some of the major environmental issues humankind is currently driving (WWF, 2020). Anthropogenic pollution has been a concern since Rachel Carson published her book "Silent Spring" more than a half a century ago, where she documented the adverse effects of pesticides on natural ecosystems, specifically the widespread use of the insecticide dichlorodiphenyl-trichloroethane (DDT) in agriculture in the United States (Carson, 1962). The worldwide use of DTT in agriculture, as well as the production and use of several other persistent organic pollutants (POPs), was later banned in the Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention, 2009). However, as legacy pollutants slowly disappear from biota, new chemicals are manufactured, and about 100 000 different substances are currently used in the European Union and European Economic Area countries (Miljødirektoratet, 2020). For most of these compounds, also known as "chemicals of emerging concern", insufficient knowledge exists about their potential negative effects on both human and environmental health (Salimi et al., 2017).

The aquatic environment is the ultimate sink for many pollutants, and the visually striking and detrimental effects of plastics on marine wildlife have increased the societal consciousness of environmental pollution during the last years. Mankind, and especially younger generations, is realizing of the negative impacts of environmental pollution and the value of nature, which Carson described eloquently:

"Those who contemplate the beauty of the earth find reserves of strength that will endure as long as life lasts"-(Carson, 1962).

## 1.2 Transcription factors as important parts of the chemical defensome

The term “chemical defensome” was introduced by Goldstone et al., 2006 and was defined as the integrated network of genes and protein families that together constitute the cellular defence mechanisms for protecting organisms against toxic chemicals. The chemical defensome consists of phase I-III biotransformation enzymes, antioxidant proteins, heat-shock proteins, and importantly, transcription factors that are able to sense xenobiotics and coordinate the expression of genes allowing organisms to cope with these harmful stressors (ibid).

The bHLH-PAS (basic helix-loop-helix Per-Arnt-Sim) protein family includes transcription factors that play roles in e.g. cellular signaling during development, regulation of circadian rhythms, and sensing of environmental stressors, such as changes in redox status or the presence of xenobiotics or changes in redox status (Furness et al., 2007; Gu et al., 2000; Kewley et al., 2004). The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and member of the bHLH-PAS family (see 1.4.1 section) (ibid). AHR is an important protein in the chemical defensome (Goldstone et al., 2006), acting as a key environmental sensor that binds and interacts with different environmental pollutants (see 1.6.1 section) (Denison & Nagy, 2003) and regulates the transcription of important genes encoding enzymes involved in the biotransformation of xenobiotics, including cytochrome P450 1A1 (*Cyp1a1*) (see 1.6.3 section) (Nebert et al., 2004; Whitlock, 1999). In fish, expression of *cyp1a* and the enzymatic activity of the translated Cyp1a protein are widely used as biomarkers of exposure to environmental contaminants (Celander, 2011; Goksøyr, 1985, 1995; Goksøyr & Förlin, 1992; Nilsen et al., 1998; Schlenk et al., 2008; Stegeman & Lech, 1991; Stegeman & Hahn, 1994). The nuclear receptors (NR) pregnane-X-receptor (PXR), the constitutive androstane receptor (CAR), and the nuclear factor erythroid 2 related factor (Nrf2) are also considered as important chemical sensors in vertebrates (Goldstone et al., 2006).

## 1.3 The aryl hydrocarbon receptor - historical perspectives

### 1.3.1 Aryl hydrocarbon hydroxylase (AHH) induction models

The first studies of enzyme induction by foreign chemicals date to more than five decades ago. A series of papers reported an increased enzyme-mediated hydroxylation activity in the liver and gastrointestinal tract of rats treated with the polycyclic aromatic hydrocarbons (PAHs) benzo[a]pyrene (B[a]P) and 3-methylcholanthrene (3-MC), denoted as the "benzpyrene hydroxylase" enzyme system (Conney et al., 1956, 1957; Wattenberg et al., 1962). This enzyme activity, where B[a]P is hydroxylated to 3-hydroxybenzo[a]pyrene, was located to the microsomes (i.e. the fraction containing fragments of the endoplasmatic reticulum) after differential centrifugation of liver homogenates and it was considered as a protective mechanism against PAH exposure. Few years later, it was observed that this enzyme system could be activated in hamster fetus cell cultures by a diverse set of PAHs, and the name was changed to the "aryl hydrocarbon hydroxylase" (AHH) system (Nebert & Gelboin, 1968b, 1968a). In 1968, Daniel Nebert developed an *in vitro* induction model in order to measure and monitor AHH activity (ibid), and further studied 3-MC-induced AHH activity *in vivo* in different tissues of rodents (Nebert & Gelboin, 1969). Notably, the *in vivo* experiments showed distinct differences in the induction of hepatic AHH enzyme activity between two mice strains, and a more inducible AHH activity in fetal cells derived from C57BL/6 mice compared to cells obtained from DBA/2 mice was reported the year after (Nebert & Bausserman, 1970). The AHH was later named as the "cytochrome $P_1$-450" enzyme based on its spectrophotometric properties, which were different than the cytochrome P450 previously described by Omura et al. (Omura & Sato, 1964, 1962; Parli & Mannering, 1970), and eventually renamed to "CYP1A1" based on the establishment of a gene nomenclature system for cytochrome P450 genes (Nelson, 2009).

### 1.3.2 The *Ah* locus

Breeding studies showed that an autosomal recessive trait predominantly based on a single gene was responsible for the differences in AHH/CYP1A1 activity between the C57BL/6 and DBA/2 mice strains. This gene was denoted the *Ah* locus, meaning aromatic hydrocarbon responsiveness. C57BL/6 mice (possessing the $Ahr^{b1}$ allele in current nomenclature) was named the ‘‘responsive’’ strain, while DBA/2 mice (possessing the $Ahr^{d}$ allele in current nomenclature) the ‘‘nonresponsive’’ strain. Studies testing a variety of xenobiotic chemicals in mice confirmed that the sensitivities were influenced by genetic differences in the *Ah* locus (Nebert, 1989; Nebert et al., 1972; Robinson et al., 1974; Thomas et al., 1972).

### 1.3.3 The induction-receptor hypothesis

In 1974, Poland et al. found that the dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was a ~ 30.000 times more potent inducer of AHH/CYP1A1 activity than 3-MC (Poland & Glover, 1974). After this finding, Poland and co-workers decided to expose six different mice strains (C57BL/6J, BALB/cJ, A/J (responsive) and DBA/2J, AKR/J and SJL/J (nonresponsive)) to TCDD, where TCDD produced AHH/CYP1A1 induction in five mice strains that were previously characterized as “nonresponsive” to 3-MC. This landmark study demonstrated that the “nonresponsive” strains also possessed the genes necessary for expression and induction of AHH/CYP1A1 (Poland et al., 1974). The first dose-response curve of induced AHH/CYP1A1 activity as a function of TCDD exposure was presented in a review written by Daniel W. Nebert in 1975 (Figure 1) (Nebert et al., 1975).

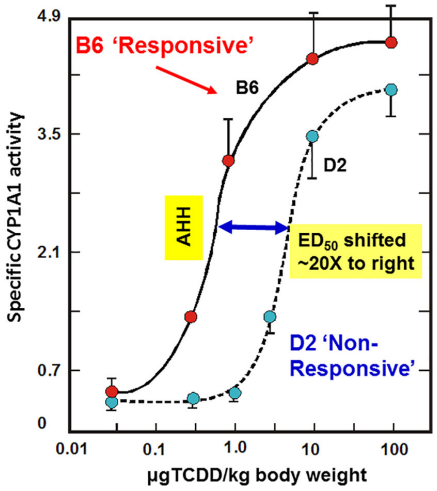


**Figure 1: AHH/CYP1A1 activity**. Dose-response curve of induced AHH/CYP1A1 activity as a function of TCDD exposure. The curves were drawn from data obtained in the TCDD exposure experiment with two mouse strains performed by (Poland et al., 1974) and adapted from the (Nebert et al., 1975) paper. C57BL/6J (B6) was named the responsive strain and DBA/2J (D2) the nonresponsive strain. Reprinted from (Nebert, 2017).

Inducibility of AHH/CYP1A1 in "nonresponsive" mice strains led to the postulation of two plausible hypotheses: 1) TCDD interacts with a cellular receptor different from the one interacting with PAHs, which is not defective and is the same in both genetically "responsive" and "nonresponsive" mice, and 2) TCDD and PAHs interact with the same cellular receptor and the greater avidity of TCDD for the receptor site present in the defective receptor permits sufficient binding to induce AHH/CYP1A1 in "nonresponsive" mice (Poland et al., 1974). The first hypothesis would imply that AHH/CYP1A1 activity in "responsive" and "nonresponsive" mice should be equally sensitive to TCDD, but that was not the case. It was required more TCDD to induce AHH/CYP1A1 activity in the "nonresponsive" mice, which supported that the second hypothesis postulated was the correct one. The main conclusion of this study was that a mutation in genetically "nonresponsive" inbred strains resulted in a defective receptor with diminished binding affinity for inducing chemicals (ibid). Another study by Poland et al. in 1975 found that heterozygous offspring from "responsive" and "nonresponsive" mice strains had an intermediate sensitivity to TCDD, confirming the conclusion of the existence of a defective receptor as previously stated

(Poland & Glover, 1975). Taken together, these studies were the first evidence of the existence of an induction-receptor that was able to modulate AHH/CYP1A1 activity in mice.

### 1.3.4 Discovery of the aryl hydrocarbon receptor (AHR)

Poland et al. tested experimentally the induction-receptor hypothesis by injecting mice with radiolabelled [$^{14}$C]TCDD (Poland & Glover, 1976). They found greater hepatic accumulation in the "responsive" strain, compared to the "nonresponsive" and the heterozygous strains, which mirrored their sensitivity to induce AHH/CYP1A1 after TCDD exposure described a year earlier (Poland & Glover, 1975). Furthermore, [$^{3}$H]TCDD binding affinity in the hepatic cytosol of mice was also assessed *in vitro*, revealing a small pool of TCDD-specific binding sites in the "responsive" mice (Poland & Glover, 1976). This landmark study by Poland and co-workers confirmed the existence of an inducer-receptor protein that was named the AHR. After this discovery, mutant hepatoma cell lines were developed as a new and potent tool to study the function and regulation of AHR (Hankinson, 1979; Legraverend et al., 1982; Miller et al., 1983). The use of this type of cell assay eventually led to the identification of important genes and proteins involved in the AHR signaling pathway, such as the aryl hydrocarbon receptor nuclear translocator (ARNT) (Hoffman et al., 1991; Reyes et al., 1992), which is required for AHR function.

### 1.3.5 Cloning of the *ahr* gene

Isolation of the AHR protein was difficult, and the early AHR characterization assays were based on observations using the reversible but high binding-affinity of [$^{3}$H]TCDD. However, development of the compound 2-azido-3[125I]iodo-7,8-dibromodibenzo-p-dioxin as a photoaffinity ligand by Poland et al. facilitated the successful purification of the AHR protein in mouse (Poland et al., 1986). This ligand coupled to AHR with a covalent bond and possessed very high specific binding. Purification of AHR from the liver of C57BL/6 mice was finally achieved using this photoaffinity ligand in combination with other conventional methods of protein

fractionation (Bradfield et al., 1991). The N-terminal amino acid sequence obtained from this purified protein led subsequently to the successful cloning of AHR cDNA from mouse (Burbach et al., 1992; Ema et al., 1992; Schmidt et al., 1993).

## 1.4 Protein sequence features of AHR

### 1.4.1 AHR is a member of the bHLH-PAS gene family

The deduced AHR amino acid (aa) sequence was expected to resemble that of steroid receptors since the AHR mode of action was similar to those. However, the AHR aa sequence was different (Okey, 2007). In fact, AHR was similar to the ARNT protein, which was cloned a year earlier (Hoffman et al., 1991). Both AHR and ARNT shared domains with high sequence similarity to two Drosophila proteins, i.e. the “periodic” (Per) and “single-minded” (Sim) proteins (Burbach et al., 1992; Ema et al., 1992).

AHR, ARNT, Per and Sim became founding members of the bHLH-PAS protein family, a subgroup of the bHLH superfamily (Gu et al., 2000; Kewley et al., 2004; Okey, 2007). bHLH proteins are involved in various important biological processes and are characterized by a basic DNA binding region adjacent to a helix-loop-helix region, which both are required for formation of functional DNA binding complexes (Kewley et al., 2004). The PAS region is an ancient domain involved in sensing and adapting to changes in the environment, and the ability of binding to a variety of cofactors and ligands is a remarkable characteristic of the PAS domain (Henry & Crosson, 2011). The PAS domain is defined as a single-input protein domain of about 250-300 aa containing two 51-aa repeats; denoted as the PASA and PASB subdomains (Figure 2) (Gu et al., 2000; Hoffman et al., 1991; Kewley et al., 2004; Nambu et al., 1991).

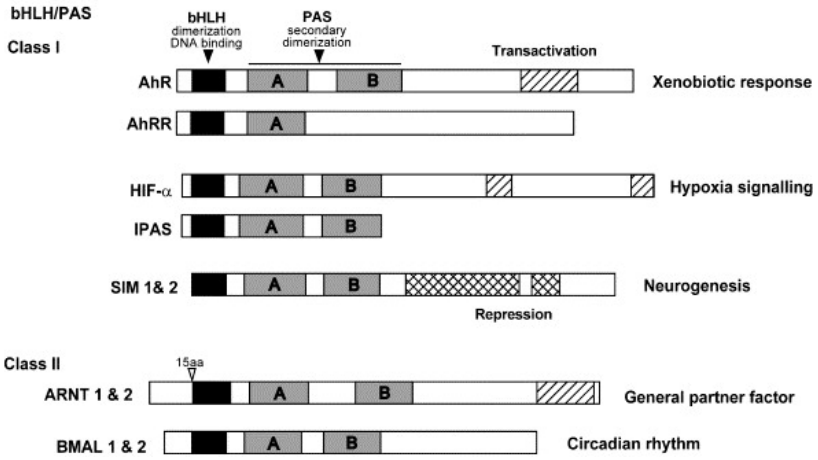


**Figure 2: The bHLH-PAS protein family.** Domain composition of different members of the bHLH-PAS protein family. bHLH-PAS Class I transcription factors neither homodimerize nor heterodimerize with other Class I members, whereas Class II transcription factors are needed to form active complexes that can bind to DNA (i.e AHR-ARNT heterodimer). Aryl hydrocarbon receptor (AHR); aryl hydrocarbon repressor (AHRR); hypoxia-inducible factor α (HIFα); inhibitory PAS domain protein (IPAS); single-minded proteins 1 and 2 (SIM 1&2); aryl hydrocarbon receptor translocator (ARNT), and circadian rhythm proteins Brain- and Muscle ARNT-Like (BMAL 1&2). Reprinted from (Kewley et al., 2004).

bHLH-PAS proteins have diverse regulatory functions (mentioned previously in section 1.2), and their dimerization is necessary in order to bind to DNA and become functional transcriptional units (Gu et al., 2000; Kewley et al., 2004). The formation of the AHR-ARNT complex is necessary for AHR to become functionally active, and the AHR-ARNT dimer was the first example identified of a bHLH-PAS heterodimer (Hoffman et al., 1991; Reyes et al., 1992). Other proteins, such as AHR repressor (AHRR), clock circadian regulator (CLOCK), and the egl-9 family hypoxia-inducible factor (previously HIF, now EGLN1) are also part of the bHLH-PAS family (Gu et al., 2000; Kewley et al., 2004; Nebert, 2017).

### 1.4.2 Domains and structure of AHR

Cloning of AHR from numerous species revealed that both the bHLH- and the PAS domains in the N-terminal region are highly conserved across vertebrates, whereas the aa composition in the C-terminal part of AHR varies to a much larger extent among species (Hahn, 2002; Okey, 2007). Aa involved in DNA-binding, as well as the nuclear localization sequences (NLS) are located in the basic region of the bHLH domain. The bHLH domain is also involved in binding to heat shock protein 90 (HSP90) and in dimerization to ARNT. Both of the PAS domains are also involved in the dimerization to ARNT, while ligand recognition and binding, in addition to HSP90 interaction are ascribed to the PASB domain. The transactivation domain, which is important for the initiation of gene transcription, is a large region towards the C-terminal end (Figure 3) (Denison et al., 2002; Hankinson, 1995; Okey, 2007).

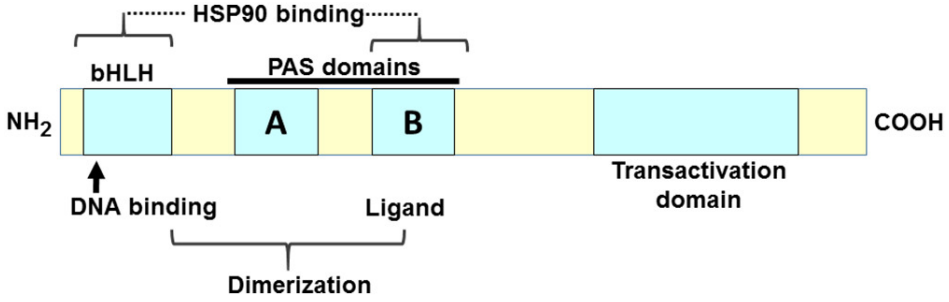


**Figure 3: Schematic illustration of the domain composition in AHR and their associated functions.** HSP90 proteins bind to both the bHLH and PASB domains; the ligand binding pocket resides in the PASB domain; dimerization to ARNT is regulated by both the bHLH domain and the PAS domains; DNA-binding takes place in the basic region of the bHLH domain; the transactivation domain is a large segment located in the C-terminus. Reprinted from (Nebert, 2017) (modified drawing found in (Okey, 2007) and references therein).

## 1.5 AHR evolution and function

### 1.5.1 AHR evolution

Several studies have identified AHR and AHR homologous genes in different animal phyla (Hahn, 2002; Hahn et al., 2006, 2017). Early studies suggested that AHR was a vertebrate protein, but identification of AHR homologs in *Caenorhabditis elegans* (Hahn et al., 1997; Powell-Coffman et al., 1998) and *Drosophila melanogaster* (Duncan et al., 1998) revealed a broader evolutionary distribution of AHR.

AHR is an ancient protein, but the exact time of the origin of AHR is not known. However, genome analyses have revealed the presence of different bHLH-PAS proteins in filastereans, choanoflagellates and porifera phyla (Hahn et al., 2017). The oldest predicted AHR homologous gene has been identified in the placozoan *Trichoplax*, whereas an AHR homolog has been confirmed in the cnidaria starlet sea anemone *Nematostella vectensis* (Hahn et al., 2017; Reitzel et al., 2014). The presence of an AHR homolog has also been identified in some present-day invertebrate species, and the biological function of AHR in the nematode *C. elegans* and the fruit fly *D. melanogaster* has been widely studied. Notably, the lack of an AHR-encoding gene was revealed in the appendicularian *Oikopleura dioica* (Yadetie et al., 2012), although it is present in the ascidian *Ciona intestinalis* (Sekiguchi et al., 2020), both members of the subphylum Tunicata. On the other hand, in the vertebrate chordate lineage, several different AHR forms have been identified (Figure 4). (Hahn et al., 2017). The existence of different AHR genes in vertebrate animals indicates that the first chordates possessed one AHR gene. Hahn et al. suggested that different vertebrate- and teleost-specific whole genome duplication events, as well as an early tandem gene duplication of AHR have led to the evolution of the AHR family in vertebrates, which is composed of AHRR, AHR1, AHR2 and AHR3 (Figure 5) (Hahn, 2002; Hahn et al., 2006).

