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MOLECULAR AND FUNCTIONAL PROPERTIES OF THE ATLANTIC COD (*GADUS MORHUA*) ARYL HYDROCARBON RECEPTORS AHR1A AND AHR2A

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Abstract

The aryl hydrocarbon receptor (Ahr) is a ligand-activated transcription factor that mediates the toxicity of halogenated and polycyclic aromatic hydrocarbons in vertebrates. Atlantic cod (*Gadus* morhua) has recently emerged as a model organism in environmental toxicology studies, and increased knowledge of Ahr-mediated responses to xenobiotics is imperative. Genome mining and phylogenetic analyses revealed two Ahr-encoding genes in the Atlantic cod genome, *gmahr1a* and *gmahr2a*. *In vitro* binding assays showed that both gmAhr proteins bind to TCDD, but stronger binding to gmAhr1a was observed. Transactivation studies with a reporter gene assay revealed that gmAhr1a is one order of magnitude more sensitive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) than gmAhr2a, but the maximal response of the receptors were similar. Other well-known Ahr agonists, such as β-naphthoflavone (BNF), 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) and 6-formylindolo[3,2-b]carbazole (FICZ), also activated the gmAhr proteins, but gmAhr1a was in general the more sensitive receptor and produced the highest efficacies. Induction of *cyp1a* in exposed precision-cut cod liver slices confirmed the activation of the Ahr signaling pathway *ex vivo*. In conclusion, the differences in transcriptional activation by gmAhrs with various agonists, the distinct binding properties with TCDD and BNF, and the distinct tissue-specific expression profiles, indicate different functional specializations of the Atlantic cod Ahrs.

Graphical Abstract

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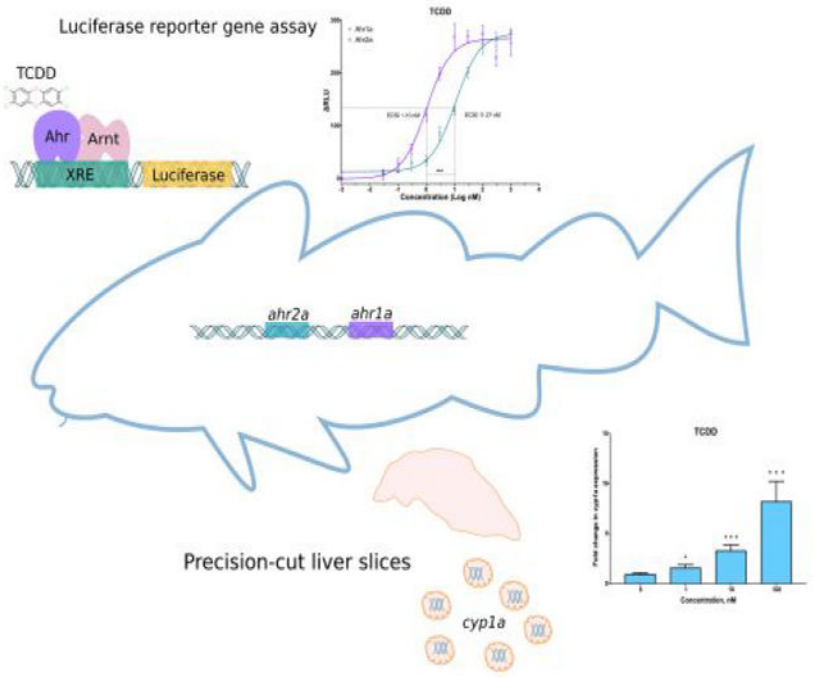
Supporting information

Method details, cloning primers, COS-7 transfection details, cytotoxicity/viability measurements, multiple sequence alignments, amino acid composition analyses, and quantitative real-time PCR analyses.

The authors declare no competing financial interest.



Introduction

The ligand-activated transcription factor aryl hydrocarbon receptor (AHR) is a member of the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) superfamily, and has been widely studied because of its important role in mediating cellular responses to environmental pollutants. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) has been established as the most potent exogenous AHR ligand, but also planar polycyclic aromatic hydrocarbons (PAHs), dioxin-like polychlorinated biphenyls (dl-PCBs) such as 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), the synthetic flavonoid β-naphthoflavone (BNF), and certain endogenous compounds such as the tryptophan derivative 6-formylindolo[3,2-b]carbazole (FICZ) have been shown to bind to and activate AHR[1–5]. Recently, many investigations have focused on elucidating the physiological roles of AHR. It is now known that besides acting as a xenosensor and modulating the transcription of genes that encode proteins involved in the biotransformation of xenobiotic compounds, AHR participates in different signalling pathways and functions in physiological systems such as the cardiovascular-, reproductive- and immune systems[6–8]. In fact, it has been suggested that the original function of AHR started as a developmental regulatory gene in invertebrates, and that the ability to mediate xenobiotic responses is an evolved adaptative response mechanism present in vertebrates[9,10].

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)[11–14]. Upon ligand activation, AIP dissociates from the protein complex and it is suggested that only the AHR-HSP90 complex translocates into the nucleus[15–19]. AHR disassociates from HSP90 and heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This heterodimer binds to xenobiotic response elements (XRE) upstream of AHR target genes,

modulating the transcription of a battery of genes encoding enzymes involved in the biotransformation of xenobiotics, including cytochrome P450 1A (CYP1A)[14,20–23].

A tandem gene duplication prior to the divergence of cartilaginous and bony fish lineages led to the appearance of two Ahr clades; Ahr1 and Ahr2[10,24]. Further, due to the teleost-specific whole genome duplication event, fish may posses both an *ahr1a-ahr2a* and an *ahr1b-ahr2b* tandem pair, although the number of paralogous genes that have been retained throughout evolution varies among different fish species[25]. In contrast to teleosts, humans and rodents have retained only one AHR-encoding gene that was thought to be the ortholog of the teleost *ahr1*, but it is now thought to represent a different evolutionary lineage[10]. Ahr has previously been described in several fishes, including zebrafish (*Danio rerio*)[24,26,27], mummichog (*Fundulus heteroclitus*)[28], red seabream (*Pagrus major*)[29], Atlantic salmon (*Salmo salar*)[30–32], Japanese medaka (*Oryzias latipes*)[33], Japanese pufferfish (*Takifugu rubripes*)[34] and Atlantic tomcod (*Microgadus tomcod*)[35]. Expression patterns of *ahr* genes vary among fishes, and *ahr2* is often more abundant and has wider tissue distribution, whereas expression of *ahr1* is mainly found in brain and heart[28–30,36]. Loss-of-function studies using morpholino-modified antisense oligonucleotides or genome editing have shown that Ahr2 has a primary role in mediating toxic responses to TCDD and dl-PCBs in some fishes[37–45]. The exact function of Ahr1 has not yet been elucidated. A possible physiological role in early development of zebrafish has been hypothesized[24], but loss-of-function studies have not yet revealed such a role[44–46].