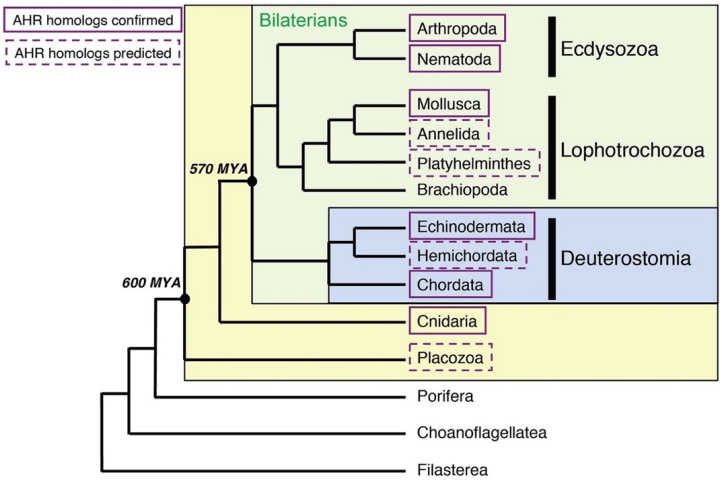


**Figure 4: The presence of AHR across animal phyla.** Phylogenetic tree showing AHR homologous genes across different animal phyla. Bilaterian protostome (ecdysozoa and lophotrochozoan) and deuterostomes animals (blue box) are indicated within the green box. Solid boxes indicate AHR homologous genes confirmed by cloning. Dashed boxes indicate predicted AHR homologs based on genome sequence analyses. The yellow box indicates all the taxa in which AHR has been identified to date. Reprinted from (Hahn et al., 2017).

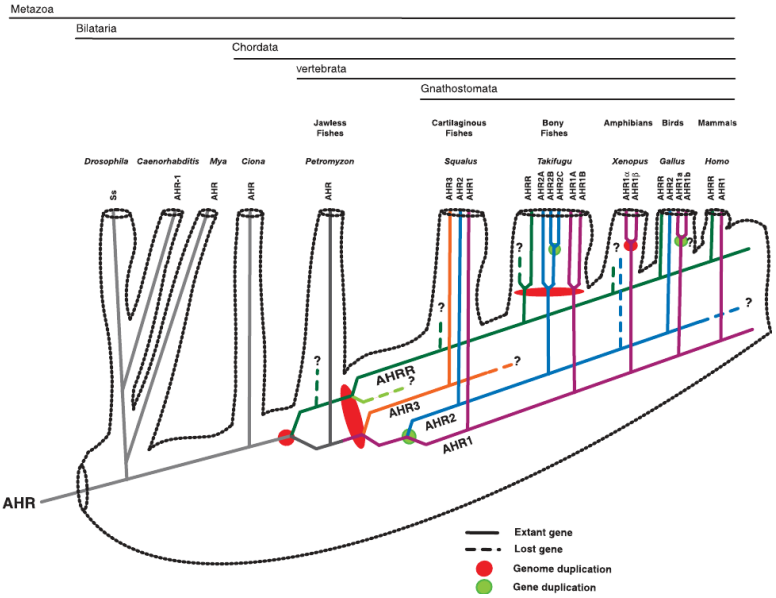


**Figure 5: Evolutionary history of AHR.** Different gene- and whole-genome duplication events together with lineage-specific gene losses have determined AHR diversity across different animal phyla. In fish, it is thought that a tandem gene duplication prior to the teleost-specific whole-genome duplication event is responsible for the multiple Ahr genes present in fish. Reprinted from (Hahn et al., 2006).

### *Ahr1 and Ahr2 in teleosts*

Identification of Ahr1 and Ahr2 in different fish species supported the hypothesis suggesting that a tandem gene duplication event occurred before the divergence of bony and cartilaginous fishes (Figure 5) (Hahn, 2002; Hahn et al., 1997). *ahr1-ahr2* tandem pairs have now been identified in cartilaginous fishes, bony fishes, coelacanths, birds and reptiles (Hahn et al., 2017). However, *ahr1* and *ahr2* genes are not found in mammalian species, such as rodents and humans. Initially, Ahr1 was thought to be orthologous to mammalian AHR, but recent sequence analyses have shown that these two genes represent different evolutionary lineages (ibid). It was also postulated that the fish *ahr2* and mammalian AHRR genes were orthologs. However, studies of *ahrr* genes from mummichog (*Fundulus heteroclitus*) and zebrafish revealed that these were non-orthologous genes to each other (Andreasen et al., 2002; Hahn, 2001; S. I. Karchner et al., 2002). Notably, only the zebrafish *ahr1a* appears to be an ortholog of the human AHR and other AHR genes (Hahn, 2002; Hahn et al., 2006). Teleost genomes often contain both *ahr1a-ahr2a* and *ahr1b-ahr2b* tandem pairs, which are thought to have arisen after the subsequent teleost-specific whole-genome duplication event (Glasauer & Neuhauss, 2014). An additional *ahr3* gene has been found in elasmobranchs, a subclass of cartilaginous fishes (Figure 5) (Hahn et al., 2006).

## 1.5.2 AHR function

Initially, AHR was identified as a receptor involved in mediating responses to pollutants such as benzo[a]pyrene and TCDD, and being able to induce expression and synthesis of drug-metabolizing enzymes in vertebrates. Creation of the *Ahr*-null (knockout) mice was essential in order to further understand the role and function of AHR (Mimura et al., 1997; Schmidt et al., 1996).

Studies on *Ahr*-null mice revealed that this genetically modified strain was resistant to hepatotoxicity, cardiotoxicity, and teratogenesis commonly observed after TCDD exposure (Fernandez-Salguero et al., 1995; Lin et al., 2001; Mimura et al., 1997; Peters et al., 1999; Thurmond et al., 1999). Knockout studies also helped to elucidate

the physiological role of AHR during normal development. *Ahr*-null mice showed abnormalities in liver ontogenesis, vascular development, and immune function, but the absence of a functional AHR is not lethal to mice (Fernandez-Salguero et al., 1995; Harstad et al., 2006; Lahvis et al., 1998; Mimura et al., 1997; Schmidt et al., 1996). All of these studies indicated that AHR mediated the typical teratogenic effects produced upon exposure to TCDD and that AHR as well had an endogenous role in different physiological processes.

Studies on the invertebrate model species *C. elegans* and *D. melanogaster* also aided the understanding of the physiological function of AHR. AHR homologous genes in *C. elegans* and *D. melanogaster* lack the ability to bind to typical vertebrate AHR agonists, but are involved in the development and function of sensory structures and neural systems (Hahn et al., 2017). AHR-1 in *C. elegans* was shown to be involved in development of touch receptor neurons, GABAergic motor neurons, interneurons and sensory neurons (Qin & Powell-Coffman, 2004), and its function was dependent on its co-factor AHA-1 (ARNT homolog) (Huang et al., 2004). The AHR homolog, *spineless* (*ss*), is also involved in development of *D. melanogaster*. Loss-of-function mutations demonstrated that *ss* regulates formation of distal antennae identity, as well as tarsal regions of the legs and mechanosensory bristles (Duncan et al., 1998). Emmons et al. also showed that the fruit fly ARNT homolog tango (*tgo*) functions as a heterodimer for *ss* in the development of these structures (Emmons et al., 1999). Furthermore, it has been shown that *ss* controls dendrite morphology in sensory neurons during early development and photoreceptor cell fate later in development, and *tgo* is also required in both processes (Crews & Brenman, 2006; Kim et al., 2006; Thanawala et al., 2013). Functional characterization of the anemone *N. vectensis* AHR also showed that this protein do not bind to common vertebrate AHR ligands and it does neither interact with ARNT (Reitzel et al., 2014). Hahn et al., 2006 proposed that AHR initially had a role in development (invertebrates) and has later evolved into a ligand-activated transcription factor (vertebrates).

A role of Ahr2 in mediating xenobiotic responses in ELS of fish is well established (Clark et al., 2010; Garcia et al., 2018; Goodale et al., 2012; Incardona et al., 2011; Jönsson et al., 2007, 2009; Prasch et al., 2003; Souder & Gorelick, 2019; Sugden et al., 2017; Van Tiem & Di Giulio, 2011; Yin et al., 2008). Differences in tissue-specific expression patterns, and ligand- and target gene specificities observed between Ahr1 and Ahr2 indicated both physiological and xenobiotic roles of Ahrs in fish (Hahn et al., 2017). Although the role of Ahr1 is not yet well elucidated it has been suggested a role of zebrafish Ahr1b in embryonic development, where expression of *ahr1b* and localization of Ahr1b protein was observed in the developing eye of zebrafish (Karchner et al., 2017; Karchner et al., 2005; Sugden et al., 2017). It has been hypothesized that Ahrs in fish might have acquired functional specialization through subfunction partitioning (Hahn et al., 2017).

In the last years, more research has been focused on enhancing our understanding of the physiological role of the AHR. A role of the AHR signaling pathway in mediation of several critical life processes, as well as cellular functions has now become evident (Denison et al., 2011; Mulero-Navarro & Fernandez-Salguero, 2016; Nebert, 2017). The role of AHR in immune functioning has been described. High levels of constitutive AHR expression is found in liver and in immune cells constituting barrier tissues such as skin, lung, gut, and mucosal epithelia, as well as in the placenta (Esser & Rannug, 2015). Implications of AHR in cell proliferation, differentiation, pluripotency, stemness, adhesion and migration are also well characterized (Larigot et al., 2018; Mulero-Navarro & Fernandez-Salguero, 2016). Elucidation of the endogenous roles of AHR has also supported the idea of AHR as a therapeutic target to treat diseases, including specific tumors, immune disorders, inflammatory diseases, and to enhance production of hematopoietic stem cells (Mulero-Navarro & Fernandez-Salguero, 2016).

## 1.6 AHR-mediated responses to xenobiotics

### 1.6.1 AHR ligands

Initially, the ligand-binding site of AHR was thought to have rigid dimensions and to only accommodate ligands that were highly planar. However, *in vitro* competition assays and high-throughput screening assays revealed that a variety of ligands are able to bind to AHR (Okey, 2007). AHR is now considered a promiscuous transcription factor because of its ability to bind and be activated by a diverse group of xenobiotic and endogenous compounds (Denison & Nagy, 2003; Denison et al., 2011). Planar halogenated aromatic hydrocarbons (HAHs) and PAHs are among the "classical" xenobiotic AHR ligands originating from anthropogenic sources. HAHs represent the most potent class of AHR ligands (with binding affinities in the pM to nM range), and the dioxin TCDD is part of this group. In fact, AHR has also been named "the dioxin receptor" because of its high affinity to TCDD. HAHs are very stable ligands possessing a high binding affinity to AHR, which produce a persistent activation of the AHR-dependent signaling pathway and target gene expression across most vertebrate species. In contrast, PAHs (with binding affinities in the nM to μM range), produce a transient induction of AHR, but are also able to activate AHR-dependent gene expression, such as Cyp1a (Denison & Nagy, 2003;Denison et al., 2011). A major difference between these classes of chemicals is that HAHs are poorly metabolized, whereas PAHs and most other AHR ligands are readily degraded by xenobiotic metabolism (biotransformation). Hence, it has been stated that the ability of a ligand to produce AHR-dependent toxicity is associated to its resistance to metabolism and ability to persistently activate AHR (Denison et al., 2011). Polychlorinated biphenlys (PCBs), such as dioxin-like PCBs can also bind to AHR and produce AHR-mediated toxicity (Nguyen & Bradfield, 2008).

A variety of naturally occurring dietary chemicals have also been shown to directly activate and/or inhibit the AHR signaling pathway (Denison & Nagy, 2003). The most widely investigated dietary AHR ligands are plant compounds, such as the phytochemical indole-3-carbinol (I3C). I3C is in itself a weak AHR agonist, but one

of its derivatives produced in the mammalian digestive tract, the indolo-(3,2,-b)-carbazole (ICZ), exhibits high affinity (low nM range) for binding to AHR, similar to that of TCDD, and is thus a potent AHR agonist (Bjeldanes et al., 1991; Gillner et al., 1993). Flavonoid compounds ubiquitously found in fruits and vegetables, such as beta-naphthoflavone (BNF), can also bind to AHR and are considered the most abundant naturally occurring dietary AHR ligand. In general, the majority of the dietary AHR ligands identified to date have been shown to have weak AHR activity (Ashida et al., 2008; Denison & Nagy, 2003; Nguyen & Bradfield, 2008).

Numerous endogenous AHR ligands, including arachidonic acid and leukotrienes, heme metabolites and UV photoproducts of tryptophan, have been discovered and characterized during the last years (Denison & Nagy, 2003;Nguyen & Bradfield, 2008). The 6-formylindolo[3,2-b]carbazole (FICZ) has been determined as the most active tryptophan derivative produced after ultraviolet light (UV) exposure. FICZ has a high affinity for AHR and is a strong activator of *Cyp1a1* transcription (Rannug et al., 1987;Rannug et al., 1995; Wei et al., 1998). Recently, another tryptophan metabolite, kynurenine, has been shown to bind to AHR in human tumor cells (Opitz et al., 2011).

### 1.6.2 The AHR protein complex

The unliganded AHR is located in the cytoplasm in a protein complex with two HSP90 proteins, a co-chaperone protein (p23), and AHR-interacting protein (AIP) (Carver & Bradfield, 1997; Denison et al., 2011; Kazlauskas et al., 1999; Perdew, 1988). HSP90 is necessary for proper AHR folding and stability (Petrulis & Perdew, 2002). AIP interacts with both HSP90 and enhances transcriptional activity, folding, and stabilization of AHR (Meyer et al., 1998; Meyer & Perdew, 1999; Petrulis & Perdew, 2002). AIP also promotes the cytoplasmic localization of AHR by inhibiting interaction between the AHR protein complex and the protein that mediates AHR transport into the nucleus, i.e. importin-β (Petrulis et al., 2003; Ramadoss et al., 2004). P23 prevents ubiquitylation-mediated degradation of AHR (Kazlauskas et al., 1999; Kudo et al., 2017; Pappas et al., 2018). In general, the protein complex ensures

cytoplasmic localization of AHR and keeps it in a high ligand affinity conformational state (Kudo et al., 2017; Petrulis & Perdew, 2002).

### 1.6.3 The classical AHR genomic pathway

Recognition and binding to an AHR agonist produces a conformational change in the AHR protein, resulting in exposure of its NLS. AHR dissociates then from AIP, and it is proposed that the AHR-HSP90 protein complex translocates into the nucleus (Ikuta et al., 2000; Rothhammer & Quintana, 2019; Tsuji et al., 2014). In the nucleus, AHR heterodimerizes with ARNT, and the ligand-AHR-ARNT complex transforms into its high affinity DNA binding form. The ligand-AHR-ARNT complex binds to xenobiotic response elements (XRE) containing the 5′-KNGCGTGM-3′ consensus sequence in the promoter region upstream of AHR target genes and modulate the transcription of a battery of genes encoding enzymes involved in the detoxification of xenobiotics, where CYP1A1 is among the most characterized (Figure 6) (Denison et al., 2011; Pollenz et al., 1993; Soshilov & Denison, 2008; Swanson, 2002; Whitlock, 1999).

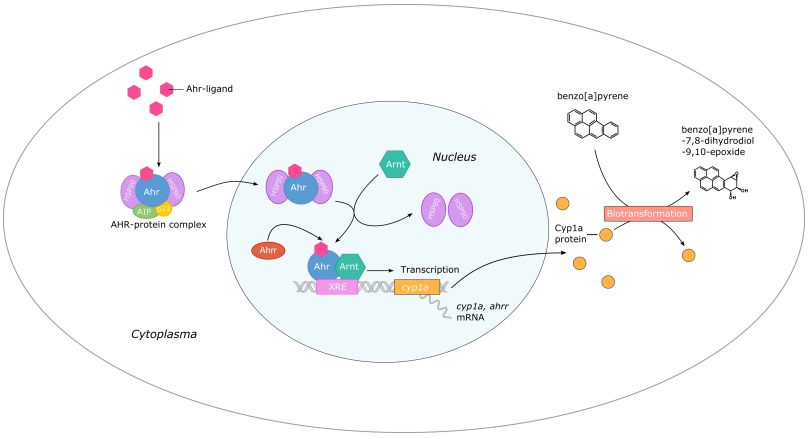


**Figure 6: Theoretical Ahr genomic pathway in vertebrates.** Illustration describing the Ahr signaling pathway in vertebrates. Activation of the Ahr pathway is produced after ligand binding and translocation of Ahr to the nucleus. Ahr binds to Arnt in the nucleus and the Ahr-Arnt heterodimer binds to XRE and produces transcription of a battery of genes, such as *cyp1a* and *ahrr*. The Cyp1a enzyme is involved in biotransformation of pollutants, which together with the epoxide hydrolase enzyme converts B[a]P to benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide as indicated. Ahrr regulates Ahr transcriptional activity and is part of a negative feedback-loop (modified figure that was originally made by Alexander K. Madsen).

### *The cytochrome P450 1A (Cyp1a)*

The CYPs constitute a superfamily of heme containing enzymes that catalyse oxidative and reductive transformations of both endogenous and exogenous chemicals. CYP families 1-4 include enzymes involved in drug and xenobiotic metabolism, which convert lipophilic chemicals into more water-soluble products for detoxification and excretion (Denison & Whitlock, 1995; Nebert & Dalton, 2006; Schlenk et al., 2008). CYP1 enzymes such as CYP1A1 play important roles in catalysing oxidation of many environmental pollutants such as dioxins, dioxin-like PCBs, and some PAHs (Nebert & Dalton, 2006). Mammalian CYP1 genes are regulated by AHR (Nebert et al., 2004), and Fernandez-Salguero et al., 1995 demonstrated that *Ahr*$^{-/-}$ mouse lacks inducible CYP1 expression and dioxin-inducible phenotypic traits. CYP1 enzymes are also involved in the metabolic activation (toxification) of certain PAHs. An example is the CYP1A1-mediated biotransformation of B[a]P into the reactive and carcinogenic metabolite benzo[a]pyrene-7,8,-dihydrodiol-9,10-epoxide (Figure 6), which can covalently bind to DNA and form DNA adducts that may lead to mutagenesis (Grover & Sims, 1968; Daniel W. Nebert & Dalton, 2006).

Vertebrate CYP1 genes are divided into two major subclades: the CYP1As and the CYP1B/1Cs (Goldstone & Stegeman, 2006). In humans, there are two *CYP1A* paralogous genes (*CYP1A1* and *CYP1A2*), while in fish so far only one *cyp1a* gene has been discovered (Goldstone & Stegeman, 2006). *CYP1A1* is the best-studied AHR target gene and is involved in phase I of the biotransformation (Denison & Whitlock, 1995; Nebert & Dalton, 2006). Induction of *cyp1a* has been widely studied in fish and is used as a biomarker of exposure to environmental pollutants (Celander, 2011; Goksøyr, 1995; Goksøyr & Förlin, 1992; Nilsen et al., 1998; Schlenk et al., 2008;Stegeman & Lech, 1991; Stegeman & Hahn, 1994).

### *The aryl hydrocarbon repressor (Ahrr)*

AHRR is a key target gene that regulates AHR transcriptional activity (Mimura et al., 1999). AHRR binds to ARNT, and the AHRR-ARNT heterodimer binds to XREs, but the AHRR-ARNT dimers are not transcriptionally active (Baba et al., 2001; S. I. Karchner et al., 2002; Mimura et al., 1999). The exact mechanism of AHRR-mediated repression of AHR transcriptional activity has not yet been elucidated, but it has been suggested that AHRR suppresses AHR function in a negative feedback loop by either competing with AHR for ARNT, or by the binding of AHRR-ARNT to XRE and thus replacing the AHR-ARNT-XRE binding (Hahn, 2002; Hahn et al., 2009). A recent crystallography study showed the formation of an AHRR-ARNT heterodimer (Sakurai et al., 2017). The N-terminal AHRR protein structure is similar to AHR, but the PASB domain is lacking, resulting in the absence of a ligand-binding domain in AHRR and the inability to bind to typical AHR ligands (Baba et al., 2001; Karchner et al., 2002; Mimura et al., 1999). In accordance with what is described in mammals, Ahrr in fish also lacks the PASB domain and does not possess a ligand-binding site (Evans et al., 2005; Karchner et al., 2002; Roy et al., 2006). The lack of binding to mammalian AHR ligands has been demonstrated in *F. heteroclitus* Ahrr (Karchner et al., 2002). In zebrafish, both Ahrra and Ahrrb control the TCDD-activated Ahr signaling pathway, but the exact mechanisms of how these proteins interact with zebrafish Ahrs is not known (Evans et al., 2008; Jenny et al., 2009).

## 1.6.4 The AHR non-genomic pathway

In the early 1980s, different scientists pointed out the difficulty in explaining toxicity responses produced by various AHR ligands based solely on the model producing the induction of CYP proteins (Matsumura, 2009). The classical AHR genomic action model could not explain the inflammatory responses observed in cells exposed to TCDD, which lead to the hypothesis of the existence of an independent non-genomic AHR pathway (ibid). Matsumura et al. reported previously a significant up-regulation of certain types of kinases in isolated hepatocyte plasma membranes from rats exposed *in vivo* to TCDD (Matsumura et al., 1984). The development of the *src*-null (knockout) mice by Dunlap et al. demonstrated that this type of mice were less

susceptible in acquiring wasting syndrome phenotypes associated to TCDD exposure in comparison to the wild-type exposed mice (Dunlap et al., 2002; Dunlap & Matsumura, 2000). Other studies also observed a rapid increase in intracellular concentration of $Ca^{2+}$ after TCDD exposure. The increase in intracellular concentration of $Ca^{2+}$ has been hypothesized to be the triggering event initiating the activation of inflammatory responses after TCDD exposure, and to be mediated by the non-genomic AHR pathway (Matsumura, 2009). It has been observed that an increase in intracellular $Ca^{2+}$ produces an activation of protein kinase C (PKCα), which is a very early action of TCDD exposure that does not depend on Src kinase or ARNT. PKCα phosphorylates a cytosolic enzyme, phospholipase A2 (cPLA2), with the subsequent release of arachidonic acid. Src kinase activation is thought to be a down-stream signaling event from cPLA2, which regulates transcription of cyclooxygenase 2 (COX2). COX2 uses arachidonic acid to produce prostaglandin, leading to inflammatory responses (Figure 7) (Larigot et al., 2018; Matsumura, 2009).

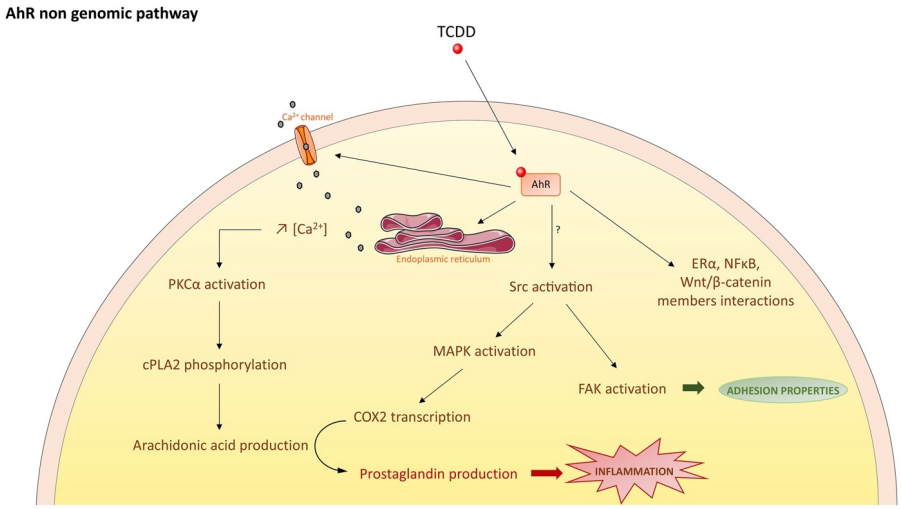


**Figure 7: The AHR non-genomic pathways.** An independent pathway involved in mediating inflammatory responses different from the well-known genomic AHR pathway based on CYP regulation has been postulated. Exposure to AHR ligands, such as TCCD, produces an increase in intracellular $Ca^{2+}$ and this event is considered the triggering mechanism regulating the non-genomic pathway. $Ca^{2+}$ can modulate production of arachidonic acid, which is transformed into prostaglandins. Metabolism of arachidonic acid is mediated by cyclooxygenase 2 (COX2) enzyme, in which transcription is initiated after

activation of Src kinase. AHR may also interact with other signaling pathways. Reprinted from (Larigot et al., 2018). Figure license: https://creativecommons.org/licenses/by-nc-nd/4.0/.

Cross-talks between AHR and several intracellular signaling pathways, cellular functions, and nuclear hormone receptors (NR) have also been characterized (Beischlag et al., 2008; Denison et al., 2011). One of the most studied is the cross-talk between the AHR and the NR estrogen receptor (ER). Liganded AHR can repress ER signaling through a number of different mechanisms and produce an antiestrogenic effect (Beischlag et al., 2008; Denison et al., 2011; Matthews & Gustafsson, 2006; Ohtake et al., 2003, 2007; Yadetie et al., 2018). Interactions between AHR and WNT/β-catenin, and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kβ), have also been observed (Beischlag et al., 2008; Bock, 2019; Denison et al., 2011; Procházková et al., 2011). Early studies also linked AHR and Nrf2 pathways (Nguyen et al., 2003). The Nrf2 transcription factor regulates transcription of drug-metabolizing and antioxidant enzymes by binding to antioxidant response elements (ARE) in DNA (Kensler et al., 2007; Nguyen, Sherratt et al., 2003; Qiang Ma, 2015). It was later shown that a cross-talk between AHR and Nrf2 is required for induction of conjugating enzymes. A bidirectional genetic cross-talk also appears to exist between these two proteins (Hayes et al., 2009; Miao et al., 2005; Shin et al., 2007), and it has been shown to be required for induction of conjugating enzymes in mice exposed *in vivo* to TCDD (Yeager et al., 2009). In fish, the cross-talk between Ahr and Er is also characterized (Bemanian et al., 2004; Celander, 2011).

## 1.7 Crude oil toxicity

Crude oil is a complex mixture consisting of thousands of different chemicals that can be toxic to marine life and produce long-lasting effects on marine ecosystems in the event of an oil spill (Barron et al., 2020). PAHs are assumed to be the most toxic components since they are considered the most bioavailable compounds present in the water soluble fraction (WSF) of crude oil (Carls et al., 1999; Carls & Meador, 2009). Crude oil is especially harmful to the vulnerable ELS of fish, and can lead to acute mortality and produce numerous sublethal effects, including formation of heart and yolk sac edema, alterations in heart morphogenesis, and craniofacial and spinal deformities (Carls et al., 1999; Cherr et al., 2017; Hodson, 2017; Incardona & Scholz, 2017).

Several studies have postulated that tricyclic PAHs present in the WSF are the main responsible compounds for crude oil-induced toxicity (Hodson, 2017; Incardona, 2017). However, this theory has recently been questioned (Meador & Nahrgang, 2019) due to the relative low toxicity of these tricyclic PAHs when tested in low concentrations without being part of a crude oil WSF (Butler et al., 2016; Sørensen et al., 2019). Meador & Nahrgang, 2019 proposed an alternative hypothesis to the receptor specific toxic event proposed by Incardona et al., where bioaccumulation of compounds in crude oil disrupts calcium homeostasis and may produce nonspecific baseline toxicity. The authors also claimed that crude oil toxicity should not only be ascribed to PAHs as they are just a small portion of the WSF and that it cannot be excluded that other toxic compounds present in crude oil act through mechanisms that are still unidentified (ibid). Even though the aromatic fraction does clearly not contain the only toxic compounds in crude oil, it is still well documented that PAHs are strongly correlated to the toxicity of oil and petroleum products (Adams et al., 2014; Kamelia et al., 2019; Kang et al., 2014; Radović et al., 2014).

Toxicity of crude oil may also be increased by exposure to sunlight, or specifically ultraviolet (UV) radiation (Barron, 2017). Photo-enhanced toxicity of crude oil is linked to PAHs, and mainly 3-5 ring PAHs, as well as their oxygen, sulfur, and nitrogen analogs (Arfsten et al., 1996; Barron, 2017). There are two known mechanisms leading to photo-enhanced toxicity: photomodification and photosensitization. Photomodification is produced by photooxidation of compounds present in crude oil, which may in some cases be more toxic. In photosensitization, bioaccumulated PAHs absorb UV and the energy transfer from an excited state to oxygen molecules may lead to formation of reactive oxygen species (ROS) and free radicals within the organism, leading to a state of oxidative stress (Barron, 2017; Roberts et al., 2017). Photosensitization is believed to be the dominant mechanism in photo-enhanced toxicity affecting ELS of fish (Barron, 2017), and excess of ROS can produce DNA damage, degradation of proteins and lipids, mitochondrial dysfunction, alterations in $Ca^{2+}$ homeostasis, and subsequent mitochondria-dependent apoptosis (Ermak & Davies, 2001; Görlach et al., 2015; Ott et al., 2007; Simon et al., 2000).

## 1.8 Atlantic cod (*Gadus morhua*)

### 1.8.1 Distribution and ecology

Atlantic cod (*Gadus morhua*) is an economically, culturally, and ecologically important teleost that is widely distributed in the northern parts of the Atlantic Ocean. Two distinct Atlantic cod ecotypes exist in Norwegian waters: Norwegian coastal cod (NCC) and Northeast Arctic cod (NEAC or Skrei) (Rollefsen, 1933). NCC is a non-migratory and demersal fish that inhabits coastal areas and fjords along the Norwegian coast, and spawn close to the shore in shallow waters. In contrast to NCC, the NEAC is a migratory and pelagic fish that undertakes migrations over long distances between the feeding grounds in the offshore areas of the Barents Sea and the various spawning locations along the coast of Norway. Genomic analyses have found distinct genomic regions and chromosomal rearrangements patterns that separate the two behavioral cod ecotypes (Berg et al., 2016; Hemmer-Hansen et al., 2013; Karlsen et al., 2013; Kirubakaran et al., 2016).

Vesterålen, Senja and Lofoten in Northern Norway are the main spawning areas for the world´s largest cod population (the NEAC stock) (Bogstad, 2009; Olsen et al., 2009). Spawning takes place mainly in March and April, and a female cod can spawn up to several million eggs during this period. NEAC eggs are buoyant near the surface, and eggs and larvae drift with the northbound currents back to the Barents Sea (Olsen et al., 2009).

### 1.8.2 Sequencing of the cod genome

Star et al. published the first Atlantic cod genome sequence in 2011 (Star et al., 2011), and the annotated "gadMor1" version was deposited in the Ensembl database. Later, two new improved versions of the cod genome ("gadMor2" (Tørresen et al., 2017) and "gadMor3") have been produced with state-of-the-art sequencing technology (NCBI ac. no. GCF_902167405.1). Interestingly, the genome sequencing revealed that Atlantic cod lack several genes important for the adaptive immune system in vertebrates, including the major histocompatibility complex (Mhc) II, Cd4, and invariant chain (II), and low coverage genome sequencing of 66 teleost species demonstrated the absence of *mhcII* across the entire Gadiformes order (Malmstrøm et al., 2016; Star et al., 2011). In addition to the lack of important components in the defence system towards pathogens like bacteria and viruses, Eide et al. further reported the loss of the xenobiotic sensor NR Pxr in the cod genome. Pxr is an important regulator of the defence system towards chemical stressors in most other vertebrates (Eide et al., 2018), and this finding led to the hypothesis that Ahr may have evolved a broader compensatory functional role as a xenosensor and regulator of *cyp* gene transcription in Atlantic cod, including *cyp3a* gene regulation. Notably, increased numbers of putative XREs in the promoter region of cod *cyp3a166* and *cyp3a169* in comparison to orthologs in zebrafish, human, and mouse were revealed, suggesting that the transcriptional regulation of Atlantic cod *cyp3a* genes are more complex and may include extended coordinated signaling pathways and receptor crosstalk (ibid).

### 1.8.3 Cod as a bioindicator and toxicological model species

Atlantic cod has been used as a bioindicator species for monitoring programs and field studies assessing marine pollution for several decades (Beyer et al., 1996; Dale et al., 2019; Goksøyr et al., 1994; Husøy et al., 1996; Hylland et al., 2008; OSPAR, 2010; Sundt et al., 2012). Furthermore, cod has been commonly used as a model in toxicological studies assessing the toxicity of crude oil and oil related compounds (Aas et al., 2000; Goksøyr et al., 1991; Hansen et al., 2019; Holth et al., 2014; Meier et al., 2010; Sørensen et al., 2017, 2019; Sturve et al., 2006). This has recently been accentuated with the proposed offshore oil activities outside Lofoten, Vesterålen, and Senja in Northern Norway, which are crucial spawning and nursing areas for several North Atlantic fish species, including the NEAC. Sequencing of the cod genome has also facilitated the possibility of interdisciplinary approaches using transcriptomics, metabolomics and proteomics as a first step towards a systems biology understanding of genomic responses to environmental stressors (toxicogenomics or environmental genomics). This has placed Atlantic cod in a position as a unique marine teleost model organism for exploratory or hypothesis-generating research focused on in-depth understanding of effects of chemicals with different modes of action and response profiles of genes, proteins and metabolites (Bratberg et al., 2013; Dale et al., 2020; Karlsen et al., 2011; Khan et al., 2020; Lie et al., 2009; Olsvik et al., 2011; Yadetie et al., 2013, 2017, 2018).

# 2. Aims of the study

AHR has an important role in mediating xenobiotic responses to environmental pollutants in vertebrates, including fish. However, the Ahr signaling pathway in Atlantic cod has previously only been studied with regard to induction and localization of *cyp1a* expression and Cyp1a activity (Husøy et al., 1994, 1996). Almost nothing was known about the Ahr receptor itself, including basal knowledge such as the number of Ahr-encoding genes in the cod genome. Atlantic cod is an important fish species, both economically and ecologically, and during the last years, governmental authorities in Norway have considered oil activities in important spawning grounds. In order to enhance our knowledge of how Atlantic cod senses and responds to environmental contaminants, the overall aims of this PhD thesis were to functionally characterize the cod Ahrs proteins and to investigate their physiological roles, including a possible subfunctional partitioning. Furthermore, as limited knowledge exists about the photo-enhanced toxicity of crude oil to ELS of Atlantic cod, an important aim of this thesis was to assess the molecular mechanisms and phenotypic traits underlying this type of toxicity. The latter could also provide valuable knowledge to aid future risk assessment of a possible oil spill in sensitive spawning and nursery grounds of Atlantic cod.

The aims of this PhD thesis were divided into the following objectives:

- Functionally characterize the Atlantic cod *ahr1a* and *ahr2a* genes and proteins using genome mining, cloning, phylogenetic analyses, ligand-binding and ligand-activation assays, and tissue-specific expression analyses (Paper I)

- Assess a putative subfunctional partitioning of Ahr1a and Ahr2a by mapping their gene expression in ELS of Atlantic cod using *in situ* hybridization and quantitative polymerase chain reaction (qPCR) analyses (Paper II)

- Investigate molecular mechanisms involved in photo-enhanced toxicity of crude oil to ELS of Atlantic cod with transcriptome analysis, and visualization of adverse phenotypic outcomes (Paper III).

# 3. Results

## 3.1 Atlantic cod aryl hydrocarbon receptors: Ahr1a and Ahr2a (Paper I)

Homology searches revealed the existence of two Ahrs in the Atlantic cod genome. The coding sequences of the two *ahr* genes were cloned, and based on phylogenetic clustering and synteny analysis, these genes were classified and named as *ahr1a* (MN329012) and *ahr2a* (MN329013). The N-terminal parts, including the bHLH and PAS domains, were well conserved between cod Ahr1a and Ahr2a, and orthologs from other teleost species, whereas the C-terminal part, containing the transcriptional transactivation domain, was poorly conserved as observed in other studies. The aa residues constituting the characteristic "TCDD-binding-fingerprint" present in mammalian AHR, as well as aa showed to be involved in binding of TCDD in other teleosts, were also conserved in both cod Ahrs.

Tissue-specific expression of *ahr1a*, *ahr2a*, as well as *arnt1* and *arnt2* genes, was analysed in different tissues from juvenile Atlantic cod. *ahr2a* was ubiquitously expressed in most tissues, and highest *ahr2a* expression was found in heart, liver, gill and eye. On the contrary, expression of *ahr1a* was in general less abundant and only observed in some tissues, including liver, brain, and eye. *arnt1* and *arnt2* also demonstrated distinct expression patterns. Expression of *arnt1* was ubiquitous in most tissues, and highest levels were found in the brain, liver, stomach and heart. *arnt2*, on the other hand, was highly expressed in the brain, gill and eye. Ligand activation and ligand binding affinities were assessed *in vitro* using luciferase reporter gene assays and velocity sedimentation with well-known AHR ligands, including FICZ, TCDD, B[a]P, PCB126 and BNF. Velocity sedimentation revealed that both [$^3$H]TCDD and [$^3$H]BNF bound with higher affinity to Ahr1a than to Ahr2a. Transactivation assays in COS7-cells also demonstrated differences between the two receptors. FICZ was the most potent ligand producing $EC_{50}$ values in the picomolar

range for both Ahr1a and Ahr2a. As expected, TCDD was also a highly potent agonist, but the activation differed between the receptors. In accordance with the affinity assay, Ahr1a demonstrated the lowest $EC_{50}$ value ($EC_{50}$ 1 nM) with TCDD, which was one order of magnitude lower compared to Ahr2a ($EC_{50}$ 11.27 nM). Ahr1a and Ahr2a differed also by producing distinctive efficacies ($E_{max}$) in transactivation assays with B[a]P, PCB126 and BNF, where Ahr1a was the most responsive receptor for most of the compounds. Activation of the Ahr signaling pathway by the various Ahr agonists was also confirmed *ex vivo* by exposure of precision-cut liver slices (PCLS) prepared from juvenile Atlantic cod and measuring induction of *cyp1a* expression with quantitative polymerase chain reaction (qPCR) analyses. TCDD and FICZ induced *cyp1a* expression at very low concentrations, whereas B[a]P and PCB126 produced stronger transcriptional activation at the highest concentrations used.

## 3.2 *ahr1a* and *ahr2a* are expressed during early life stages of Atlantic cod (Paper II)

Using B[a]P-infused silicone rods in a passive-dosing exposure experiment, the spatial and temporal expression of *ahr1a* and *ahr2a* at early developmental stages of Atlantic cod were characterized. The expression of the Ahr target genes, *cyp1a* and *ahrrb* was also assessed and quantified as a measure of activation of the Ahr signaling pathway. Expression of *ahr1a*, *ahr2a*, *cyp1a* and *ahrrb* was detected in 8 days post fertilization (dpf) and 10 dpf embryos, and in 3 days post hatching (dph) larvae. Induction of *ahr2a* was observed in B[a]P-exposed embryos at 10 dpf, whereas *cyp1a* expression significantly increased in B[a]P-exposed embryos at all sampling stages.