Atlantic cod (*Gadus morhua*) is an ecologically and commercially important species that is widely distributed in the North Atlantic Ocean. Atlantic cod has also commonly been used as an indicator species in marine pollution monitoring programs, such as the Protection of the Marine Environment of the North-East Atlantic (OSPAR) convention, water column monitoring of offshore petroleum activities in Norway, and recently in a waste dumping site outside the city of Bergen (Norway)[47–50]. The Ahr target gene *cyp1a* has been extensively studied in cod and used as a biomarker of exposure to environmental pollutants, including PAHs, dioxins and dl-PCBs[51–57]. These studies describing *cyp1a* gene expression, as well as Cyp1a protein synthesis, immunohistochemistry, and enzymatic activity, point to a functional Ahr pathway in cod. However, the molecular basis by which this species senses and responds to contaminants is not completely understood. Our group has recently described the lack of the xenobiotic sensor, pregnane X receptor (Pxr), belonging to the nuclear receptor superfamily of transcription factors, in Atlantic cod and other members of the Gadiformes order[58]. A hypothesis that Ahr has evolved a broader compensatory functional role as a xenosensor in Atlantic cod was therefore raised (ibid), emphasizing the need for a better understanding of the diversity and functional properties of the Ahr signalling pathway in this species. In the present study, we describe for the first time the primary structure, synteny, phylogeny, ligand binding affinities and agonist activation, as well as tissue specific expression profiles of the Atlantic cod Ahr1a and Ahr2a (denoted gmAhr1a and gmAhr2a). Our results show distinct differences in ligand binding affinities, agonist activation, and tissue-specific expression profiles of the gmAhr proteins, which indicate functional specialization.

Material and methods

1. **Fish.** Atlantic cod used in these studies were farmed fish from Austevoll Research Station (Institute of Marine Research, Bergen, Norway) and from Havbruksstasjonen in Tromsø (Nofima, Norway). All fish were kept at the Industrial and Aquatic Laboratory (ILAB, Bergen, Norway) in 500 L tanks in natural seawater at 9 °C, with a 12:12 h light/dark cycle regime and fed *ad libitum* with a commercial diet (Harmony Nature 500, EWOS, Bergen, Norway). Juvenile fish of both sexes (approx. 1.5–2 years old) were used for preparing *ex vivo* liver slices, whereas only female juvenile fish (approx. 1.5–2 years old) were used in the tissue-specific expression study. The fish were maintained and treated in accordance with the guidelines of the Norwegian Board of Biological Experiments with Living Animals.

2. **RNA isolation and cloning of *gmahr1a, gmahr2a, gmarnt1*.** Total RNA was extracted from Atlantic cod (*Gadus morhua*) heart and liver tissue following the protocol from the manufacturer (TriReagent; Sigma-Aldrich, Oslo, Norway). Complementary DNA (cDNA) was synthesized using Invitrogen Random hexamer, oligo(dT)12–18, and Superscript III/IV Reverse Transcriptases (Fisher Scientific, Oslo, Norway). *gmarnt1* and *gmahr1a* were amplified as single fragments from cDNA prepared from cod liver and heart, respectively. *gmahr2a* was amplified as two overlapping fragments from liver cDNA. Detailed information on cloning and primers are presented in Supplementary material and Table S1. Atlantic cod Ahr1a, Ahr2a, and Arnt1 cDNA sequences were deposited in GenBank with the following accession numbers: Ahr1a; MN329012, Ahr2a; MN329013, and Arnt1; MN329014.

3. **Synteny mapping, sequence alignments, and phylogenetic analyses.** Synteny analyses of the genomic regions containing *ahr* genes of different fishes were based on genome data present in Ensembl. Multiple sequence alignments of N-terminal Ahr regions were performed in Clustal-Omega (EMBL-EBI) and edited in Jalview. The phylogenetic tree was inferred using AHR N-terminal amino acid sequences of fishes, mammals, reptiles and birds obtained from Genbank. Amino acid sequences were aligned with MUSCLE, and Bayesian inference analysis was conducted in MrBayes v3.2.7a (see Supplementary material for details).

4. ***In vitro* protein expression and velocity sedimentation assays.** [^{35}S]methionine-labeled gmAhr proteins and *Fundulus heteroclitus* Ahr2a were synthesized *in vitro* using the TnT-Quick Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufactureŕs instructions. The [^{35}S]methionine-labeled TnT reactions were assessed with SDS-polyacrylamide gel electrophoresis and the proteins were visualized by radiography. [^{3}H]TCDD (2 nM) and [^{3}H]BNF (10 nM) binding affinity to gmAhr1a, gmAhr2a and fhAhr2a was measured by velocity sedimentation with sucrose gradients in a vertical tube rotor as described in Karchner et al.[28] (details in Supplementary material).

5. **Transfection, exposure and luciferase reporter gene assay.** COS-7 simian kidney cells were cotransfected with pcDNA3.1/Zeo(+) based gmAhr1a, gmAhr2a and gmArnt1 plasmids, luciferase reporter plasmid (pGudLuc6.1) and Renilla luciferase or β-galactosidase normalization plasmids (pRT-TK and pCMV- βGAL, respectively). For details on cell culturing, transfections and ligand exposure, see Table S2 and Supplementary material. Cytotoxicity was evaluated with two fluorescent dyes, resazurin and 5-carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM), as described by Pérez-Albaladejo et al.[59] (Fig. S1, Fig. S2, protocol details in Supplementary material).

6. **Tissue-specific expression of *ahr1a, ahr2a, arnt1* and *arnt2*.** Tissue samples from ovaries, muscle, head kidney, skin, mid intestine, spleen, heart, stomach, liver, brain, gill and eye (n=3) were collected and snap frozen in liquid nitrogen. RNA was extracted from tissue samples using the TRI Reagent® protocol, and 500 ng of RNA was reverse transcribed to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA). Quantitative real-time polymerase chain reaction analyses were performed using SYBR Green Master I (Roche Applied Sciences, Basel, Switzerland) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) (for primers and protocol details, see Table S3 and Supplementary material). Expression of *gmahr1a, gmahr2a*, *gmarnt1* and *gmarnt2* was normalized across tissues by using *beta-actin* (*actb*) as the reference gene (GenBank: EX739174)[60] and the method described by Livak et al.[61].