There were no visible phenotypic traits indicating cardiotoxicity in B[a]P-exposed larvae. However, it was visible expression of *ahr2a* in the cardiovascular system of cod B[a]P-exposed embryos and larvae, and expression of both *ahr2a* and *cyp1a* was observed in the liver of B[a]P-exposed larvae. Expression of *ahr1a* was only detected

in the eye of cod embryos and larvae, where its expression was persistent and did not differ between control and B[a]P-exposed samples at any of the developmental stages assessed. Furthermore, tissue sections from 3 dph larvae revealed expression of *ahr1a* in the eye ganglion cell and inner nuclear layers (Figure 8-9). Differences in the expression of *ahr2a* between treatments were already observed at 8 dpf, where B[a]P-exposed embryos had visible *ahr2a* expression in the cardiovascular system. This pattern of *ahr2a* expression was also visible in B[a]P-exposed 10 dpf embryos and 3 dph larvae. Expression of *ahr2a* was also detectable in the jaws, heart and fin nodes from both control and B[a]P-exposed larvae (Figure 8-9). At 8 dpf and 10 dpf, expression of *cyp1a* was clearly visible in the skin and vasculature of B[a]P-exposed embryos, indicating an early effect of B[a]P-exposure. Moreover, *cyp1a* was also expressed in the heart and in the area where the liver develops in both control and B[a]P-exposed embryos. Strong expression of *cyp1a* in the skin and vasculature, as well as in the liver and intestine was also visualized in 3 dph B[a]P-treated larvae. There was also *cyp1a* expression in the heart and presumably the head kidney of 3 dph larvae from both groups. Interestingly, both *ahr2a* and *cyp1a* transcripts were observed in the liver of 3 dph B[a]P-exposed larvae, and in the heart of larvae from both groups (Figure 8-9).

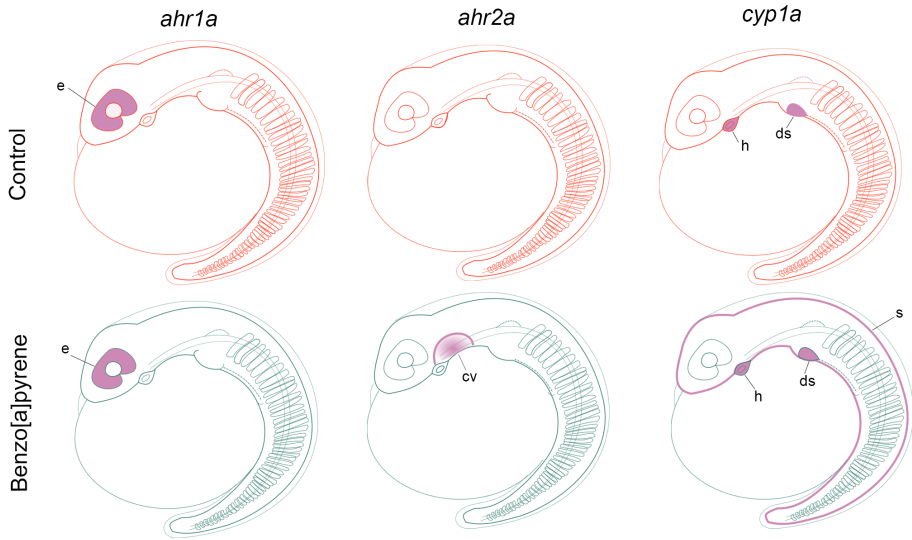


**Figure 8: Schematic illustration indicating *ahr1a*, *ahr2a* and *cyp1a* expression in 8- and 10-days post fertilization (dpf) in control and B[a]P-exposed embryos.** Control embryos are shown in orange and B[a]P-exposed embryos are shown in green. Gene expression was visualized (colored purple) by specific DIG-labelled RNA probes in cardiovascular system (cv), digestive system (ds), eye (e), heart (h) and skin (s).

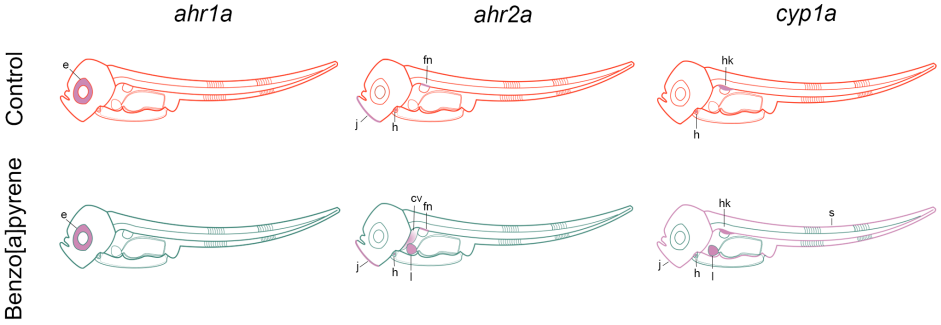


**Figure 9: Schematic illustration indicating *ahr1a*, *ahr2a* and *cyp1a* expression in 3 days post hatching (dph) in control and B[a]P-exposed larvae.** Control larvae are shown in orange and B[a]P-exposed larvae are shown in green. Gene expression was visualized (colored purple) by specific DIG-labelled RNA probes in cardiovascular system (cv), eye (e), fin nodes (fn), heart (h), head kidney area (hk), jaws (j), liver (l), and skin (s).

## 3.3 UV radiation produces photo-enhanced crude oil toxicity in ELS of Atlantic cod (Paper III)

Atlantic cod embryos were exposed to different crude oil concentrations alone, or in combination with UV radiation for 72h. At 6 dpf, embryos were harvested for chemical and transcriptome analysis. Embryos from the co-exposure experiment continued to be exposed to UV radiation for five more days until hatching. In addition, heart, craniofacial and body deformities were assessed in 3 dph larvae.

Expression of *cyp1a* was used as a biomarker for PAH exposure. The effect of measured total sum of PAHs (ΣPAH) in water on *cyp1a* expression was similar between experiments (with and without UV radiation co-treatment). In contrast, different effects of ΣPAH water concentration on ΣPAH body burden levels were observed between experiments, where body burden accumulation of 2- and 3-ring PAHs was lower in embryos co-exposed to crude oil with UV radiation. However, ΣPAH body burden had a stronger effect on *cyp1a* expression in the co-exposed embryos. Increased mortality was observed in both experiments, but higher mortality was detected in co-exposed embryos at the higher doses.

Craniofacial malformations and heart deformities were clearly visible in larvae from both experiments. However, larvae co-exposed with UV radiation had a higher percentage of spinal curvature deformities at the high crude oil doses, where the scoliosis phenotype was predominant. The number of differentially expressed genes (DEGs) and enriched pathways were also affected by the light regime, where higher numbers of DEGs were revealed in the co-treatment with UV radiation. Gene ontology, network, and pathway analyses revealed enrichment of muscle contraction and vasoconstriction pathways in embryos exposed only to crude oil. Some genes involved in myocyte differentiation and proliferation, as well as in jaw formation were downregulated in co-exposed embryos. The Ahr signaling pathway and induction of *ahr2a*, *cyp1a*, *ahrrb* gene expression was detected in embryos from both experiments. Notably, pathways such as circadian rhythm, oxidative stress and

mitochondrial function related pathways, the Nrf2 signaling pathway, tryptophan metabolism, and vitamin A metabolism were enriched in datasets following interaction analyses between crude oil and UV radiation**,** which indicated a photo-enhanced effect of crude oil on these processes.

# 4. Discussion

## 4.1 *ahr1a* and *ahr2a* genes in Atlantic cod

Atlantic cod has two paralogous *ahr* genes, i.e. *ahr1a* and *ahr2a*, which are located in a tandem pair on linkage group 20 in the Atlantic cod genome. The number of retained *ahr* genes varies significantly across teleost species, as demonstrated in e.g. zebrafish (*ahr1b-ahr2b* tandem, chromosome 22; *ahr1a*, chromosome 16, GRCz11, Ensembl), Japanese pufferfish (*Takifugu rubripes*) (*ahr1a-ahr2a* tandem, chromosome 1, *ahr1b-ahr2b* tandem, chromosome 8; *ahr2c*, chromosome 6, FUGU5, Ensembl), Japanese medaka (*Oryzias latipes*) (*ahr1a-ahr2a* tandem, chromosome 21; *ahr1b-ahr2b tandem,* chromosome 2, ASM223467v1, Ensembl), mummichog (*ahr1a-ahr2a* tandem*,* chromosome 1; *ahr1b-ahr2b* tandem, chromosome 18), *Fundulus_heteroclitus*-3.0.2, (Miller et al., 2019)) and Atlantic herring (*ahr1b-ahr2* tandem, chromosome 21, Ch_v2.0.02, Ensembl). However, the well-conserved structural features in the *ahr1a* and *ahr2a* primary sequences, including the bHLH and PAS domains, support the notion that the basic functions of Ahr are conserved in the Atlantic cod Ahr proteins.

## 4.2 Differences in ligand binding affinities and transactivation activity

Binding studies with *in vitro* translated cod Ahr proteins demonstrated stronger binding of [$^3$H]TCDD to Ahr1a than Ahr2a. Differences in binding to [$^3$H]TCDD have also been observed between Ahr paralogs in other teleost species. Of the three zebrafish Ahrs, Ahr2 and Ahr1b bound to [$^3$H]TCDD with comparable affinities, whereas Ahr1a did not bind at all to this compound (Andreasen et al., 2002; Karchner et al., 2005). In Atlantic salmon (*Salmo salar*), different affinities to [$^3$H]TCDD were also observed between the paralogous Ahr2 proteins (Hansson & Hahn, 2008).

Moreover, differences in sensitivity between cod Ahr1a and Ahr2a towards TCDD exposure were revealed with the luciferase transactivation assay. In accordance with the discrepancies observed in [$^3$H]TCDD binding affinity, the lowest $EC_{50}$ value was determined for Ahr1a, which was about one order of magnitude lower than that of Ahr2a. Greater sensitivity of Ahr1 to TCDD in comparison to Ahr2 has previously been reported in lake sturgeon (*Acipenser fulvescens*), Atlantic sturgeon (*Acipenser oxyrinchus*) and red seabream (*Pagrus major*) (Bak et al., 2013; Doering et al., 2015; Roy et al., 2018). Furthermore, Ahr1a also bound with highest affinity to [$^3$H]BNF, which also produced higher transactivation activity with Ahr1a in comparison to Ahr2a. Andreasen et al., 2002 also observed differences in binding to [$^3$H]BNF in zebrafish, where Ahr2a in contrast to Ahr1a was capable of binding to this compound.

The differences in binding affinity and transactivational activity towards TCDD between Atlantic cod Ahr1a and Ahr2a could not be ascribed to the absence of key aa, since residues constituting the mammalian “TCDD binding finger-print”, as well as other aa shown to be important for binding of TCDD in other teleosts species, were positionally conserved in the ligand-binding domain of both Ahr proteins (Bisson et al., 2009; Doering et al., 2015; Fraccalvieri et al., 2013; Pandini et al., 2009). Hence, discrepancies in ligand binding affinity and sensitivities between Ahr1a and Ahr2a must be attributed to other structural features present in these protein sequences.

## 4.3 Tissue-specific expression profiles

*ahr2a* was ubiquitously expressed across the different tissues analysed from juvenile Atlantic cod. On the other hand, *ahr1a* was mainly expressed in liver, brain, gill and eye. In other fishes, *ahr2a* is also the most abundant and widely expressed gene, whereas *ahr1* expression is mainly located to the brain and heart (Abnet et al., 1999; Hansson & Hahn, 2008; Karchner et al., 1999; Yamauchi et al., 2005). The abundant expression of *ahr2a* in the liver indicated that Ahr2a is most likely the main protein involved in mediating hepatic xenobiotic responses. In ELS of Atlantic cod, *ahr1a* and *ahr2a* were also expressed in both 6 and 10 dpf embryos and 3 dph larvae. Expression of *ahr1a* was focused in the eye of both embryos and larvae, whereas *ahr2a* was visible in the heart, jaws and fin nodes of cod larvae. Our results indicated physiological roles of cod Ahrs in the ontogenesis of these organs.

## 4.4 Subfunction partitioning of Atlantic cod Ahr1a and Ahr2a

The different ligand binding affinities, transactivation activities, and tissue expression profiles supported the idea that a subfunction partitioning of Ahr1a and Ahr2a has occurred in Atlantic cod (Paper I). This hypothesis was further explored by mapping the expression and localization of *ahr1a*, *ahr2a* and *cyp1a* in Atlantic cod embryos and larvae exposed to either the Ahr agonist B[a]P and or to seawater control. An evolved specialized role of Ahr2a in possibly mediating xenobiotic responses, as well as a physiological role of both Ahr1a and Ahr2a in ontogenesis during development was indicated (Paper II).

As previously described in section 1.5.2, the ability of AHR to bind pollutants and mediate xenobiotic responses is believed to be an adaptive function that evolved in the vertebrate lineage (Hahn et al., 2017). The initial role of AHR was suggested to regulate the development of sensory structures and neuronal systems in invertebrates (ibid), and an endogenous role of AHR in vertebrates has later been described during

the last decades (Denison et al., 2011; Mulero-Navarro & Fernandez-Salguero, 2016; Nebert, 2017). In fish, differences in tissue-specific expression patterns, as well as ligand- and target gene specificity between retained Ahrs paralogs, suggest that the multiple forms of Ahr found in teleosts may have acquired different functional specializations through subfunctional partitioning (Hahn et al., 2017).

### 4.4.1 Ahr2a is likely involved in mediating xenobiotic responses

The presence of ~10 times more *ahr2a* transcripts than *arh1a* transcripts in juvenile liver tissue suggests that Ahr2a is a major form in the hepatic Ahr signaling pathway involved in mediating xenobiotic responses. Moreover, the predominant expression of *arnt1* in the liver indicates similarly that Arnt1 is most likely the heterodimeric partner protein of gmAhr2, although this must be confirmed by other means. Additionally, possible Ahr2-mediated responses to xenobiotics are also indicated by the following results presented in this thesis:

1) Exposure to B[a]P led to an increase in *ahr2a* expression in 10 dpf cod embryos and visible expression of *ahr2a* was observed in the cardiovasculature system of B[a]P-exposed embryos and larvae (Paper II).

2) In B[a]P-exposed cod larvae, expression of both *ahr2a* and *cyp1a* was revealed in the developing liver (Paper II).

3) Expression of *ahr1a* was only revealed in the eye of cod embryos and larvae, and there were no quantitative or qualitative differences in *ahr1a* expression or expression patterns between control and B[a]P-exposed samples (Paper II).

Mutagenesis (knock-out), genetic knock-out lines and morpholino (knock-down) studies have demonstrated that Ahr2 is the main subtype involved in mediating xenobiotic responses in ELS of zebrafish and mummichog (Clark et al., 2010; Garcia et al., 2018; Goodale et al., 2012; Incardona et al., 2011; Jönsson et al., 2007, 2009; Prasch et al., 2003; Souder & Gorelick, 2019; Sugden et al., 2017; Van Tiem & Di Giulio, 2011; Yin et al., 2008). Morpholino studies have also showed that the Arnt1 protein is required for TCDD-induced toxic responses in developing zebrafish

(Antkiewicz et al., 2006; Prasch et al., 2006). A role for Ahr2 in mediating toxicity responses in ELS of red seabream and medaka has also been suggested (Hanno et al., 2010; Yamauchi et al., 2006). Even though knock-down or knock-out analyses were not part of this study, our results support the notion that Ahr2a is the main protein involved in mediating xenobiotic responses in teleosts.

### 4.4.2 Ahr1a may also be involved in mediating toxicity

Even though *ahr2a* was the most abundantly expressed gene in the liver, Ahr1a was the receptor exhibiting the strongest binding and highest sensitivity and efficacies for most of the compounds tested *in vitro*. Our results therefore suggest that Ahr1a may still be involved in mediating xenobiotic responses in tissues where this receptor is expressed. In zebrafish, Ahr1a does not bind to TCDD and lacks transactivation activity after TCDD exposure (Andreasen et al., 2002; Karchner et al., 2005), whereas Ahr1b is a fully functional receptor (Karchner et al., 2005). A possible role of both Ahr1 and Ahr2 in mediating dioxin toxicity has also been postulated for red seabream and white sturgeon (Bak et al., 2013; Doering et al., 2014, 2015), but the function of Ahr1 in mediating toxicity in fish has not yet been fully elucidated. In birds, sensitivity to TCDD and dioxin-like compounds has been linked to Ahr1 (Farmahin et al., 2012, 2014; Head et al., 2008; Karchner et al., 2006; Manning et al., 2012). In contrast to fish, Ahr1 mediates ELS mortality in birds, while AHR2 is not believed to have a role in mediating toxicity responses (Farmahin et al., 2012, 2014; Manning et al., 2012)

Doering et al., 2018 compared linear relationships between sensitivities and ELS mortality data obtained from Ahr transactivation studies and exposures of fishes and birds to dioxin-like compounds. In fish, it was only found a significant linear relationship between Ahr2 activation and early life stage mortality, supporting the hypothesis that Ahr2 mediates ELS mortality in fish.

### 4.4.3 Endogenous roles of Ahrs during early development of Atlantic cod

Focused expression of *ahr1a* in the eye at all developmental stages independent of B[a]P exposure, suggests a role of this protein in the development of eye and vision in Atlantic cod. In zebrafish, high levels of *ahr1b* transcripts in embryos led to the hypothesis of a possible physiological role of *ahr1b* in embryonic development (Karchner et al., 2005). Accordingly, *in situ* hybridization studies also revealed expression of zebrafish *ahr1b* in the developing eye, and it did not change after exposure to TCDD or BNF (Karchner et al., 2017; Sugden et al., 2017). Furthermore, the zebrafish Ahr1b protein was also localized in the developing eye, specifically to the retinal inner and outer plexiform layers (Karchner et al., 2017).

*ahr2a* expression was visible in jaws and fin nodes of cod larvae independent of B[a]P exposure, suggesting a physiological role of *ahr2a* in the ontogenesis of jaw and fins. This is largely in agreement with observations made by Garcia et al., 2018, demonstrating that Ahr2-null zebrafish adults had malformed cranial skeletal bones and severely damaged fins, and also mainly infertile. Souder & Gorelick, 2019 also showed craniofacial malformations and irregularities in the development of fins in Ahr2 knock-out zebrafish embryos. Exposure to some PAHs led to the development of an ectopic caudal fin fold perpendicular to the normal caudal fin fold in zebrafish, and this phenotype has been named “X-fin” (Geier et al., 2018). Benzo[k]fluoranthene (BkF) was the most potent compound of the class of PAHs producing this phenotype and this phenomenon has been shown to be Ahr2-dependent (Garland et al., 2020). A role of Ahr2 in inhibiting fin regeneration after TCDD exposure was also shown in zebrafish embryos (Diamond et al., 2006).

The high sensitivity of both cod Ahr1a and Ahr2a to the endogenous AHR ligand FICZ is another indication of a role of both receptors in physiological processes. Exposure to FICZ also induced *cyp1a* expression *ex vivo* in cod liver slices. FICZ binds also to zebrafish Ahr1b and Ahr2 and induce *cyp1a* expression in an Ahr2-dependent manner. Cyp1a has been shown to regulate the metabolism and biological effects of FICZ (Jönsson et al., 2009; Wincent et al., 2016).

## 4.5 Crude oil cardiotoxicity

### 4.5.1 Heart morphogenesis in Atlantic cod

The heart is the first organ to form and function during vertebrate embryogenesis (Stainier, 2001). Morphogenesis of the Atlantic cod heart has previously been described by (Hall et al., 2004). Around 6 dpf, Atlantic cod is in the 20-somite stage and the heart is starting to be visible. At this time the myocardial and endocardial cells have already differentiated and are beginning to fuse to form the cardiac ring. By the 25-somite stage, it is possible to see two mesodermal tubular primordia on both ventral midline sides, which fuse into a cone by the 30-somite stage (the cardiac ring). The Atlantic cod bell-shaped heart is formed over the next 24 h, containing two epithelial layers, an outer myocardial layer that will form the heart muscles and a very thin, endocardial layer forming the inner lining. Looping of the heart and demarcation of the atrium and the ventricle chambers takes place between 45- and 50-somites stage. After 50-somite stage, the two minor chambers, the sinus venosus and the bulbous arteriosus, are delineated, and the heart is finally formed around 9 dpf.

Continuous exposure to B[a]P started at 5 dpf and cod embryos were thus exposed to B[a]P during cardiac cone fusion and full heart formation (Paper II). Crude oil exposure started at 3 dpf and lasted until 6 dpf, meaning that cod embryos were exposed to crude oil during myocardial and endocardial cell differentiation until right before cell fusion and cardiac cone formation (Paper III).

### 4.5.2 AHR-dependent toxicity

Even though the characteristic cardiac and craniofacial malformations observed in ELS of fish after exposure to crude oil and PAHs were not clearly visible in B[a]P-exposed cod larvae, there were some indications of presence of cardiotoxicity at the molecular level. The expression of *ahr2a* in the cardiovascular system of B[a]P-exposed embryos and larvae, and also in the heart of B[a]P-exposed larvae suggested Ahr-dependent cardiotoxicity. Benzo[a]pyrene is a 5-ring PAH that is a constituent of crude oil, and is known to produce Ahr-dependent cardiotoxicity in ELS of fish (Incardona, 2017; Incardona et al., 2011). In zebrafish, the developing heart and cardiovascular system were affected after exposure to a wide range of B[a]P doses (Cunha et al., 2020; Huang et al., 2012; Incardona et al., 2011). Expression of *cyp1a* was strongly visible in the skin and in the vasculature of B[a]P-exposed cod embryos and larvae. Induced Cyp1a protein expression has also been observed in the skin and cardiovascular system of zebrafish embryos exposed to high doses of B[a]P (Incardona et al., 2011). A recent systems biology approach assessing the effect of PAH in developmental toxicity of zebrafish reported induction of Cyp1a in the skin as a more robust and reliable biomarker for Ahr2 activation in developing zebrafish (Shankar et al., 2020; Shankar et al., 2019).

Activation of the Ahr signaling pathway in cod embryos exposed to crude oil with and without UV radiation was also observed and may have contributed to the craniofacial malformations and heart deformities observed in cod embryos from both experiments. As mentioned in section 1.7, crude oil is a complex mixture of different chemicals, and some PAHs that are part of this mixture have been shown to produce Ahr-dependent defects in cardiac morphogenesis (Clark et al., 2010; Incardona, 2017; Incardona et al., 2006; Scott et al., 2011; Van Tiem & Di Giulio, 2011). Moreover, craniofacial abnormalities are considered as a secondary response to the cardiac failure produced by crude oil and TCDD (Incardona et al., 2004; Lanham et al., 2014).

### 4.5.3 AHR-independent toxicity

Enrichment of transcripts involved in muscle contraction and vasoconstriction pathways was detected in cod embryos exposed to crude oil alone, which is in accordance with other studies reporting alterations in cardiac muscular contraction observed in ELS of fish exposed to crude oil (Brette et al., 2014, 2017; Incardona, 2017; Sørhus et al., 2016). As mentioned in section 1.7, it has been postulated that cardiotoxic effect of crude oil to ELS of fish is largely produced by the presence of tricyclic PAHs (Brette et al., 2014, 2017; Incardona, 2017; Sørhus et al., 2016). Tricyclic PAHs have been shown to disrupt excitation-contraction (EC) coupling in cardiomyocytes and alter cardiac muscular contraction in an Ahr-independent manner (Brette et al., 2014, 2017; Incardona, 2017).

In normal functional cardiomyocytes, $Ca^{2+}$ and $K^{+}$ ions are essential for controlling the cardiac contraction cycle. Action potentials open L-type $Ca^{2+}$ channels (Ltcc), and this leads to extracellular $Ca^{2+}$ entry and a subsequent internal release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR). An increase in intracellular $Ca^{2+}$ activates contractile actin/myosin filaments, which causes EC coupling and cardiomyocyte contraction. Action of pumps and exchangers, such as sarcoplasmic-endoplasmic reticulum calcium ATPase 2 (Serca2) and the cardiac delayed rectifier potassium ($I_{kr}$) channel, restore the baseline levels of intracellular $Ca^{2+}$ and $K^{+}$ ions afterwards (Incardona, 2017).

Incardona et al. have postulated that tricyclic PAHs may disrupt EC coupling in cardiomyocytes by blocking the influx of $K^{+}$ and $Ca^{2+}$ ions through the $I_{kr}$ and Ltcc membrane channels, respectively. Alterations in these membrane channels may lead to depletion of intracellular $Ca^{2+}$ stores from the SR mediated by the Serca2 channel and the RyR located in the SR membrane (ibid). Brette et al., 2014 showed that crude oil cardiotoxicity in yellowfin tuna (*Thunnus albacares*) and the Pacific bluefin tuna (*Thunnus orientalis*) was produced through blocking of the $I_{kr}$ channel, and a decrease of both $Ca^{2+}$ cycling and current disrupted EC coupling in cardiomyocytes. An additional experiment by Brette et al., 2017 demonstrated the impairment of EC coupling in Pacific mackerel (*Scomber japonicas*), yellowfin tuna and the Pacific

bluefin tuna cardiomyocytes after exposure to phenanthrene, and established this phenomenon as a key determinant of cardiotoxicity of crude oil.

The craniofacial malformations and heart deformities observed in cod embryos exposed to crude oil may be also explained by disruptions in cardiomyocyte muscle contraction produced by tricyclic PAHs acting in an Ahr-independent manner. Even though muscle contraction and vasoconstriction pathways were not enriched in embryos exposed to crude oil with UV radiation, downregulation of genes involved in myocardial cell differentiation and proliferation were observed, as well as craniofacial and heart deformity phenotypes.

## 4.6 Photo-enhanced toxicity of crude oil

### 4.6.1 Possible photomodification of PAHs

A stronger effect of ΣPAH body burden on *cyp1a* expression in cod embryos exposed to crude oil with UV radiation was revealed, even though the ΣPAH body burden levels were lower when UV radiation was present. This may indicate photomodification of absorbed PAHs in co-exposed embryos, where PAHs can be transformed into new oxygen-containing and more polar molecules that were not identified in the chemical analyses (Maki et al., 2001; Ray et al., 2014; Schemeth et al., 2019; Yu, 2002). 2- and 3-ring PAHs accumulated to a larger extent in embryos exposed to crude oil alone, whereas there were no differences in 4+-ring PAH accumulation between the experiments. In crude oil, the PAH content is usually predominantly 2- and 3-ring structures (Bence et al., 1996; Carls et al., 1999), which are in general poor *cyp1a* inducers (Incardona, 2017). However, these type of PAHs are more readily photomodified (Ankley et al., 1997; Arfsten et al., 1996; Wernersson, 2003), and oxygen-substituted PAHs have been shown to be inducers of *cyp1a* (Wincent et al., 2015; Wincent et al., 2016). Furthermore, in transactivation assays using cod Ahr1a and Ahr2a, hydroxylated phenanthrene and chrysenes, including 1,2-dihydrophenanthrene-1,2-diol and chrysene-1-ol, chrysene-2-ol,

chrysene-3-ol, and chrysene-4-ol are potent Ahr agonists, while their unsubstituted parent compounds are not able to activate the cod Ahrs (Lille-Langøy et al. in prep.).

### 4.6.2 Increased mortality and spinal curvature deformities

Increased mortality of embryos exposed to crude oil with UV radiation at high doses is in accordance with other studies reporting photo-enhanced toxicity of crude oil and PAHs to ELS of fish, as well as juvenile fish (Alloy et al., 2016; Barron et al., 2003, 2005, 2018; Diamond et al., 2006; Farwell et al., 2006; Hatlen et al., 2010; Incardona et al., 2012; Little et al., 2000). Moreover, UVB is considered to be a major environmental stressor to pelagic marine fish eggs and larvae, and increased mortality has been observed in a variety of fish species after exposure to UVB (Zagarese & Williamson, 2001), including Atlantic cod where UVB radiation has been shown to cause DNA damage (Béland et al., 1999; Browman & Vetter, 2002; Kouwenberg et al., 1999; Lesser et al., 2001).

Increased abundance of spinal curvature deformities was observed in the embryos exposed to crude oil with UV radiation at high doses. This is an adverse outcome often associated to the photo-enhanced toxicity of crude oil and PAHs on ELS of fish (Barron et al., 2003; Diamond et al., 2006; Farwell et al., 2006). Exposure to UV radiation also produced spinal abnormalities in developing zebrafish, and curvature of the notochord was observed in medaka and African sharptooth catfish (*Clarias gariepinus*) embryos exposed to UVA (Mahmoud et al., 2009; Sayed & Mitani, 2016; Torres Nuñez et al., 2012).

### 4.6.3 Cellular pathways affected by the effect of UV radiation on crude oil

Several of the enriched pathways found in the transcriptome of embryos exposed to crude oil with UV radiation were also enriched in interaction analyses assessing the effect of the co-treatment on gene expression, indicating a photo-enhanced effect of crude oil on these cellular processes.

### *Circadian rhythm*

Upregulation of genes involved the light responsive circadian rhythm pathway in co-exposed embryos is consistent with previous reports assessing expression of genes and mechanisms involved in light regulation (Reppert & Weaver, 2001; Weger et al., 2011). Light regulates the circadian clock, which controls physiological changes during the day-night cycle in most organisms (Reppert & Weaver, 2001).

### *Oxidative stress*

Enrichment of mitochondrial iron dependent pathways, such as iron-sulfur clusters and heme synthesis, mitochondrial respiratory chain, and ferroptosis pathways indicates mitochondrial dysfunction in embryos exposed to crude oil with UV radiation. Mitochondrial dysfunction is an indication of oxidative stress, where overproduction of ROS can cause impairment of mitochondrial respiration, and subsequent shut-down of ATP production and mitochondria-dependent apoptosis (Ott et al., 2007; Simon et al., 2000).

Reactive oxygen species are molecules that can be generated by various sources, such as by-products of mitochondrial respiratory chain activity, and can control signaling pathways and physiological processes such as cell proliferation and cell death (Schieber & Chandel, 2014). Excess of ROS can damage important enzymes in the mitochondrial respiratory chain (Ott et al., 2007) or affect mitochondrial $Ca^{2+}$ homeostasis by for example inducing the opening of the mitochondrial permeability transition pore (mPTP) located in the inner mitochondrial membrane, or by altering $Ca^{2+}$ transport between the sarcoplasmic/endoplasmic reticulum (SR/ER) and mitochondria (Ermak & Davies, 2001; Gordeeva et al., 2003; Görlach et al., 2015). Alterations in $Ca^{2+}$ homeostasis can result in mitochondrial $Ca^{2+}$ accumulation and mitochondria-dependent apoptosis (Ermak & Davies, 2001; Görlach et al., 2015). Furthermore, disruption of $Ca^{2+}$ transport between the sarcoplasmic/endoplasmic reticulum (SR/ER) and mitochondria has been described to cause neuronal dysfunction and death in neurodegenerative diseases in a state of oxidative stress (Görlach et al., 2015; Guo et al., 2013). In fish, mitochondrial dysfunction and apoptosis has been shown to be associated to ROS overproduction and oxidative

stress in ELS of zebrafish (Jiang et al., 2019; Pan et al., 2018; Zhao et al., 2016) and fathead minnow (*Pimephales promelas*) (He et al., 2012) after exposure to environmental pollutants.

### *Antioxidant responses*

Presence of ROS and oxidative stress was also indicated by enrichment of the Nrf2 pathway, which modulates antioxidant responses and has a major role in maintaining the redox cell homeostasis (Kensler et al., 2007; Nguyen et al., 2003; Qiang Ma, 2015). Reactive oxygen species can regulate the Nrf2 pathway and induce transcription of antioxidant enzymes and of proteins participating in detoxification of xenobiotics, such as glutathione S-transferases (GSTs) (ibid). Antioxidant responses are also mediated by the Nrf2 signaling pathway in cadmium-exposed zebrafish and in jian carp (*Cyprinus carpio*) where cellular oxidative stress was observed (Jiang et al., 2014; Wang & Gallagher, 2013). Upregulation of different *gst* genes has also previously been observed in cod larvae after exposure to crude oil (Olsvik et al., 2010), further supporting antioxidant responses in ELS of Atlantic cod.

The transcriptomic patterns indicating an increased state of oxidative stress produced by the UV co-treatment may explain the spinal curvature deformities observed only in UV co-exposed cod embryos, where cell death in the spinal cord may have produced this phenotypic outcome.

### *Retinoid metabolism*

Alterations in retinoid metabolism pathway in co-exposed embryos suggested disruption of embryogenesis. Retinoids, including Vitamin A and its metabolites, control numerous key events such as limb patterning, craniofacial- and eye development (Ross et al., 2000). Disruption of vitamin A metabolism can cause developmental abnormalities in both fish and mammals (Colbert, 2002; Haga et al., 2002; Isken et al., 2008; Le et al., 2012; Lie et al., 2016) and several environmental pollutants have been shown to alter the vitamin A signaling pathway (Beníšek et al., 2008; Novák et al., 2008). In ELS of haddock, exposure to crude oil revealed disruption of retinoid metabolism and signaling (Lie et al., 2019). Alterations in the

retinoid metabolism might have contributed to the teratogenic effects observed on co-exposed cod larvae.

### *Vitamin D signaling pathway*

Upregulation of *cyp2r1* and *cyp24a1* genes indicated modulation of the vitamin D signaling pathway, possibly affecting $Ca^{2+}$ homeostasis and bone formation in embryos exposed to crude oil with UV radiation. In mammals, vitamin D is synthesized through UV-mediated photochemical conversion in the skin, whereas in fish this transformation is not likely to occur under natural conditions (Lock et al., 2010; Pierens & Fraser, 2015). The active form of Vitamin D is calcitriol, which is metabolized by *cyp2r1* and *cyp24a1*. Calcitriol regulates $Ca^{2+}$ homeostasis and bone formation in fish (Lock et al., 2010; Sundell et al., 1993; Sundell & Björnsson, 1990), and CYP24A1 enzyme degrades and modulates excess calcitriol (Dzik & Kaczor, 2019; Jones et al., 2012). Overexpression of *cyp24a1* may lead to vitamin D deficiency and can putatively have affected bone formation in co-exposed cod larvae.

### 4.6.4 Possible Ahr-cross talk in embryos exposed to crude oil with UV radiation

Tryptophan photoproducts are endogenous AHR ligands, and FICZ is one of the most potent tryptophan derivatives (Denison & Nagy, 2003; Nguyen & Bradfield, 2008). *In vivo* exposure to UVB has been shown to induce AHR-mediated stress responses using AHR knockout mice via transcription of *cyp1a* (Fritsche et al., 2007). Enrichment of the tryptophan metabolism pathway, which also include *cyp1a*, suggests a role of the Ahr signaling pathway in mediating UV responses in co-exposed cod embryos.

The Ahr signaling pathway has also been suggested to interact with the retinoid signaling pathway in fish through increased Cyp induction that leads to increased clearance of retinoids (Berntssen et al., 2015; Berntssen et al., 2016). Moreover, a cross-talk between AHR and VDR has been observed in human monocyte/macrophage-derived cells (Makishima, 2014; Matsunawa et al., 2012). Benzo[a]pyrene-induced transcription of *Cyp1a1* was enhanced through activation of VDR by calcitriol in human cell lines, indicating a possible role of VDR in benzo[a]pyrene-mediated toxicity (Makishima, 2014; Matsunawa et al., 2012). However, the exact molecular mechanism involved in the AHR-VDR cross-talk is yet not known. In Atlantic cod, some PAHs are able to interact with the Vdra and Vdrb receptors and modulate their activities *in vitro* (Goksøyr et al. in prep).

Production of ROS and oxidative stress has been shown to be increased by up-regulating the production of CYP enzymes (Dalton et al., 2002; Nebert et al., 2000). Activation of AHR and CYP activity is considered an important underlying cause of dioxin-induced toxicity and oxidative stress (Stohs, 1990). AHR disruption has previously been shown to contribute to mitochondrial ROS production and mitochondrial dysfunction (Carreira et al., 2015; Senft et al., 2002; Tappenden et al., 2011). On the other hand, activation of the Ahr pathway induces antioxidant responses though a cross-talk with Nrf2. AHR and Nrf2 interact at the genetic level, and this cross-talk has been shown to be necessary for induction of conjugating enzymes in mice exposed *in vivo* to TCDD (Yeager et al., 2009).

# 5. Conclusions

The Ahr1a and Ahr2a receptors were for the first time characterized in Atlantic cod. Well-conserved structural features in their primary sequences were indentified, including the bHLH and PAS domains, as well as important aa residues for binding of TCDD in mammals and fish. Different binding affinities and transactivation activities towards several classical AHR ligands, in addition to distinct tissue specific expression profiles, suggested that a subfunction partitioning of these proteins had occurred in this teleost species. More abundant expression of *ahr2a* in liver in comparison to *ahr1a* indicated further that this gene is most likely involved in mediating xenobiotic responses in Atlantic cod, as observed in other fishes. However, the high sensitivity of Ahr1a to the different AHR ligands tested may also suggest that pollutants can modulate Ahr1a activity *in vivo*.

Subfunction partitioning of Ahr1a and Ahr2a was observed in ELS of Atlantic cod. Expression of *ahr2a* was mainly localized in the cardiovascular system, whereas *cyp1a* was mainly induced in the skin and vasculature in B[a]P-exposed embryos and larvae. Both *ahr2a* and *cyp1a* expression was observed in the liver of B[a]P-exposed larvae, further supporting a role of Ahr2a in mediating responses to xenobiotics in ELS of Atlantic cod. Specific roles of these proteins in ontogenesis during early development were indicated by persistent expression of *ahr1a* in the eye of both embryos and larvae, and the presence of *ahr2a* in the jaws and find nodes of larvae independent of the exposure regimes.

Mechanisms mediated by crude oil photo-enhanced toxicity to ELS of Atlantic cod were identified. Phenotypic outcomes and the underlying transcriptomic changes were enhanced by the co-treatment to crude oil, such as enhanced oxidative stress. These results are of relevance for risk assessment when evaluating the possible effects of an oil spill on the ELS of Atlantic cod and possible impacts on fish stocks in important spawning areas.