7. ***Ex vivo* exposure assays with precision-cut liver slices (PCLS) and analyses of *cyp1a* expression.** PCLS were prepared as described previously with some modifications (details in Supplementary material)[62]. Liver slices were exposed to TCDD (n=5), FICZ (n=6), B[a]P (n=6) and PCB126 (n=7) (DMSO 0.01%). The viability and cytotoxicity of the liver slices were assessed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay[63] and the Cytotoxicity Detection Kit (LDH) (Sigma-Aldrich, Missouri, United States) (Fig. S3 and Fig. S4, protocol details in Supplementary material). Total RNA was isolated from frozen slices (two slices pooled per sample) using the RNeasy® Plus Universal Mini Kit (QIAGEN, Hilden, Germany). cDNA synthesis and qPCR analyses were performed as described in Supplementary material. Reference gene primers were reported previously[60].

Results

Sequencing, phylogenetic analyses, and synteny of cod Ahrs.

Homology searches in the Atlantic cod genome (Ensembl, gadMor1) identified two putative Ahr-encoding genes organized in a tandem pair (ENSGMOG00000004709 and ENSGMOG00000004692). However, when compared to *ahrs* from other teleost species, neither ENSGMOG00000004709 nor ENSGMOG00000004692 appeared to encode complete Ahr protein sequences. In addition, sequence gaps introduced from inadequate genome sequencing and/or genome assembly were present in both gene models. To obtain the full protein encoding sequences, transcripts encoded by ENSGMOG00000004709 and

ENSGMOG00000004692 were cloned from cDNA prepared from Atlantic cod heart and liver tissue, respectively. Sequencing of the cloned DNA revealed that ENSGMOG00000004709 and ENSGMOG00000004692 constitute two open reading frames consisting of 2874 bp and 3384 bp, encoding proteins with calculated molecular weights of 104.3 and 122.7 kDa. These cDNA sequences have been deposited in the National Center for Biotechnology information (NCBI) with accession numbers MN329012 and MN329013.

A phylogenetic tree was made based on the deduced amino acid sequences and representative Ahr sequences obtained from a diverse set of vertebrates, including several teleost species (Fig. 1). Importantly, the phylogeny shows a distinct clustering of MN329012 and MN329013 in clade 1a and clade 2a of the Ahr protein family, respectively. Based on the phylogenetic clustering, and supported by the tandem organization in the Atlantic cod genome, we have classified and named ENSGMOG00000004709 and ENSGMOG00000004692 as gm*ahr1a* and gm*ahr2a*, respectively. Analyses of the genomic region surrounding *gmahr1a* and *gmahr2a* revealed that the genes *ndufa* (NADH dehydrogenase ubiquinone 1 alpha subcomplex subunit 10; ENSGMOG00000004781) and *cntnap5* (contactin-associated protein like 5; ENSGMOG00000004709) are localized adjacent and upstream of *gmahr2a*, oriented in the opposite and same direction, respectively (Fig. S5a). However, no genes were found immediately downstream of *gmahr1a*. Although the individual directions of the genes may vary, this syntenic relationship is well conserved among many teleost species, including mummichog (*Fundulus heteroclitus*), medaka (*Oryzias latipes*), green spotted puffer (*Tetraodon nigroviridis*), and Japanese puffer (*Takifugu rubripes*) (Fig. S5a). Genome mining in a recent and more comprehensive cod genome sequence assembly (gadMor2) revealed that both *gmahr1a* and *gmahr2a* consist of 11 exons and confirmed their co-localisation in a tandem pair in linkage group 20 in the Atlantic cod genome (Fig. S5b)[64,65].

gmAhr1a and gmAhr2a have sequence identity and sequence similarity of 39.24% and 46.34%, respectively (Fig. S6). The sequence identity in the N-terminal part is high, while the C-terminal part suggested to be responsible for transcriptional transactivation is poorly conserved among the gmAhrs. Furthermore, the N-terminal parts of gmAhr1a and gmAhr2a were compared with Ahr1 and Ahr2 sequences obtained from a selected set of other teleosts (Fig. S7). The multiple sequence alignments revealed a high degree of conservation among these species, including the bHLH and PAS domains, which participate in ligand binding, HSP90 and AHR/ARNT dimerization, as well as DNA binding. The N-terminal part of Ahr1a from Japanese pufferfish (*Takifugu rubripes*) has the greatest sequence identity to gmAhr1a (84.35%) (Fig. S7a), while Ahr2 from another gadiform species, the Atlantic tomcod (*Microgadus tomcod*), shares extensive sequence identity (95.33%) with gmAhr2a in the N-terminal region (Fig. S7b). Interestingly, the identity between tomcod Ahr2 and gmAhr2 extends further C-terminally and includes an apparently repetitive sequence region present from amino acid 735 to 804 in gmAhr2, which may be characteristic for members of the gadiform order (Fig. S8). Furthermore, the nuclear localization signal (NLS), as well as the nuclear export signal 1 (NES1) and NES2 appear to be well-conserved in both gmAhr proteins. The LxxLL motif present before NES1 and the cysteine residue present in NES2, which are known to be critical for nuclear localization, are also conserved (Fig. S7)[66–68]. All of the amino acid residues part of the “TCDD-binding-fingerprint” characteristic of

mammalian AHR, in addition to amino acids previously shown to be involved in binding of TCDD in other teleost species, including A388, H296, and Q388, are conserved in gmAhr1a and gmAhr2a (Fig. S7, Fig. S9, Fig. S10)[69,70]. Furthermore, the amino acid residues (P34, S35, R37, H38 and R39) found in the basic region 2 essential for binding to xenobiotic response elements (XRE) are conserved in both proteins (Fig. S7)[22,71–73]. The C-terminal part of mammalian AHR contains the transactivation domain that has an acidic (D/E) domain, as well as a high content of glutamine (Q), and proline, serine, and threonine-rich (P/S/T-rich) subdomains[74]. To reveal such putative subdomains in the gmAhr proteins, the percentages of these characteristic amino acids were plotted for every 20 bases as previously done for Ahr1 and Ahr2 from red seabream (*Pagrus major*) (Fig. S11)[29]. A putative Q-rich domain is present in both gmAhr1a and gmAhr2a, but the frequencies of glutamine were not as high as observed in mammalian AHRs. The other transactivation domains, such as acidic and P/S/T-rich domains, were found to be present in the C-terminal half of both gmAhr proteins.

Tissue-specific expression profiles of Atlantic cod *ahr*s and *arnt*s.