# 6. Future perspectives

## 6.1 Has Ahr taken over the role of Pxr in Atlantic cod?

The lack of Pxr in the Atlantic cod genome led to the hypothesis that Ahr may have adopted its functional role (Eide et al., 2018). Pxr is a xenobiotic receptor that has an important position in the chemical defensome in vertebrates. It is a key-regulator of the transcription of a number of genes involved in the biotransformation of xenobiotics, such as cytochrome P450 3A (CYP3A) (Goldstone et al., 2006). Regulation of *cyp3a166* by Ahr in Atlantic cod was indicated by the increased number of XREs present in its promoter region (Eide et al., 2018). Induction of *cyp3a166* was in this work revealed in Atlantic cod embryos after exposure to crude oil with and without the presence of UV radiation, and previously in liver slices after *ex vivo* exposure to the AHR agonist BNF (Eide et al., 2018). Notably, zebrafish *cyp3* genes appear to be regulated by both Pxr and Ahr2 (Kubota et al., 2015), where regulation of the orthologous gene *cyp3a65* has previously been found to be modulated by Ahr2 (Chang et al., 2013; Kubota et al., 2015; Tseng et al., 2005).

More studies are needed to elaborate if Ahr1a or Ahr2a have adopted a broader compensatory and functional role in the absence of Pxr. For instance, additional Ahr transactivation and binding affinity assays using prototypic PXR ligands could further elucidate if such compounds also act as ligands and agonists for the cod Ahrs. Furthermore, DNA-protein interaction analyses can be used to assess binding of cod Ahrs to the promoter region of *cyp3a166*, or to other Pxr target genes present in the cod genome using chromatine immunoprecipitation assays and cod Ahrs specific antibodies.

## 6.2 Further characterization of subfunction partitioning of Atlantic cod Ahrs

Based on the data presented in this PhD thesis, a subfunction partitioning of Atlantic cod Ahr1a and Ahr2a was strongly indicated. The original idea to further explore the subfunction partitioning of Ahr1a and Ahr2a was to treat Atlantic cod embryos with Ahr-paralog specific morpholinos and expose them to the AHR ligand B[a]P. However, due to technical challenges this knock-down experiment was not successful and morpholino studies did therefore not become part of this PhD work. Studies using Ahr1a and Ahr2a morpholinos, or the generation of cod Ahr1a and Ahr2a knock-out embryos using CRISPR/Cas9 technology would be valuable to elucidate the specific physiological roles of the cod Ahrs. Moreover, homology modelling, ligand docking analyses, and mutagenesis studies could help to clarify the identity of important aa in the ligand binding domain of cod Ahr1a and Ahr2a, and aid the understanding of their discrepancies in ligand binding and transactivation profiles, which could be linked to their functional roles.

## 6.3 Further characterization of the photo-enhanced toxicity of crude oil to ELS of Atlantic cod

Molecular mechanisms underlying the photo-enhanced toxicity of crude oil, as well as the phenotypic outcomes to ELS of Atlantic cod were characterized. A more comprehensive study assessing the effect of crude oil with UV radiation at the transcriptomic level at several embryonic developmental stages of cod would help to better understand the relationship between the gene expression profiles and the phenotypic outcomes observed. Proteomics analyses could also be applied to complement the transcriptomic data, and further substantiate and confirm the findings at a higher biological level. Assays for measuring and detecting oxidative stress could confirm altered cellular redox status, such as the presence of ROS, lipid peroxidation

or increased glutathione levels in tissue sections of exposed embryos. Presence of apoptosis and cell death could also be identified with specific cell dyes and using confocal microscopy or flow cytometry. Chemical analysis identifying photo-oxidized PAHs could also confirm photo-modification as one of the mechanisms involved in crude oil photo-toxicity.

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# Paper I

I

**Aranguren-Abadía, L.,** Lille-Langøy, R., Madsen, A. K., Karchner, S. I., Franks, D. G., Yadetie, F., Hahn, M.E, Goksøyr, A., & Karlsen, O.A (2020).

Molecular and Functional Properties of the Atlantic Cod (*Gadus morhua*) Aryl Hydrocarbon Receptors Ahr1a and Ahr2a.
Environmental Science & Technology, 54(2), 1033–1044.

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# Paper II

**Aranguren-Abadía, L.,** Donald, C.E., Eilertsen, M., Gharbi, N., Tronci, V., Sørhus E., Mayer, P., Nilsen T.O., Meier S., Goksøyr, A., & Karlsen, O.A (2020).

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II

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# Expression and localization of the aryl hydrocarbon receptors and cytochrome P450 1A during early development of Atlantic cod (*Gadus morhua*)

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ABSTRACT

The aryl hydrocarbon receptor (Ahr) is a ligand-activated transcription factor that mediates the toxicity of dioxins and dioxin-like compounds (DLCs) in vertebrates. Two clades of the Ahr family exist in teleosts (Ahr1 and Ahr2), and it has been demonstrated that Ahr2 is the main protein involved in mediating the toxicity of dioxins and DLCs in most teleost species. Recently, we characterized the Atlantic cod (*Gadus morhua*) Ahr1a and Ahr2a receptors. To further explore a possible subfunction partitioning of Ahr1a and Ahr2a in Atlantic cod we have mapped the expression and localization of *ahr1a* and *ahr2a* in early developmental stages. Atlantic cod embryos were continuously exposed in a passive-dosing exposure system to the Ahr agonist, benzo[*a*]pyrene (B[a]P), from five days post fertilization (dpf) until three days post hatching (dph). Expression of *ahr1a*, *ahr2a*, and the Ahr-target genes, *cyp1a* and *ahrrb,* was assessed in embryos (8 dpf and 10 dpf) and larvae (3 dph) with quantitative real-time PCR analyses (qPCR), while *in situ* hybridization was used to assess the localization of expression of *ahr1a*, *ahr2a* and *cyp1a*. Quantitative measurements showed an increased *cyp1a* expression in B[a]P-exposed samples at all sampling points, and for *ahr2a* at 10 dpf, confirming the activation of the Ahr-signalling pathway. Furthermore, B[a]P strongly induced *ahr2a* and *cyp1a* expression in the cardiovascular system and skin, respectively, of embryos and larvae. Induced expression of both *ahr2a* and *cyp1a* was also revealed in the liver of B[a]P-exposed larvae. Our results suggest that Ahr2a is the major subtype involved in mediating responses to B[a]P in early developmental stages of Atlantic cod, which involves transcriptional regulation of biotransformation genes, such as *cyp1a*. The focused expression of *ahr1a* in the eye of embryos and larvae, and the presence of *ahr2a* transcripts in the jaws and fin nodes, further indicate evolved specialized roles of the two Ahrs in ontogenesis.

## 1. Introduction

The aryl hydrocarbon receptor (Ahr) is a ligand activated transcription factor that has existed for more than 600 millon years of animal evolution, and it is broadly distributed in multiple phyla (Hahn et al., 2017). Ahr is a member of the basic helix-loop-helix/Per-Arnt-Sim family and has been extensively studied in toxicology because of its role in mediating responses to many xenobiotic compounds in vertebrates (Nebert, 2017; Okey, 2007). As a result of a tandem duplication of Ahr prior to the divergence of the cartilaginous and bony fish linegaes, two Ahr clades exist: Ahr1 and Ahr2 (Hahn et al., 2017; Karchner et al., 2005). In addition, due to the teleost-specific whole genome duplication, fishes can have multiple paralogous *ahr* genes (Glasauer and Neuhauss, 2014).

A diverse group of environmental pollutants, as well as endogenous compounds, are known to bind and activate Ahr, including dioxins, dioxin-like polychlorinated biphenyls, and polycyclic aromatic hydrocarbons (PAHs) (Denison and Nagy, 2003). The toxicity of PAHs and other crude oil components has been widely studied in fishes, especially during embryonic development and early life stages (Anderson et al.,

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1974; Barron et al., 2004; Goksøyr et al., 1994; Hylland et al., 2008; Sørhus et al., 2015). Ahr2 is the main protein involved in mediating xenobiotic responses to such compounds in most fish species (Tanguay et al., 1999; Walker et al., 1991; Zanette et al., 2009), as demonstrated in loss-of-function studies using morpholino-modified antisense oligonucleotides or genome editing (Clark et al., 2010; Garcia et al., 2018; Goodale et al., 2012; Jönsson et al., 2007, 2009; Prasch et al., 2003; Sugden et al., 2017).

Unliganded Ahr is located in the cytoplasm in a protein complex with two HSP90 proteins, a co-chaperon protein p23, and Ahr-interacting protein (AIP) (Carver and Bradfield, 1997; Denison et al., 2011; Kazlauskas et al., 1999; Perdew, 1988). The recognition and binding to an agonist promote the conformational activation of Ahr and subsequent translocation into the nucleus where Ahr heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt). This heterodimer binds to xenobiotic response elements (XRE) upstream of Ahr target genes and modulate the transcription of different enzymes involved in the biotransformation of xenobiotics, including cytochrome P450 1A (Cyp1a) (Denison et al., 2011; Pollenz et al., 1993; Soshilov and Denison, 2008; Swanson, 2002; Whitlock, 1999). Another Ahr target gene is the aryl hydrocarbon receptor repressor (Ahrr), which has a role in regulating Ahr transcriptional activity. Different mechanisms of Ahrr-mediated repression of the activity of Ahr have been postulated, including a crystallography study that showed the formation of an Ahrr-Arnt heterodimer (Mimura et al., 1999; Sakurai et al., 2017).

During the last decades different studies have highlighted the role of Ahr in signalling pathways and physiological systems other than the response to xenobiotic compounds, including the cardiovascular, reproductive and immune system in vertebrates (Esser and Rannug, 2015; Mulero-Navarro and Fernandez-Salguero, 2016; Nebert, 2017). It has been suggested that the original function of Ahr was to regulate the development of neural systems and sensory structures, and that these ancestral functions are still present in modern living invertebrates (Hahn et al., 2017). Hahn et al. suggested a specialization of the different *ahr* paralogs through subfunction partitioning in some non-mammalian vertebrates (Hahn et al., 2017). Some of the partitioning mechanisms described include tissue-specific expression patterns, as well as ligand- and target gene specificity. An example is the zebrafish, whose three Ahrs are thought to have evolved different functional roles. While the Ahr2 of zebrafish (zfAhr2) is involved in mediating toxic responses to dioxin-like compounds and PAHs, zfAhr1b is suggested to have a role in embryonic development (Karchner et al., 2005). zfAhr1a, on the other hand, is not activated by any classical Ahr agonists, but may still be involved in the regulation of physiological responses by being activated by a variety of non-halogenated compounds (Chlebowski et al., 2017; Goodale et al., 2012; Incardona et al., 2006; Knecht et al., 2013). A role of zfAhr2 in the development of the nervous and sensory system has also been demonstrated (Garcia et al., 2018; Goodale et al., 2012; Souder and Gorelick, 2019).

Atlantic cod (*Gadus morhua*) has for many years been used as an indicator species in marine pollution monitoring programs and field studies (Beyer et al., 1996; Dale et al., 2019; Goksøyr et al., 1994; Hylland et al., 2008; OSPAR, 2010; Sundt et al., 2012). However, in order to obtain a better understanding of how cod mediates responses to pollutants, we recently functionally described the Ahr1a and Ahr2a receptors for the first time (Aranguren-Abadía et al., 2020). These paralogous genes differed in their tissue-specific expression in juvenile cod, as well as possessing different ligand binding affinities and transactivation activities, which may indicate a subfunctional partitioning of the Ahr receptors in this species.

The aim of the present study was to further explore a possible subfunction partitioning of Ahr1a and Ahr2a in Atlantic cod by mapping the expression and localization of *ahr1a*, *ahr2a* and *cyp1a* in early developmental stages. Activation of the Ahr-signalling pathway was confirmed by measuring expression of *cyp1a* in B[a]P-exposed samples. Our results suggest that Ahr2a is the major subtype involved in mediating responses to B[a]P in early developmental stages of Atlantic cod. Furthermore, expression of *ahr2a* in developing jaws and fin nodes of larvae, and the persistent presence of *ahr1a* transcripts in the eye of both cod embryo and larvae indicates a physiological role of these genes in the ontogenesis of these organs.

## 2. Material and methods

### 2.1. Passive-dosing exposure experiment

#### 2.1.1. Cleaning and loading of silicone rods

Water exposure of highly hydrophobic compounds like B[a]P is challenging due to the very low water solubility and high absorption to surfaces in the exposure tanks. Passive dosing was chosen to establish and maintain freely dissolved concentrations of B[a]P at the solubility limit, while avoiding co-solvent addition (Smith et al., 2010). Translucent 1-mm silicone rods (Altec Extrusions, Victoria, UK) were cut to 1 m lengths and precleaned by soaking in methanol and ultrapure water washes. All chemicals used were GC Suprasolv® analytical grade and supplied by Merck (Darmstadt, Germany). The rods were then loaded into saturated B[a]P methanol solutions for two days, whereas control-rods were immersed in methanol (> 99 % purity, Sigma-Aldrich, St. Louis, Missouri, United States) (Smith et al., 2010). After the loading, excess methanol was removed by three successive soaks in ultrapure water for 2–24 hours. One corresponding rod was allowed to equilibrate with 80 mL of autoclaved seawater in each glass beaker for two days before the start of the exposures. All glassware was heated to 400 °C overnight before use.

#### 2.1.2. Exposure of Atlantic cod eggs

Eggs and sperm were stripped from brood stocks of Atlantic cod (*Gadus morhua*), and the eggs were fertilized *in vitro* at the Institute of Marine Research (IMR), Austevoll Research station. Fertilized eggs were transferred to indoor egg incubators, and maintained at 7 ± 1 °C until transported to IMR's facilities in Bergen to begin the exposure studies at 5 days post fertilization (dpf), when the cardiac cone of cod embryos is visible. About 200 eggs were placed randomly in the prepared exposure beakers of either B[a]P or seawater (control group). There were four replicate beakers for each treatment and an additional beaker without eggs (water control) for each group. Exposure to B[a]P started at 5 dpf and lasted until 3 days post hatching (dph). The experiment took place in a cold room at 8 °C, and the temperature in the water and in the room was monitored during the experiment. Dead eggs were removed during daily inspections from all the beakers to minimize bacterial contamination. Samples of several embryos and larvae were collected at 8 dpf, 10 dpf and 3 dph for gene expression and *in situ* hybridization analyses.

#### 2.1.3. Water chemistry and body burden analyses

Water samples (1.0 mL) were taken at 5 dpf, 8 dpf, 11 dpf and 3 dph and liquid-liquid extracted with two 1-mL volumes of dichloromethane (SupraSolv®, Merck, Darmstadt, Germany). Extracts were dried with $Na_2SO_4$, and exchanged into 200 μL isopropanol (EMSURE®, Merck, Darmstadt, Germany). Twenty eggs were collected from each replicate jar at 8 dpf. These body burden samples were homogenized and extracted twice as in (Sørensen et al., 2019), with 50 % dichloromethane in *n*-hexane (SupraSolv®, Merck, Darmstadt, Germany) and subsequent clean-up with solid-phase extraction (Chromabond® silica, 3 mL, glass, Macherey-Nagel, Dueren, Germany). Water and body burden analyses for B[a]P and internal standard were performed on an Agilent 6890 gas chromatograph coupled to a 7010 triple quadrupole mass spectrometer as described in (Sørensen et al., 2016). Statistical differences in body burden concentration between control and B[a]P-exposed groups were analyzed using a Welch's *t*-test in R v1.2.1335 software (Crawley, 2012).

### 2.2. Quantitative polymerase chain reaction (qPCR) analyses

Total RNA was isolated from ten pooled samples of embryos or larvae from each beaker (n = 4) at 8 dpf, 10 dpf and 3 dph using the TRI Reagent® protocol (Sigma-Aldrich, St. Louis, Missouri, United States) as outlined by Chomczynski, 1993. 500 ng of total RNA was reverse transcribed to cDNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad, California, USA) following the provider's protocol. Quantitative real-time polymerase chain reaction analyses (qPCR) were performed using SYBR Green Master I (Roche Diagnostics, Mannheim, Germany) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, USA) as described in (Aranguren-Abadía et al., 2020). Elongation factor 1 alpha (*ef1a*) and ubiquitin (*ubi*) were used as reference genes (Table S1) for normalization of gene expression across samples, and the geNorm software (Vandesompele et al., 2002) was used to calculate the normalization factor based on the geometric mean. The geNorm stability index M for the reference genes was 0.97. Relative induction of *ahr1a*, *ahr2a*, *cyp1a* and *ahrrb* genes (Table S1) between control and B[a]P-exposed groups was analyzed at the different sampling points using a Welch *t*-test on log transformed data in R v1.2.1335 software.

### 2.3. In situ hybridization (ISH)

#### 2.3.1. Tissue preparation for in situ hybridization

Cod embryos at 8 and 10 dpf and larvae at 3 dph were fixed in 4% paraformaldehyde buffered in 1x phosphate-buffered saline (PBS) solution (pH 7.4) for 48 h at 4 °C for whole mount *in situ* hybridization. Whole mount embryos and larvae were briefly washed in 1x PBS, dehydrated in methanol, and stored in 100 % methanol at −20 °C until use. Larvae for tissue sections *in situ* hybridization were fixed in 4% paraformaldehyde-buffered in 1x PBS for 48 h at 4 °C, incubated in a solution of 25 % sucrose, 25 % Tissue Tek (Sakura Finetek, California, United States) and 50 % 1x PBS overnight at 4 °C before they were oriented and embedded in Tissue Tek and kept at −80 °C.

#### 2.3.2. Molecular cloning

Cloning of *ahr1a* and *ahr2a* genes has been previously described in (Aranguren-Abadía et al., 2020). A partial sequence of *cyp1a* was amplified as a single fragment from cDNA prepared from juvenile cod heart using Taq DNA polymerase (Invitrogen, California, USA) and specific primers (Table S1). The *cyp1a* fragment was inserted into a pCR™4Blunt-TOPO® vector (Thermo Fisher, Massachusetts, USA), and DNA sequencing was performed at the University of Bergen Sequencing Facility.

#### 2.3.3. Synthesis of ahr1a, ahr2a and cyp1a RNA probes

Preparation of digoxigenin (DIG)-labelled riboprobes for *ahr1a, ahr2a* and *cyp1a* were done following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). In the synthesis of the riboprobes, PCR products was used as template for the reactions as described in (Thisse and Thisse, 2008) (Table 1) and the synthesized probes were precipitated by LiCl and EtOH together with tRNA (Roche Diagnostics, Mannheim, Germany). Further, *ahr1a* and *ahr2a* probes were designed in the less conserved coding sequence region in order to avoid cross-hybridization of probes between the paralogous genes.

#### 2.3.4. Whole mount in situ hybridization

Whole mount *in situ* hybridization of embryos and larvae (n = 3 per gene and sampling point) were performed as described in (Valen et al., 2014). Briefly, embryos and larvae were rehydrated in methanol (75−25%) followed by dechorination of embryos. Pigmentation of embryos and larvae was removed by bleaching in 3% Hydrogen peroxide ($H_2O_2$)/0.5 % Potassium hydroxide (KOH) (Sigma-Aldrich, St. Louis, Missouri, United States). Prior to hybridization, the embryos and larvae were treated with Proteinase K (Promega, Wisconsin, United States). After hybridization, the tissue was thoroughly washed and treated with RNase A (Sigma-Aldrich, St. Louis, Missouri, United States) to remove unhybridized probe. Before applying the antibody (anti-DIG conjugated with alkaline phosphatase, Fab fragments (1:2000) (Cat. No 11093274910, Roche Diagnostics, Mannheim, Germany, RRID: AB_514497)) the embryos and larvae were incubated in 2% blocking solution (Roche Diagnostics, Mannheim, Germany) in 2x SSC with 0.05 % Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, United States). The DIG-labelled probes were visualized by 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate system (BCIP) (Roche Diagnostics, Mannheim, Germany) and sense probes were used as a control for nonspecific DIG-probe labelling (data not shown).