The tissue-specific expression of the two *gmahr* genes was assessed in juvenile Atlantic cod with quantitative real-time PCR (qPCR). The expression levels in the examined tissues were quite different between the paralogous genes, suggesting that their expression profiles appear to be gene-specific (Fig. 2a). The mRNA expression of *gmahr2* was detectable in all tissues, albeit in lower levels in ovaries and muscle. Heart, liver, gill, and eye had the highest levels of *ahr2a*. In contrast, expression of *ahr1a* was not measurable in most tissues, and only notable in liver, brain, gill, and eye. The cod genome harbours two Arnt-encoding genes, representing members of both the *arnt1* and *arnt2* subfamily. The tissue-specific expression was also assessed for the *arnt* genes, demonstrating that *arnt1* was ubiquitously expressed in most tissues, with the highest levels found in brain, liver stomach, and heart (Fig. 2b). Similarly, a*rnt2* was also expressed in high levels in brain, but to a lesser extent in other tissues with only notable mRNA levels in gill and eye (Fig. 2b).

In vitro synthesis of gmAhr and specific binding of [^{3}H]TCDD and [^{3}H]BNF.

Protein syntheses of gmAhr1a and gmAhr2a, as well as *F. heteroclitus* Ahr2a, were carried out in an in vitro transcription and translation system. [^{35}S]methionine-labelled Ahr proteins were successfully produced and migrated corresponding to their predicted molecular weight in SDS-PAGE (Fig. 3a). Unlabelled Ahr proteins were then synthesized and their ability to bind [^{3}H]TCDD (2 nM) and [^{3}H]BNF (10 nM) were determined by velocity sedimentation analyses, in which Ahr proteins are separated by sedimentation properties (size and shape) and specific binding to radioligand is measured (Fig. 3b and 3c). Specific binding of [^{3}H]TCDD was observed for all Ahrs assessed, with a distinct peak, between fractions 10 and 20, corresponding to the sedimentation behaviour typical of Ahr proteins[28]. gmAhr1a and fhAhr2a exhibited a very similar binding profile to [^{3}H]TCDD. However, gmAhr2a demonstrated significantly lower binding to [^{3}H]TCDD than both gmAhr1a and fhAhr2a. Although the reduced signal may partly reflect a weaker expression of gmAhr2a, the significant lower specific binding suggests a lower affinity of gmAhr2a for [^{3}H]TCDD. Notably, [^{3}H]BNF specific binding was also different between the Ahrs; gmAhr1a demonstrated the highest amount of specific binding, while only weak or no specific binding

to [^{3}H]BNF was observed for both gmAhr2a and fhAhr2a (Fig. 3c). Although these results are suggestive of differences in binding affinity and specificity among cod Ahr proteins, the binding assay using a single ligand concentration is only qualitative. We therefore performed more quantitative assays for ligand-dependent transactivation in cell culture.

Ahrs transcriptional activity in COS7-cells.

The abilities of gmAhr1a and gmAhr2a to activate transcription of an XRE-controlled luciferase reporter gene were assessed in COS-7 cells in which the gmAhrs were transiently expressed along with the Atlantic cod Arnt1 protein. Five well-known mammalian and piscine Ahr agonists, including TCDD, BNF, B[a]P, PCB126, and FICZ, were tested at increasing concentrations. Both gmAhr1a and gmAhr2a were activated by each of these compounds, but distinct differences in E_{max}, and EC_{50} were observed between the receptors (Fig. 4, Table S4). FICZ was found to be the most potent compound, with EC_{50} in the picomolar range for both gmAhr proteins (Fig. 4b). However, at higher FICZ concentrations, activation of gmAhr1a was significantly lowered, suggesting a bi-phasic concentration-response to this endogenous compound. Interestingly, the activation by TCDD also differed between the two receptors. Although the E_{max} was very similar, the EC_{50} values differed by one order of magnitude, where gmAhr1a displayed the lowest EC_{50} of approximately 1 nM (Fig. 4a). Differences in the activation patterns were revealed also for B[a]P, PCB126 and BNF (Fig. 4c,d,e). For these three compounds, E_{max} was significantly higher for gmAhr1a. Similarly, as for FICZ, gmAhr1a showed tendencies to a biphasic concentration-response at higher BNF concentrations.

Activation of the Ahr signaling pathway in precision-cut liver slices.

The compounds tested for transcriptional activation of gmAhrs *in vitro* were also tested *ex vivo* in liver slices using a similar concentration range as used in the *in vitro* transactivation assay (BNF presented in[62]). Activation of the Ahr signalling pathway was assessed by measuring altered mRNA expression of *cyp1a* with qPCR. The two most potent compounds in the *in vitro* transactivation assay, TCDD and FICZ, also induced *cyp1a* expression at the lowest concentrations used (1 nM) *ex vivo*, while B[a]P induced expression of *cyp1a* at 10 nM (Fig. S12a and S12b). When tested in concentrations up to 10 μM, B[a]P produced very strong transcriptional responses with approx. 400-fold increase in *cyp1a* expression (Fig. S12c). On the other hand, PCB126 was the least potent compound; inducing *cyp1a* expression only at 200 and 2000 nM (Fig. S12d). Alterations in the expression of *gmahr1a* and *gmahr2a* was also assessed in liver slices exposed to increasing concentrations of TCDD and B[a]P, but no significant differences in transcript levels were observed (Fig. S13).

Discussion

Genome mining identified two divergent gmAhr-encoding genes organized in the same orientation in a tandem pair in linkage group 20 in the Atlantic cod genome. Phylogenetic analyses revealed that these genes belong to the Ahr1a and Ahr2a clades. No other AHR genes were found in the cod genome, indicating that Atlantic cod has not retained the Ahr1b and Ahr2b paralogs that are found in some other fish. Tandem positioning of *ahr* genes exists in several different fish species, but the retention of the individual Ahr paralogs varies

greatly, such as in zebrafish (*ahr1b-ahr2(b)* tandem, chromosome 22; *ahr1a*, chromosome 16, GRCz11, Ensembl), Japanese pufferfish (*ahr1a-ahr2a* tandem, chromosome 1, *ahr1b-ahr2b* tandem, chromosome 8; *ahr2c*, chromosome 6, FUGU5, Ensembl), Japanese medaka (*ahr1a-ahr2a* tandem, chromosome 21; *ahr1b-ahr2b tandem*, chromosome 2, ASM223467v1, Ensembl) and mummichog (*ahr1a-ahr2a* tandem, chromosome 1; *ahr1b-ahr2b* tandem, chromosome 18), *Fundulus_heteroclitus*-3.0.2,[75]). The syntenic relationship in the genomic region surrounding the gmAhr genes was also well conserved among Atlantic cod and several other fish species possessing an *ahr*-tandem pair arrangement. Characteristic Ahr features were present in both gmAhr protein sequences, including the bHLH and PAS domains, as well as functionally important amino acid residues involved in cellular translocation, ligand binding and XRE binding, supporting that the basic functions of Ahr are conserved in both proteins. As observed in Ahr in other teleosts, the sequence similarity varied greatly in the C-terminal transactivation domain, although the different subdomains, including Q-rich, acidic and P/S/T-rich, were identified in both gmAhr1a and gmAhr2a.

All of the common AHR ligands tested in the reporter gene assay bound to and activated both gmAhr proteins. The endogenous compound FICZ was the most potent ligand, although a decrease in gmAhr1a activity at the highest concentrations was observed. High sensitivity to this tryptophan derivative is not surprising since FICZ has been shown to be among the most potent ligands for AHR in many animals, including rodents, human, birds, amphibians and fish[40,76–80]. Differences in transactivation between gmAhr1a and gmAhr2a were observed for TCDD, B[a]P, PCB126 and BNF. While the two Atlantic cod paralogs had comparable sensitivity to FICZ and B[a]P, gmAhr1a demonstrated the highest sensitivity to TCDD and PCB126. The EC_{50} values of TCDD differed by one order of magnitude and were calculated to be ~1 nM and ~10 nM for gmAhr1a and gmAhr2a, respectively. The TCDD EC_{50} value of gmAhr1a is comparable to those observed for Ahr1 in white sturgeon, lake sturgeon, and red seabream, as well as for zebrafish Ahr2 and all of the Atlantic salmon Ahr2 proteins[24,30,70,81]. A greater sensitivity of Ahr1 to TCDD in fish possessing both Ahr1 and Ahr2 has previously been reported in lake sturgeon, Atlantic sturgeon and red seabream. Significant differences in E_{max} values between gmAhr1a and gmAhr2a were observed for B[a]P, PCB126, and BNF, where gmAhr1a produced higher maximum responses for PCB126 and BNF. Higher transactivation activity of Ahr1a compared to Ahr2a after PCB126 exposure is in line with previous findings in Atlantic sturgeon and shortnose sturgeon[82].

Differences in gmAhr1a and gmAhr2a binding to [^{3}H]TCDD and [^{3}H]BNF were found in *in vitro* expressed proteins. In accordance with the luciferase reporter gene assay with TCDD and BNF, gmAhr1a was the receptor with the highest binding to both compounds. Differences in binding to [^{3}H]TCDD have been reported previously with Ahr in other teleosts, and appear to vary among different species and Ahr paralogs. Zebrafish Ahr2 and Ahr1b bound with similar magnitude to [^{3}H]TCDD, while the zebrafish Ahr1a did not bind to this compound[24,26]. Both mummichog Ahr1a and Ahr2a bound [^{3}H]TCDD in a similar manner, but there were some differences in sensitivity to Ahr activation among the four Atlantic salmon Ahr2 proteins[24,28,30]. Andreasen et al. also showed that zebrafish Ahr2 was capable of binding to [^{3}H]BNF, in contrast to zebrafish Ahr1a that was not[26].

Homology modelling and *in silico* ligand docking of the zebrafish Ahr1a, Ahr1b and Ahr2 suggested that H296 and A386 are important amino acids for binding of TCDD[69,83]. These predictions were confirmed with *in vitro* mutagenesis, where replacement of Y296H and T386A restored the ability of zebrafish Ahr1a to bind to both TCDD and DNA[69]. *In silico* protein modelling and ligand docking analyses with Ahr1 and Ahr2 from lake sturgeon and white sturgeon suggested a higher sensitivity of white sturgeon Ahr2 to TCDD and dioxin-like compounds due to the presence of the amino acid A388 in its ligand-binding domain[70]. The presence of A388 would result in a larger binding cavity, which was suggested to provide a more optimal orientation for such compounds. Notably, the residues important for binding and coordination of TCDD in zebrafish and white sturgeon Ahr2, in addition to the amino acids constituting the mammalian "TCDD binding-fingerprint"[84], are positionally conserved in gmAhr1a and gmAhr2a. Thus, the discrepancies in ligand binding, sensitivities, and efficacies of gmAhr1a and gmAhr2a observed in this study must be attributed to other structural features present in these protein sequences.

While *in vitro* ligand activation assays demonstrated that Atlantic cod Ahrs could be ligand activated, activation of the Ahr signalling pathway by FICZ, TCDD, B[a]P, and PCB126 was also confirmed *ex vivo* in Atlantic cod liver slices. These data are in agreement with previous *in vivo* and *in vitro* studies that have reported induced CYP1A protein activity and expression in Atlantic cod exposed to BNF, TCDD, B[a]P and PCB105[53,55–57,85,86]. In line with the low EC_{50} values determined for FICZ and TCDD in the Ahr transactivation assay, induction of *cyp1a* was observed in slices exposed to these two ligands in the low nanomolar range. Interestingly, a greater fold change induction of *cyp1a* was observed in liver slices exposed to B[a]P and PCB126 as compared to TCDD. The reduced viability of liver slices exposed to 100 nM TCDD may explain the lower induction of *cyp1a* (Fig. S3). However, the liver of Atlantic cod differs from other fishes due to its high content of lipids[87]. Lipid droplets present in the hepatocytes might sequester lipophilic compounds and make them less available for the cytosolic receptors[55,56]. Hence, different distribution and accumulation of these compounds in liver slices may contribute to the differences in *cyp1a* induction produced by TCDD, B[a]P, and PCB126.

In general, *gmahr2a* was the most abundant and widely expressed gene in the different tissues sampled, while *gmahr1a* expression was only detectable in liver, brain, gill and eye. In other fishes, *ahr2* is also the most abundant and widely distributed gene, whereas expression of *ahr1* is mainly found in brain and heart[28–30,36]. The opposite case was seen for the *arnt* transcripts, where *arnt1* was the most abundant and detectable gene in many tissues. The almost absence of *arnt2* in the liver indicates that gmArnt1 is most likely the heterodimer partner of gmAhr2. The presence of ~10 times more *gmahr2a* transcripts than *gmarh1a* in liver tissue suggests an important role of *gmahr2a* in the recognition of xenobiotics and controlling transcription of biotransformation enzymes.