#### 2.3.5. Tissue sections in situ hybridization

*In situ* hybridization for *arh1a*, *arh2a* and *cyp1a* transcripts (sense and antisense) was performed on serial sections for n = 6 larvae per gene as described in (Ebbesson et al., 2011). By using a Cryostat (Leica CM 3050, Leica Microsystems, Wetzlar, Germany), 12 µm transversal sections were performed for each larvae and deposited into Superfrost Ultra Plus slides (Menzel-Gläser, Braunschweig, Germany). Proteinase K (Sigma-Aldrich, St. Louis, Missouri, United States) treatment was applied for 3.5 min and tissue sections were left 1 h at room temperature for drying prior to hybridization. Sections were treated with RNase (Roche Diagnostics, Mannheim, Germany) prior to incubation in antibody solution (1x maleate buffer + 1% block solution + 0,3% Triton X-100) with anti-digoxigenin alkaline phosphatase FAB-fragment (1:2000, (Roche Diagnostics, Mannheim, Germany)). Chromogen substrate (NBT/BCIP) (Roche Diagnostics, Mannheim, Germany) was applied for visualization of DIG-labelled probes and then mounted in 70 % glycerol for microscopy.

### 2.4. Imaging, craniofacial and cardiac analyses

Leica DMLB and M420 microscopes (Leica Microsystems, Wetzlar, Germany) with the camera Infinity 3 (Teledyne Lumenera, Ontario, Canada) and Image-Pro Premier 9.3 (Media Cybernetics, Rockville, Maryland, United States) were used for imaging of *in situ* hybridization samples. Adobe Photoshop CS5 (San Jose, California, United States) was used to adjust exposure, brightness and contrast in the pictures.

Larvae pictures and videos during the B[a]P-exposure were taken with an Olympus SZX-10 Stereo microscope equipped with a 1.2 Mp resolution video camera (Unibrain Fire-I 785c) controlled by BTV Pro 5.4.1 software (www.bensoftware.com). Larvae were immobilized in 3% methylcellulose and kept at 8 °C using a temperature controlled stage (Brook Industries, Illinois, United States). Length of mandible, ethmoid plate, area of edema and the ventricular and atrial diastolic (D)

**Table 1**
Overview of oligosequences used for probe synthesis. Promoter T3 and T7 sequences are marked in bold font.

| Gene | Forward primer (5′-3′) with T3 promoter | Reverse primer (5′-3′) with T7 promoter | Probe size (bp) |
|---|---|---|---|
| *ahr1a* | **TAATACGACTCACTATAGGG**GCTGAGATGCTGCTGCATCTGCT | **CATTAACCCTCACTAAAGGGAA**CAGTCCAACCACCCCATTCCTGGATT | 777 |
| *ahr2a* | **TAATACGACTCACTATAGGG**CATCTGGTTGGAGAGCATCTGGTTGGA | **CATTAACCCTCACTAAAGGGAA**TGGACCAGGTGTTCATGGAGAGC | 1026 |
| *cyp1a* | **TAATACGACTCACTATAGGG**TTCTGGATGTTGGAGTTCTC | **CATTAACCCTCACTAAAGGGAA**TCTACATGCTCATGAAGTTCC | 841 |
| *cyp1a* | **TAATACGACTCACTATAGGG**TTCTGGATGTTGGAGTTCTC | **CATTAACCCTCACTAAAGGGAA**TCTACATGCTCATGAAGTTCC | 841 |

and systolic diameter (S) used to estimate the fractional shortening (FS = (D–S)/D) were measured using Image J (Image J 1.52 t, National Institute of Health, Bethesda, Maryland, USA) with the object J plugin as described in (Sørhus et al., 2016). Differences in the length of jaw and ethmoid plate, edema area and FS between groups were assessed using one-way ANOVA and Tukey's multiple comparisons tests in R v1.2.1335 software.

## 3. Results

### 3.1. Water chemistry and body burden analyses

Atlantic cod eggs were kept in normal seawater or seawater with B[a]P-infused silicone rods from 5 dpf until 3 dph. The concentration of B[a]P in water was measured at four time points, and body burden of B[a]P was measured after 3 days of exposure (8 dpf). Measured B[a]P concentration in water across all analyzed samples ranged between 0.3 and 37 μg/L with an average of 7.6 μg/L in seawater at 7 °C (Fig. S1A).

The average body burden level in the B[a]P-exposed embryos was 14 ± 5 pg/embryo (range: 8–20); by wet weight, average body burden was 6.5 μg/kg wet weight (range: 3.7–9.2, Fig. S1B). Body burden in the B[a]P-exposed group was significantly greater ($p = 0.01$) than background levels present in the control group (0.36 μg/kg, Figure S1B). Although body burden samples were only collected at 8 dpf, B[a]P is presumed to have been absorbed throughout the duration of the experiment.

### 3.2. Expression of ahr1a, ahr2a, cyp1a and ahrrb in eggs and larvae

Samples of embryos and larvae were collected at 8 dpf, 10 dpf and 3 dph, and the expression of *ahr1a*, *ahr2a*, *cyp1a* and *ahrrb* genes was assessed with qPCR analyses (Fig. 1). The expression of *ahr1a* did not change notably between the control and B[a]P-exposed groups at the different sampling points, but expression of *ahr2a* increased slightly at 10 dpf ($p = 0.05$) (Fig. 1A, B). *cyp1a*-expression was induced in B[a]P-exposed samples at 8 dpf ($p = 0.01$), 10 dpf ($p = 0.001$) and 3 dph ($p = 0.00006$), reaching close to a 40-fold average induction in transcript levels at 10 dpf and 3 dph compared to the normal seawater control samples (Fig. 1C). Expression of *ahrrb* did not change between the control and B[a]P-exposed groups at the different sampling points (Fig. 1D).

### 3.3. Whole mount in situ hybridization of Atlantic cod embryo and larvae

The specific localization of *ahr1a*, *ahr2a*, and *cyp1a* expression was assessed in cod embryo and larvae using *in situ* hybridisation. At 8 dpf, the expression of *ahr1a* was evident in the eye of both controls and B[a]P-exposed embryos (Fig. 2A, B) (Fig. 2C, D). In contrast, differences in the *ahr2a* expression pattern was already detectable at this developmental stage (Fig. 2E, F), and *ahr2a* was only visible in the cardiovascular system of embryos exposed to B[a]P (Fig. 2G, H). An early effect of B[a]P-exposure on *cyp1a* expression was also visible, where its presence was detectable in the skin and vasculature of treated embryos (Fig. 2I, J). Expression of *cyp1a* was also localized in the heart and in the area where the liver develops in both control (Fig. 2L, M) and B[a]P-exposed embryos (Fig. 2O, P). There was not any visible expression of *cyp1a* in the eye of embryos from neither groups (Fig. 2K, N), as the staining present on the eye lens in B[a]P-exposed embryos corresponds to skin tissue (Fig. 2N).

At 10 dpf, *ahr1a* transcripts were present in the eye ganglion cell layer of embryos independent of their exposure regime (Fig. 3A, B, C, D). Notably, at this stage the expression of *ahr2a* was strongly visible in the cardiovascular system of exposed embryos (Fig. 3F, H) and still not detectable in embryos kept in normal seawater (Fig. 3E, G). Expression of *cyp1a* was also stronger in the skin and vasculature of B[a]P-exposed embryos (Fig. 3J) compared to the control group (Fig. 3I). *cyp1a* transcripts were also present in the heart and in the liver of embryos from both groups (Fig. 3L, M, O, P). No visible *cyp1a* expression was detected in the retinal layers of the control group (Fig. 3I, K) or the exposed group (Fig. 3J, N). However, some faint staining was seen in the lens in the control group, but this might be due to probe being trapped in the lens (Fig. 3K).

The specific expression of *ahr1a* in the eye was not clearly visible in the 3 dph whole mount larvae (Fig. 4A, B), but tissue sections revealed a persistent *ahr1a* expression in the ganglion cell and inner nuclear layers in larvae from both groups (Fig. 4C, D). At this stage, the

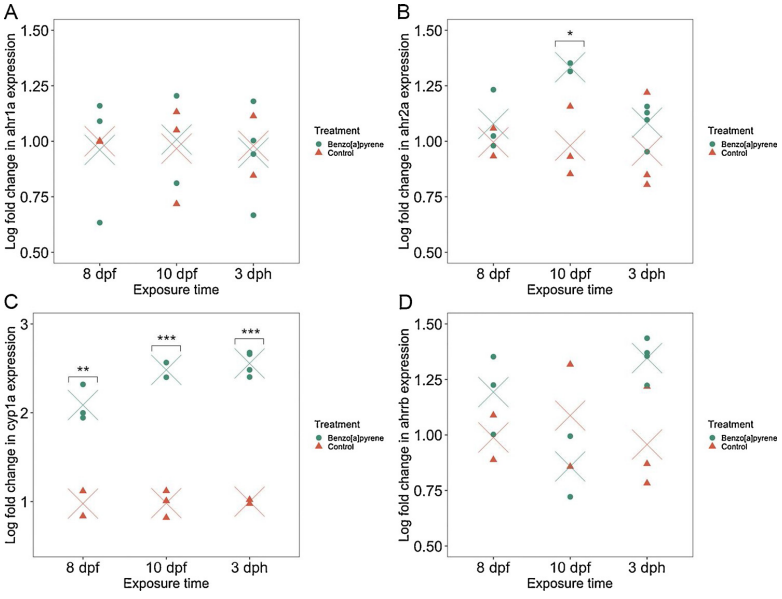


**Fig. 1.** Expression of *ahr1a, ahr2a, cyp1a* and *ahrrb* in B[a]P-exposed embryos and larvae. Atlantic cod eggs and larvae were exposed to B[a]P in a passive-dosing system using infused silicone rods. Expression of *ahr1a* (a) *ahr2a* (b) *cyp1a* (c) and *ahrrb* (d) was measured with qPCR at 8 days post fertilization (dpf), 10 dpf and 3 days post hatching (dph). Log fold change in gene expression was calculated in comparison with the control group (normal seawater). Pool of 10 embryos or larvae (n = 4) were collected from each beaker, but some samples were compromised during laboratory analysis. The data are presented as normalized values (points) and mean (cross). Statistical differences in log transformed transcript levels between control and B[a]P-exposed samples at the different sampling points were assessed by using a Welch *t*-test in R v1.2.1335 software. Level of statistical significance is indicated with *($p \leq 0.05$), **($p \leq 0.01$) or *** ($p \leq 0.001$).

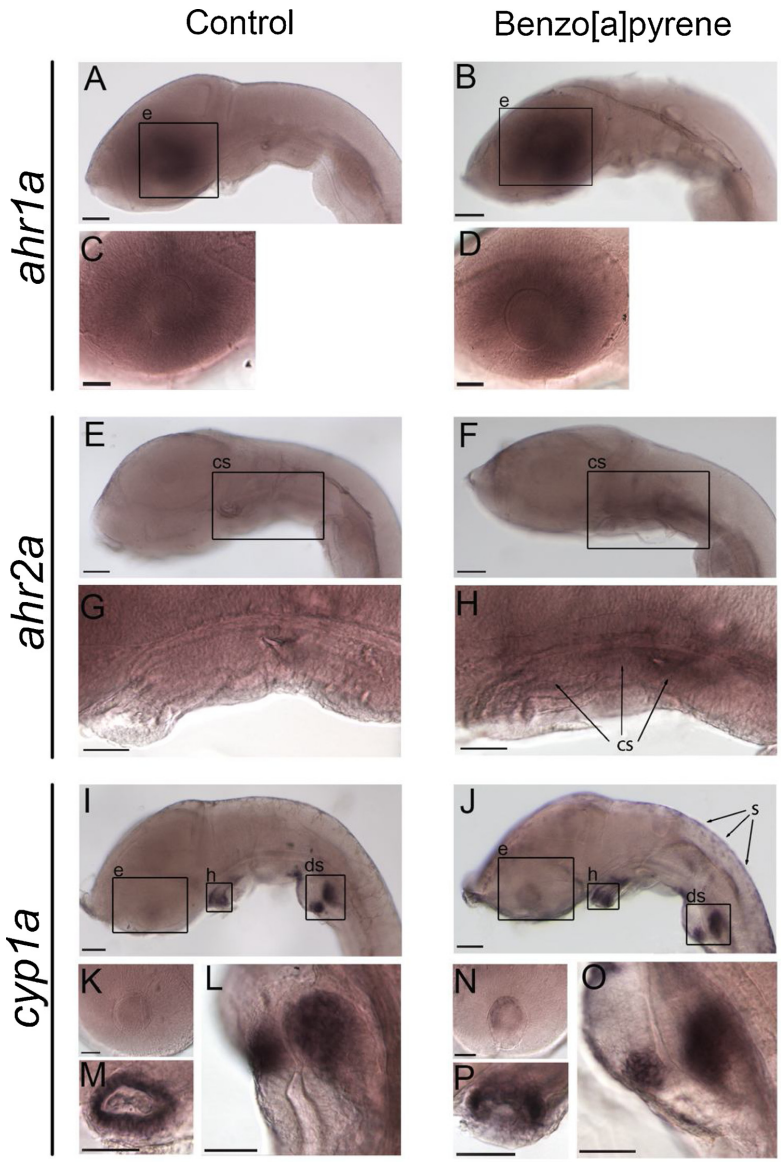


**Fig. 2.** Whole mount *in situ* hybridization of control and B[a]P-exposed Atlantic cod embryos at 8 dpf. Expression of *ahr1a*, *ahr2a* and *cyp1a* was assessed in both B[a]P-exposed and control samples. All pictures show dorsal view of either the whole embryo (A, B, E, F, I and J) or detailed areas where gene expression was visible. Pictures of the eye (C, D, K, N), cardiovascular system (G, H), digestive system (L, O), and heart (M, P) areas were imaged. Gene expression was visualized by specific DIG-labelled RNA probes. e; eye, cs; cardiovascular system, h; heart, ds; digestive system, s; skin. Scale bars: 100 μm in the whole embryo pictures and 500 nm in the detailed areas pictures.

expression of *ahr2a* was also intense in the cardiovascular system of B[a]P-exposed larvae (Fig. 4F) compared to the controls (Fig. 4E). In addition, expression of *ahr2a* was also detectable in the jaws, heart and fin nodes in larvae from both groups (Fig. 4G, H, J, K, M) and mainly in the liver area bordering towards the intestine of B[a]P-exposed individuals (Fig. 4L). In contrast to control larvae, there was a strong expression of *cyp1a* in the skin and vasculature of B[a]P-treated larvae, which made it difficult to capture the inner structures with conventional microscopy (Fig. 4N, O). However, *in situ* hybridization on tissue sections confirmed the presence of *cyp1a* in the heart and presumably the head kidney of both groups (Fig. 4Q, R, T, U) and in the liver and intestine of B[a]P-exposed samples (Fig. 4S). There was no expression of *ahr2a* or *cyp1a* in the liver from control larvae (Fig. 4I, P), but both *ahr2a* and *cyp1a* transcripts was observed in the liver of B[a]P-exposed larvae (Fig. 4L, S). Expression of those genes was also present in the heart of larvae from both groups, independent of the exposure regime (Fig. 4H, K).

### 3.4. Analyses of craniofacial and cardiac anomalies

No clearly visible malformations were observed in B[a]P-exposed 3 dph larvae, but differences in the length of jaw and ethmoid plate, edema area and FS between groups were measured for assessing putative deformities that can be produced after exposure to PAHs in early life stages (Table S2). Jaw lengths did not differ significantly between groups, and no consistent deviations in the length of the ethmoid plate

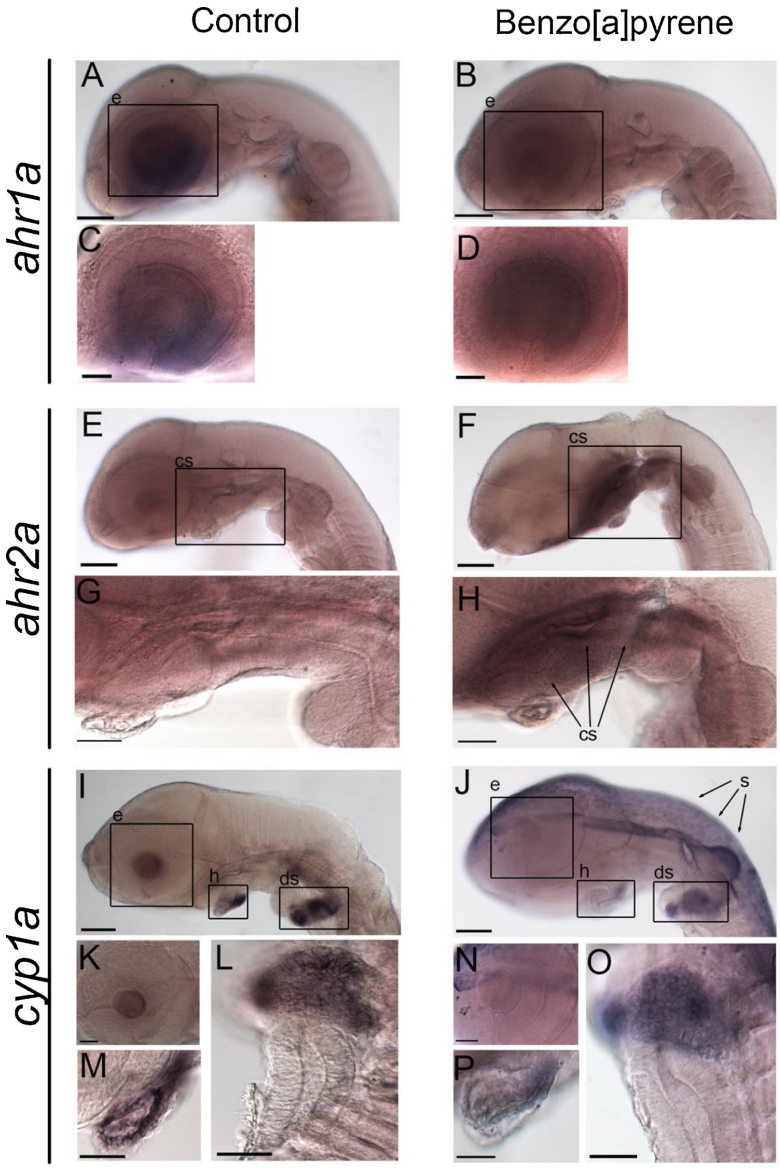


**Fig. 3.** Whole mount *in situ* hybridization of control and B[a]P-exposed Atlantic cod embryos at 10 dpf. Expression of *ahr1a*, *ahr2a* and *cyp1a* was assessed in both B[a]P-exposed and control samples. All pictures show dorsal view of either the whole embryo (A, B, E, F, I and J) or detailed areas where gene expression was visible. Pictures of the eye (C, D, K, N), cardiovascular system (G, H), digestive system (L, O), and heart region (M, P) were imaged. Gene expression was visualized by specific DIG-labelled RNA probes. e; eye, cs; cardiovascular system, h; heart, ds; digestive system, s; skin. Scale bars: 100 μm in the whole embryo pictures and 500 nm in the detailed areas pictures.

was revealed across the whole sample set (Table S2). Edema was not detected in 3 dph B[a]P-exposed larvae, nor any alterations in FS between control and B[a]P-exposed groups.