Toxic responses to TCDD, PCB126 and B[a]P in zebrafish and mummichog have been demonstrated to be mediated by Ahr2[37–45,88]. Other studies also suggested a role of Ahr2 in mediating TCDD toxicity in red seabream, medaka, and Atlantic salmon[30,33,89]. In spite of *gmahr2a* being the highest expressed gene in the liver, gmAhr1a was the receptor that demonstrated the strongest binding and highest sensitivity for most of the compounds tested

in vitro. This suggests that gmAhr1a may be involved in mediating toxicity responses in tissues where this receptor is expressed. Studies with red seabream and white sturgeon suggested also a role for both Ahr proteins in mediating dioxin toxicity[81,90]. Moreover, in birds and white sturgeon, a greater sensitivity to dioxin-like compounds has been linked to Ahr1 activity[91–93]. However, the involvement of Ahr1 in mediating toxicities is still not clear. A recent study compared Ahr1 and Ahr2 EC_{50} values obtained from transactivation studies and early life stage mortality data from fishes and birds exposed to dioxin-like compounds. Importantly, only a significant linear relationship between Ahr2 activation and early life stage mortality was revealed[94].

It is suggested that the ability of AHR to regulate transcription of xenobiotic-metabolizing enzymes in vertebrates is an adaptive function evolved in the vertebrate lineage[10]. In invertebrates, AHR is involved in development of sensory structures and neural systems (ibid). The physiological roles of AHR in vertebrates is less well understood, but it is now known that AHR participates in different signalling pathways and physiological systems such as the cardiovascular-, reproductive- and immune system[6–8]. In addition, the multiple Ahr paralogs found in some non-mammalian vertebrates, like fish, may have acquired Ahr protein specialization through subfunction partitioning. Tissue-specific expression patterns, and ligand and target gene specificity are among some partitioning mechanisms. Zebrafish is an example of evolution leading to development of different physiological roles of the Ahrs. As discussed earlier, zfAhr2 has been shown to be involved in mediating toxicity of dioxin-like compounds and PAHs, as well as a suggested a role of in the development of the nervous system[43,44,95]. On the other hand, Karchner et al. found high expression levels of *zfahr1b* in zebrafish embryos compared to the other two *zfahr*s, suggesting a role of zfAhr1b in embryonic development[24].

Although zfAhr1a was originally thought to be non-functional (unable to bind TCDD or activate transcription *in vitro*), it was later shown to be involved in responses to a variety of compounds, including leflunomide, pyrene, and xanthone[42,96,97]. In a study in zebrafish embryos, the endogenous compound FICZ was shown to bind both zfAhr1b and zfAhr2, but *cyp1a* induction was largely mediated by zfAhr2[40]. Atlantic cod gmAhr1a and gmAhr2a receptors are also very sensitive to FICZ, which could indicate that both receptors are involved in controlling physiological responses. Importantly, the different tissue expression profiles, ligand binding affinities, and transactivation activities also support the idea that subfunction partitioning of Ahr has occurred in Atlantic cod. Higher levels of *gmahr2a* expression in the liver indicate that this protein is most likely involved in mediating xenobiotic responses in Atlantic cod, as it is in other fishes. Moreover, the high sensitivity of gmAhr1a to the different ligands tested does not exclude the possibility of gmAhr1a activity being modulated by certain pollutants. Atlantic cod's chemical defensome is slightly different compared to several other fishes because of the lack of Pxr. Hence, a role of both paralogs in mediating responses to pollutants may be a compensatory functional role to modulate xenobiotic responses, as previously suggested[58]. Further studies, such as additional Ahr transactivation studies using PXR ligand compounds, expression and localization patterns in Atlantic cod embryos, as well as mutagenesis and gene knock-out studies, may help to elucidate the specific roles of the gmAhrs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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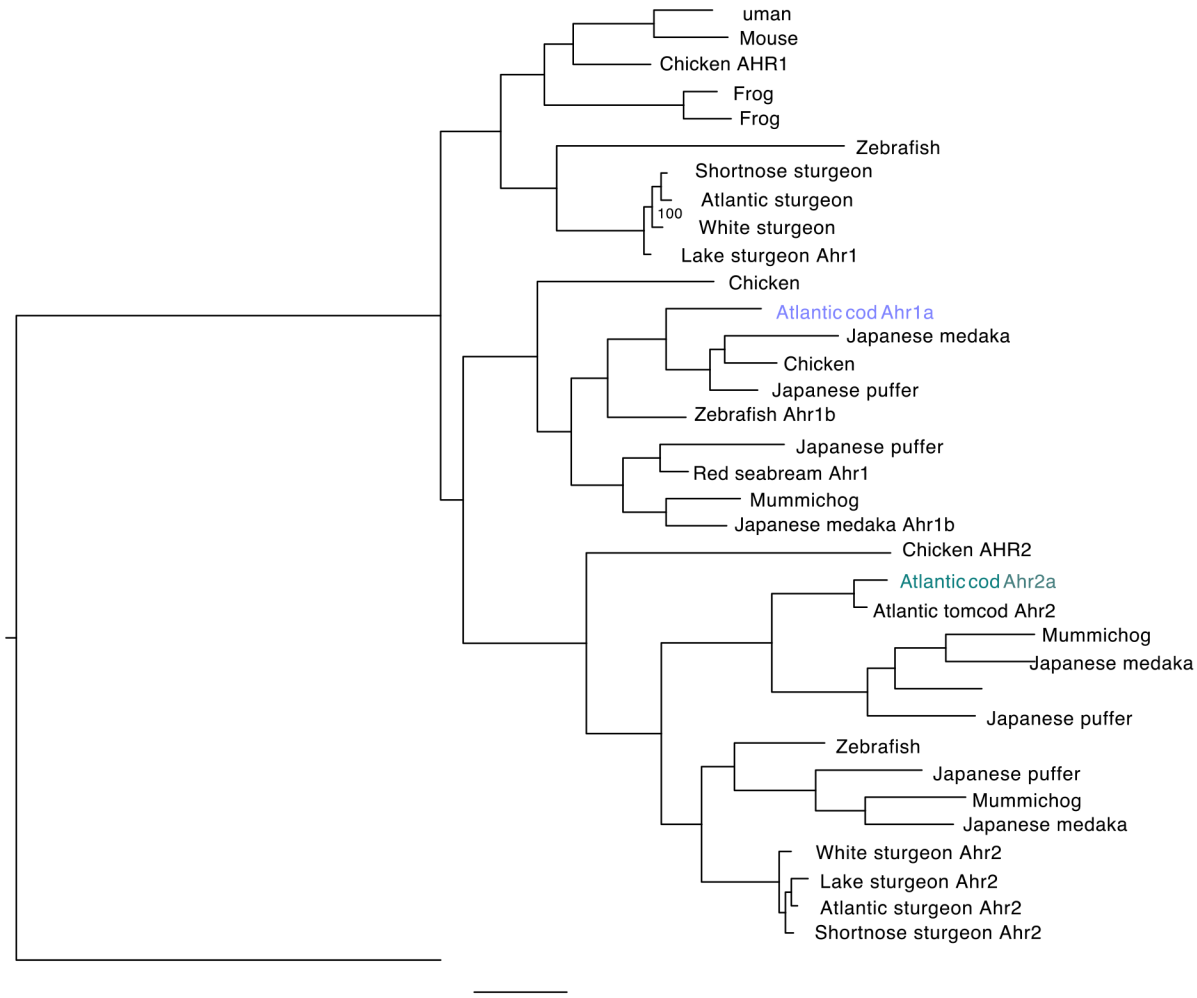

Figure 1. Phylogenetic analyses of Atlantic cod Ahr proteins and other vertebrate Ahr homologs.

The phylogenetic tree was made with MrBayes v3.2.7a using a BLOSUM substitution model. A selected set of AHR N-terminal amino acid sequences from fish, birds, reptiles and mammalian species were used. Alignment positions with gaps were not included. Bayesian inference analysis was conducted and Markov chain Monte Carlo (MCMC) analysis was run for 300,000 generations for each 1000 samples with a 25% burn-in. Four chains were used with a heating parameter of 0.1.

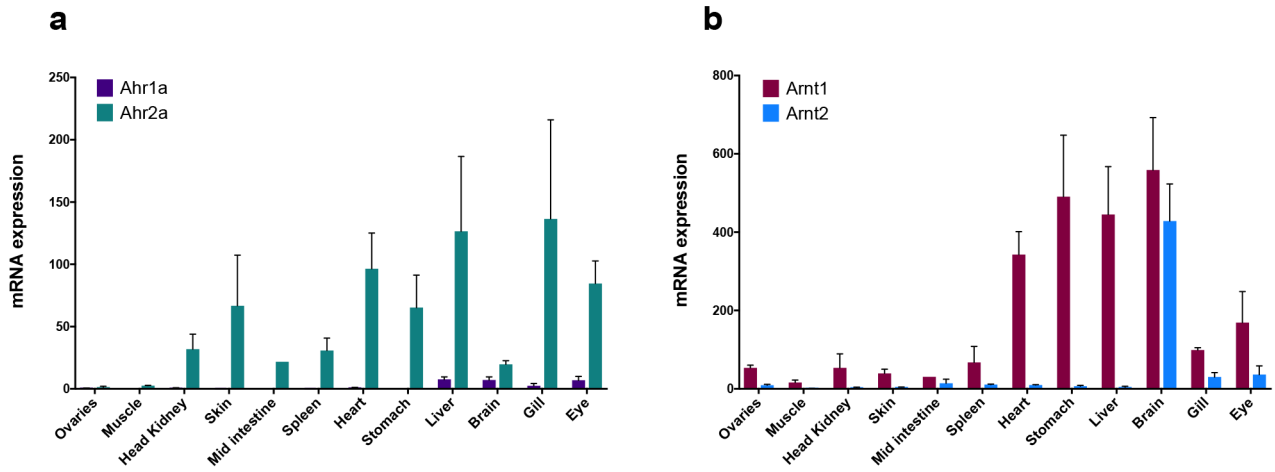

Figure 2. Tissue-specific expression of Atlantic cod *ahrs* and *arnts*.

Expression levels of *ahrs* **(a)** and *arnts* **(b)** were assessed in gonads, muscle, head kidney, skin, mid intestine, spleen, heart, stomach, liver, brain, gill and eye obtained from female juvenile Atlantic cod (n=3). Expression levels were assessed with qPCR and normalized against the reference gene *actb*. Original data were multiplied by 10000; results are expressed as mean±SEM.