## 4. Discussion

*In situ* hybridization was used to study a possible subfunction partitioning of Ahr1a and Ahr2a in Atlantic cod by mapping the expression and localization of *ahr1a*, *ahr2a* and *cyp1a* during early development. Quantitative measurements showed an induction of the Ahr-target gene *cyp1a* at the different developmental stages assessed, confirming the activation of the Ahr-signaling pathway after B[a]P-exposure. An effect of B[a]P-exposure was also clearly visible in the skin of embryos and larvae where *cyp1a* was strongly expressed. Furthermore, the induction of *ahr2a* transcripts in the cardiovascular system of B[a]P-exposed embryos further supports an early effect of B[a]P on the Ahr-signaling pathway.

The purpose of the passive-dosing technique was to establish and maintain maximum aqueous B[a]P exposure. The measured concentrations of B[a]P in the exposure water had high variation but were reasonable in the context of solubility limits and anticipated deviations arising from experimental conditions. The published solubility of B[a]P in pure water is 1.52 μg/L at 25 °C (Schwarzenbach et al., 2003). Our measurements were somewhat higher than the published value, despite

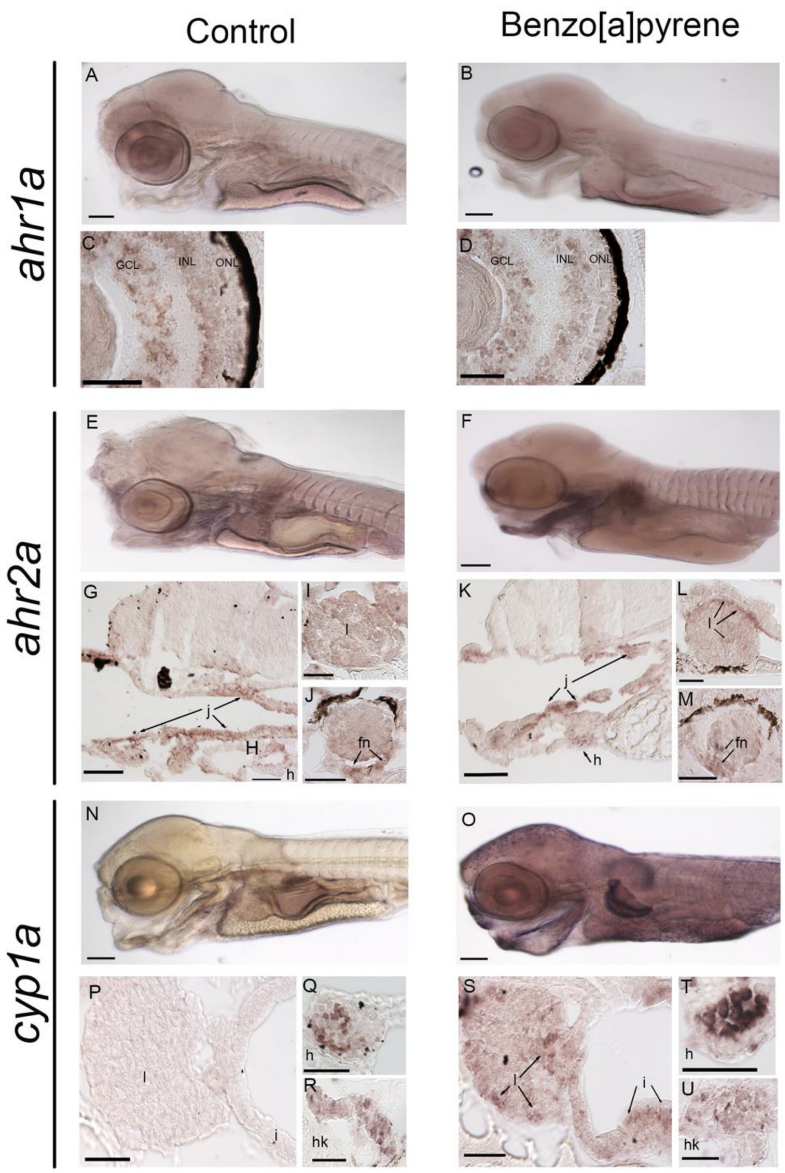


**Fig. 4.** *In situ* hybridization of whole mount and tissue sections prepared from control and B[a]P-exposed Atlantic cod larvae at 3 dph. Expression of *ahr1a*, *ahr2a* and *cyp1a* was assessed in both B[a]P-exposed and control samples. All pictures show dorsal view of either the whole larvae (A, B, E, F, N, O) or sectioned larvae. Pictures of the sectioned eye (C, D), jaws (G, K), heart (H, K,Q,T), liver (I, L, P, S), intestine (P, S), fin nodes (J, M), and presumably head kidney region (R, U) were imaged. Gene expression was visualized by specific DIG-labelled RNA probes. gcl; ganglion cell layer, inl; innear nuclear layer, onl; outer nuclear layer, j; jaws, h; heart, l; liver, fn; fin nodes, i; intestine, hk; head kidney. Scale bars: 100 μm in whole mount larvae and 500 nm in tissue sections except, 100 μm in G and K.

colder temperature and higher salinity, both of which should lower solubility. Binding of B[a]P to dissolved and particulate organic matter was the likely cause for the elevated and varying concentration measurements. The body burden analyses confirmed uptake of B[a]P into exposed fish embryos, while the actual body burdens were rather low. The uptake of B[a]P from aqueous media is limited by its very low solubility that is the combined result of a high hydrophobicity and high melting enthalpy (Kwon et al., 2016; Mayer and Reichenberg, 2006). However, the limited uptake is in line with previous passive-dosing exposure studies with *Daphnia magna* (Smith et al., 2010) and *Lumbriculus variegatus* (Agbo et al., 2013). Low body burdens can also be the results of biotransformation of B[a]P. At this developmental stage the liver bud is visible in Atlantic cod (Hall et al., 2004), and hepatic Cyp1a-mediated metabolism of B[a]P in fish has been demonstrated previously, including at early life stages during ontogenesis of the liver (Hornung et al., 2007; Kocan and Landolt, 1984; Stegeman et al., 1984; Stein et al., 1984; Varanasi et al., 1985). Low body burdens and efficient metabolism of B[a]P may also explain why the characteristic craniofacial and cardiac malformations often observed in early life stages of fish exposed to PAHs (Incardona, 2017) were not clearly visible in B[a]P-exposed cod larvae 3 dph.

Molecular mechanisms and physiological impacts of PAH-toxicity have been shown to differ among individual PAHs, and three possible modes of action have been described previously: Ahr-independent, Ahr-

dependent, and Cyp1a metabolism-dependent (Billiard et al., 2006; Clark et al., 2010; Incardona et al., 2005, 2006). B[a]P is a 5-ring PAH and well-known environmental pollutant with thoroughly characterized cytotoxic, genotoxic, mutagenic and carcinogenic properties (Juhasz and Naidu, 2000; Uno et al., 2001; Verma et al., 2012). B[a]P toxicity has previously been shown to affect the developing heart and cardiovascular system of zebrafish embryos (Huang et al., 2012; Incardona et al., 2011) by modulating the activity of Ahr2 and induce *cyp1a* expression in the heart in an Ahr-dependent mode of action (Incardona et al., 2011). Similarly, expression of *ahr2a* was mainly visible in the cardiovascular system of exposed cod embryos and larvae, and in the heart of 3 dph larvae, indicating cardiotoxicity responses to B[a]P-exposure at the different developmental stages assessed. Notably, the heart is the first organ to become functional in fish, and this process is finalized in Atlantic cod around the golden eye stage. Around 8 dpf (40-somite stage) the tubular heart is present, but looping, demarcation of the atrium and ventricle chambers, and formation of the two minor chambers (sinous venosus and the bulbous arteriosus) occurs later in development (Hall et al., 2004). The heart is fully formed at 10 dpf (golden stage) as a result of an increase in the myocardial mass, which is produced by cell differentiation or proliferation (de Pater et al., 2009). Even though expression of *ahr2a* in the heart of B[a]P-exposed embryos was not clearly visible when using whole mount *in situ* hybridization, the presence of *ahr2a* in the cardiovascular system indicates an expression of this gene also in the heart of cod embryos. Moreover, the slight quantitatively increase in *ahr2a* mRNA expression observed in 10 dpf B[a]P-exposed embryos may be linked to the increase in myocardial cells during embryonic development.

Strong induction of *cyp1a* transcripts was revealed with quantitative measurements, and abundant expression of this gene was localized to the skin and vasculature in B[a]P-exposed individuals. Induction of *cyp1a* after B[a]P exposure has been previously observed in Atlantic cod and several other teleost species (Aranguren-Abadía et al., 2020; Carlson et al., 2004; Incardona et al., 2011; Van Veld et al., 1997; Yadetie et al., 2018). Induced Cyp1a protein expression in the skin, vasculature and cardiovascular system was also reported in zebrafish embryos exposed to high doses of B[a]P (Incardona et al., 2011) and other PAHs (Shankar et al., 2019). Furthermore, other exposure studies using different Ahr agonists, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or β-naphthoflavone (BNF), have also reported increased *cyp1a* gene expression and Cyp1a protein synthesis in the skin, vasculature, and cardiovascular system of zebrafish embryos and larvae (Andreasen et al., 2002; Goodale et al., 2012; Sugden et al., 2017). The skin represents the first line of defence in a water exposure system, so expression of *cyp1a* in the epidermis of Atlantic cod embryos and larvae is not surprising.

The expression of both *ahr2a* and *cyp1a* was induced in the liver of B[a]P-exposed 3 dph cod larvae. Similar observations were made by Andreasen et al., 2002, which observed expression of these two genes in the cardiovascular system and in organs such as skin, heart, liver and head kidney of zebrafish larvae after TCDD exposure (Andreasen et al., 2002). Taken together, our results indicate a role of Ahr2a in mediating toxicity to B[a]P in early life stages of Atlantic cod, which apparently involves Ahr2a-mediated induction of *cyp1a* gene expression. Developmental toxicology studies using zebrafish embryos have also previously demonstrated that Ahr2 is the main subtype involved in mediating toxic responses by inducing *cyp1a* expression (Goodale et al., 2012; Incardona et al., 2011; Prasch et al., 2003; Tanguay et al., 1999). We have recently characterized molecular and functional properties of the Atlantic cod Ahr1a and Ahr2a receptors, and our results showed transcriptional activation of both Ahr1a and Ahr2a *in vitro* after exposure to different Ahr ligands, including B[a]P. However, the high abundancy of *ahr2a* transcripts in the liver suggested that Ahr2a is the main protein involved in mediating xenobiotic responses in juvenile Atlantic cod (Aranguren-Abadía et al., 2020). Notably, the presence of *ahr2a* and *cyp1a* transcripts in the heart, liver, and presumably the head kidney, at the different developmental stages independent of the exposure regimes, indicates also a physiological role of these genes during early development of Atlantic cod. It has been hypothesized that Ahr's original function was to regulate the development of neural systems and sensory structures (Emmons et al., 1999; Hahn et al., 2017). In vertebrates, it has been shown that Ahr regulates different signalling pathways and physiological systems, such as the cardiovascular, reproductive and immune system (Esser and Rannug, 2015; Larigot et al., 2018; Mulero-Navarro and Fernandez-Salguero, 2016; Nebert, 2017). The expression of *ahr2a* in the jaws and fin nodes in both 3 dph B[a]P-exposed and control larvae suggests a physiological role of *ahr2a* in ontogenesis of these organs. Several studies have also demonstrated that zebrafish Ahr2 has a role in the development of the neuronal and reproductive systems (Garcia et al., 2018; Goodale et al., 2012; Souder and Gorelick, 2019). It was recently demonstrated that Ahr2-null zebrafish adults had malformed cranial skeletal bones and severely damaged fins (Garcia et al., 2018). Another study also showed craniofacial malformations and irregularities in the development of fins in Ahr2 knock-out zebrafish embryos (Souder and Gorelick, 2019). Hence, a role of Ahr2a in mediating the development of Atlantic cod's jaws and fins is not unlikely. In addition, a role of CYP1A in metabolizing endogenous compounds has also recently been postulated (Lu et al., 2020) and may explain the expression of *cyp1a* in control embryos and larvae tissues.

Importantly, the eye was the only tissue were *ahr1a* transcripts were detected at all developmental stages assessed. Staining was strong in the ganglion cell layer already in 8 dpf embryos, and expression of *ahr1a* was also present in the eye inner cell layer of 3 dph larvae. In juvenile Atlantic cod, *ahr1a* expression was found in liver, brain and gill tissue, in addition to the eye (Aranguren-Abadía et al., 2020). A physiological role of the zebrafish *ahr1b* in embryonic development was postulated after high levels of *ahr1b* transcripts were detected in zebrafish embryos (Karchner et al., 2005). Recently, Karchner et al. have identified zfAhr1b protein in the inner plexiform and outer plexiform layers in the developing eye of zebrafish, and a down-regulation in the expression of genes involved in phototransduction in zfAhr1b knockdown embryos (Karchner et al., 2017). Different localization between cod *ahr1a* transcripts and zebrafish Ahr1b proteins in the eye layers may be explained by a translocation of Ahr1b to the plexiform layers subsequently to protein synthesis. Importantly, our results indicate that Atlantic cod's Ahr1a protein may be involved in embryonic development of the eye.

## 5. Conclusions

The spatio-temporal expression patterns of *ahr1a, ahr2a, and cyp1a* were mapped during early development of Atlantic cod. Different expression levels and localization of *ahr2a* and *cyp1a* transcripts were observed between control and B[a]P-exposed embryos and larvae at all developmental stages assessed. In B[a]P-exposed embryos and larvae the expression of *ahr2a* was mainly localized in the cardiovascular system, whereas *cyp1a* was mainly induced in the skin and vasculature. The presence of *ahr2a* and *cyp1a* in the liver of B[a]P-exposed larvae further supports a role of Ahr2a in mediating responses to xenobiotics in early life stages. Furthermore, the expression of *ahr1a* in the eye of both embryos and larvae, and the presence of *ahr2a* in the jaws and find nodes of larvae, also suggest roles of these proteins in ontogenesis during early development. Importantly, our data support that a subfunction partitioning of Ahr1a and Ahr2a has occurred in Atlantic cod.

## Author contributions

LA-A, CED, ES, SM were involved in experimental design and sampling. LA-A performed RNA extraction and qPCR analyses, probe-design and synthesis, whole mount *in situ* hybridization analyses, whole mount and tissue section samples imaging, craniofacial and cardiac

measurements, and statistical analyses. CED analyzed water chemistry and body burden samples. ME contributed with whole mount *in situ* hybridization, and NG and VT performed *in situ* hybridization on tissue sections. ES contributed with the craniofacial and cardiac analyses, PM with expertise in passive-dosing techniques and TON and VT with *cyp1a* plasmid preparation. LA-A, CED, and OAK wrote the manuscript, with LA-A as the lead author. All authors contributed with interpretation of the results and revision of the manuscript.

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## Declaration of Competing Interest

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

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105558.

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# Supplementary Paper II

**Table S1:** Overview of oligo sequences used for amplification of *cyp1a* probe and quantitative PCR (qPCR) analyses.

| Gene | Accesion number | Forward primer (5'-3') | Reverse primer (5'-3') | Analysis |
|---|---|---|---|---|
| cyp1a | ENSGMOG00000000318 | ATCACCGTGTGTCTGATCTA | CTGGATGTTGGAGTTCTCATC | Amplification for probe syntehsis |
| *ahr1a* | MN329012 | CAAGGGCGTCTCAAGTTCCTACAT | CAGCACTATCTTCCCCTTTGCATCAC | qPCR amplification |
| *ahr2a* | MN329013 | ACAAACTGTCCGTGCTCCGACTTA | TCCATTCGGGCCATTGTGCTTCT | qPCR amplification |
| *cyp1a* | ENSGMOG00000000318 | CACCAGGAGATCAAGGACAAG | GCAGGAAGGAGGAGTGACGGAA | qPCR amplification |
| *ahrrb* | ENSGMOG00000009114 | GTGTCCCCCACAACACAAGG | GAGTGGAAGAGATTGCTCACCA | qPCR amplification |
| *ef1a* | ENSGMOG00000012005 | CATCAACATCGTGGTCATT | ATGGTTCTCTTGTCAATGC | qPCR amplification |
| *ubi* | EX735613 | GGCCGCAAAGATGCAGAT | CTGGGGCTCGACCTCAAGAGT | qPCR amplification |

**Table S2:** Cardiotoxicity and cranial parameters measured in Atlantic cod (*Gadus morhua*) 3 dph larvae exposed to benzo[a]pyrene. Differences between groups were measured using a one-way ANOVA and Tukey's multiple comparisons tests in R v1.2.1335 software. Statistical differences between groups are indicated with letters.

| Stage | Group | Edema area, % | Fractional shortening (FS), % | Ethmoid plate, µm | Mandible, µm |
|---|---|---|---|---|---|
| 3 dph | Control A | 29±8 | 15±6 | 142±5 | 420±22 |
| 3 dph | Control B | 26±3 | 16±5 | 169±12$^{A}$ | 430±9 |
| 3 dph | Control C | 17±7 | 16±4 | 147±10 | 406±21 |
| 3 dph | Control D | 25±4 | 19±6 | 157±20 | 421±19 |
| 3 dph | Benzo[a]pyrene A | 19±7 | 17±7 | 137±21 | 381±27 |
| 3 dph | Benzo[a]pyrene B | 22±8 | 10±4 | 147±22 | 410±15 |
| 3 dph | Benzo[a]pyrene C | 17±5 | 12±4 | 144±17 | 407±12 |
| 3 dph | Benzo[a]pyrene D | 22±4 | 19±6 | 128±13$^{B}$ | 354±95 |

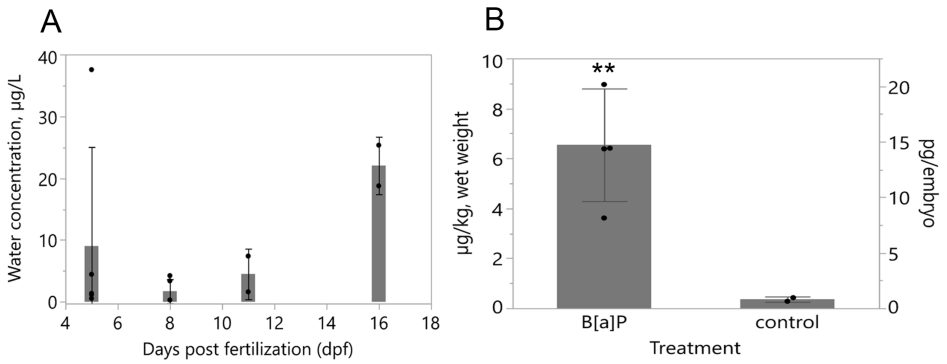


**Figure S1: Water and body burden analyses of benzo[a]pyrene.** B[a]P concentrations were measured in the exposure water at different timepoints (A) and in 8 dpf embryos (B). B[a]P concentrations are presented as average (bars) ± SD, and showing all replicate samples (points). Differences in body burden concentrations between control and B[a]P-exposed embryos were analyzed using a Welch's t-test in in R v1.2.1335 software. Level of statistical significance is indicated with ** ($p \leq 0.01$).

# Paper III

**Aranguren-Abadía, L.,** Yadetie, F., Donald, C.E., Sørhus, E., Myklatun, Xiaokang Z., Lie, K.K., Nakken, C.L., Durif, C., Shema, S., Browman, H.I., Skiftesvik, A.B., Goksøyr, A., Meier, S. & Karlsen, O.A. (2020).

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III

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