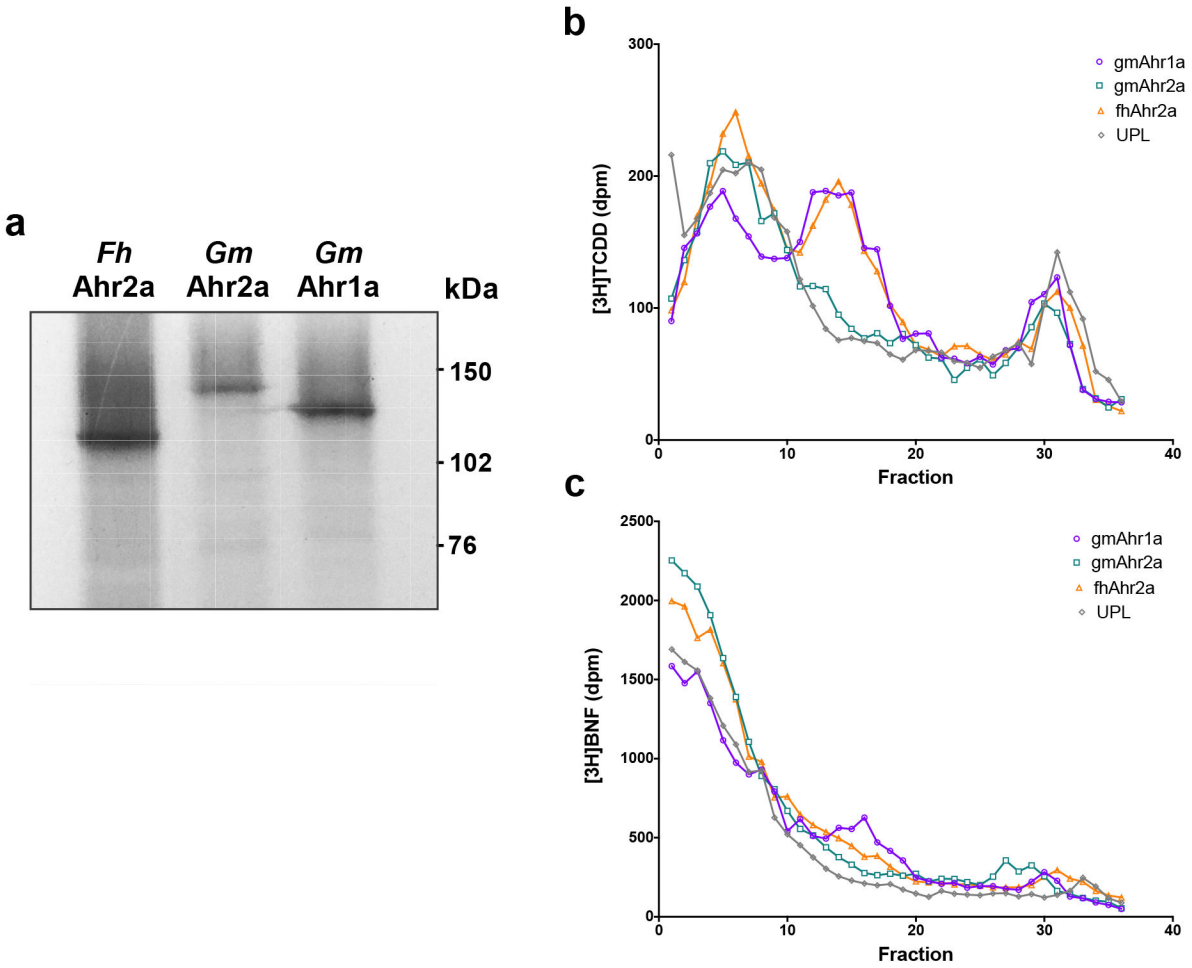

Figure 3. ***In vitro*** **protein expression and velocity sedimentation assays of Atlantic cod Ahrs.**
(a) Autoradiogram of *in vitro* translated mummichog (*Fh*) Ahr2a, Atlantic cod (*Gm*) gmAhr1a and gmAhr2a constructs labelled with [^{35}S]methionine. **(b, c)** velocity sedimentation assays on sucrose gradients using [^{3}H]TCDD **(b)** or [^{3}H]BNF **(c)**. Ahrs proteins were expressed *in vitro* and incubated over night with [^{3}H]TCDD (2 nM) or [^{3}H]BNF (10 nM). Gradients were fractionated and counted in a scintillation counter. Specific binding is the difference between total binding (expressed protein) and nonspecific binding (UPL). Mummichog Ahr2a was used as a positive control and the unprogrammed lysate containing an empty pcDNA3.1 vector (UPL) as negative control. [14C]catalase was added as an internal sedimentation marker.

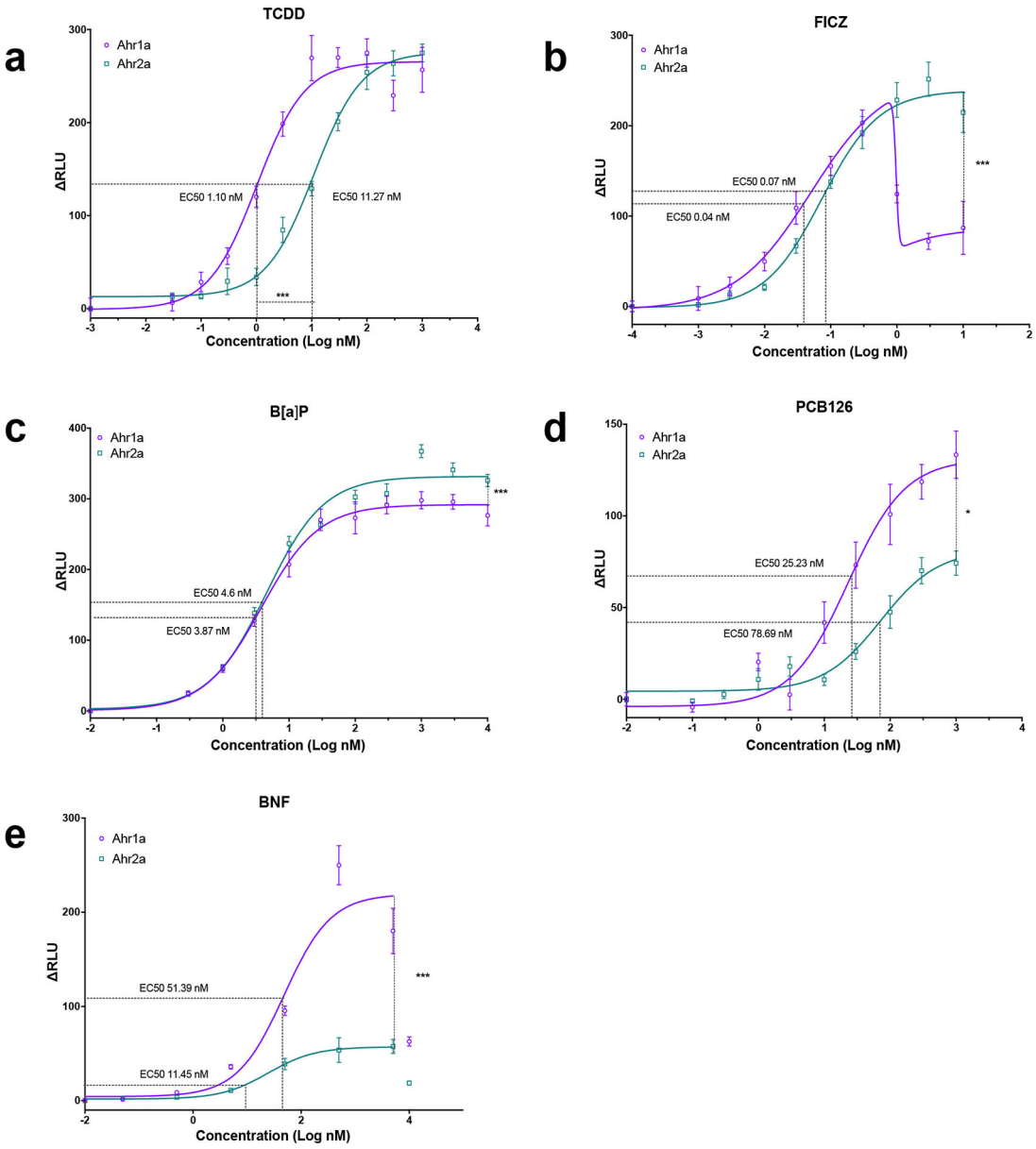

Figure 4. Luciferase reporter gene assays of Atlantic cod Ahr1a and Ahr2a exposed to FICZ, TCDD, B[a]P, PCB126 and BNF.

COS-7 cells were transfected with either the Atlantic cod Ahr1a or Ahr2a, Atlantic cod Arnt1, pGudLuc6.1 Luciferase and pRL-TK *Renilla* (control) constructs. Cells were exposed to TCDD (0.03–1000 nM) **(a)** FICZ (0.001–10 nM) **(b)** B[a]P (0.3–10000 nM) **(c)** PCB126 (0.1–1000 nM) **(d)** and **(e)** BNF (0.05–10000 nM). Relative luciferase units (ΔRLU) was calculated by normalizing the firefly luciferase activity to the transfection control *Renilla* luciferase activity and to the DMSO average of each assay. The data are presented as mean ± SEM at the different concentrations. EC_{50} values are indicated as dotted lines in the graphs. Non-linear regression analyses were performed in Prism v7. Statistical differences between EC_{50} values and maximal activation were obtained using the dose-response analyses *drc* package in RStudio v1.2.1335. Level of significance is indicated with * ($p < 0.05$) or ***

($p<0.001$). With the exception of a slight reduction in cell viability with the highest concentration of TCDD, no significant alterations in cell viabilities were observed (Fig. S2).