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Retene causes multifunctional transcriptomic changes in the heart of rainbow trout (Oncorhynchus mykiss) embryos

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Abstract

Fish are particularly sensitive to aryl hydrocarbon receptor (AhR)-mediated developmental toxicity. The molecular mechanisms behind these adverse effects have remained largely unresolved in salmonids, and for AhR-agonistic polycyclic aromatic hydrocarbons (PAHs). This study explored the cardiac transcriptome of rainbow trout (Oncorhynchus mykiss) eleuteroembryos exposed to retene, an AhR-agonistic PAH. The embryos were exposed to retene (nominal concentration 32 µg/L) and control, their hearts were collected before, at and after the onset of the visible signs of developmental toxicity, and transcriptomic changes were studied by microarray analysis. Retene up- or down-regulated 122 genes. The largest Gene Ontology groups were signal transduction, transcription, apoptosis, cell growth, cytoskeleton, cell adhesion/mobility, cardiovascular development, xenobiotic metabolism, protein metabolism, lipid metabolism and transport, and amino acid metabolism. Together these findings suggest that retene affects multiple signaling cascades in the heart of rainbow trout embryos, and potentially disturbs processes related to cardiovascular development and function.

Key words: dioxin-like toxicity, fish embryo, transcriptomics

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants. Many of them activate the aryl hydrocarbon receptor (AhR) and thereby cause dioxin-like developmental toxicity (blue sac disease, BSD) in early life stages of fish (Hawkins et al., 2002, Billiard et al., 1999, Scott et al., 2011, Incardona et al., 2006, Clark et al., 2010, Van Tiem and Di Giulio, 2011). The signs of BSD include yolk sac and pericardial sac edema, hemorrhaging, failure in erythrocyte maturation, defects in heart and vascular development, induction of cytochrome P450 enzymes, and skeletal deformities, and this condition may be lethal if severe.
The AhR is a member of the basic helix-loop-helix transcription factors, and is activated by aromatic compounds with structural characteristics similar to those of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Activated AhR translocates to the nucleus where it dimerizes with AhR nuclear translocator (ARNT), and up- or down-regulates transcription of numerous genes via binding to xenobiotic-responsive elements within the promoter sequences of these genes. The activation of the AhR precedes signs of BSD in trout embryos (Brinkworth et al., 2003).

Cardiovascular tissue is the presumed target tissue of AhR-mediated toxicity in fish. The AhR agonists disturb the development of the heart and the vasculature in fishes, and cardiovascular pathology is the first sign of dioxin-like toxicity in zebrafish (Danio rerio) and medaka (Oryzias latipes) (Henry et al., 1997, Hornung et al., 1999, Antkiewicz et al., 2005, Carney et al., 2006, Mehta et al., 2008, Plavicki et al., 2013, Scott, 2009). The most studied AhR agonist, TCDD, prevents cardiac valve formation, inhibits epicardial and proepicardial development, causes altered looping of the heart, and reduces the volume and number of cardiomyocytes in fishes (Hornung et al., 1999, Antkiewicz et al., 2005, Carney et al., 2006, Mehta et al., 2008, Plavicki et al., 2013). Similarly, other AhR agonists (e.g., PCB126, retene, benz[a]anthracene) disturb the development of the heart in zebrafish and medaka (Incardona et al., 2006, Grimes et al., 2008, Scott et al., 2011, Scott, 2009).

The molecular mechanisms of developmental toxicity of AhR agonists in fish are not well understood. AhR-ARNT-dependent transcriptional regulation is needed for toxic effects to take place, but the downstream targets are not well resolved (Antkiewicz et al., 2006, Carney et al., 2004, Prasch et al., 2003, Prasch et al., 2006). Some information has been gained from studies on zebrafish embryos exposed to TCDD, but only a little is known about the effects of AhR-agonistic PAHs (Carney et al., 2006, Chen et al., 2008, Goodale et al., 2013). Furthermore, the molecular mechanisms have to our knowledge not been studied in depth in salmonids at all.
Retene (7-isopropyl-1-methylphenanthrene) is an alkylated PAH formed from resin acids via action of anaerobic microbes, or during incomplete combustion of resinous softwood, e.g., in forest fires (Ramdahl, 1983, Tavendale et al., 1997). It has been found in sedimenting particles and the sediment surface layer in lake areas contaminated by treated pulp and paper mill effluents, and in municipal landfill soil (Leppanen and Oikari, 1999, Leppanen and Oikari, 2001, Legler et al., 2011). As an alkylphenanthrene, retene is representative of the compounds in petroleum products that cause dioxin-like developmental toxicity in fishes (Adams et al., 2014). Retene is an AhR agonist, and it causes BSD in early life stages of zebrafish, medaka, and rainbow trout (Oncorhynchus mykiss) (Billiard et al., 1999, Kiparissis et al., 2003, Scott et al., 2011). The molecular mechanism of the developmental toxicity caused by retene depends on the AhR, and thus resembles that of TCDD in zebrafish (Scott et al., 2011).

The aim of this work was to reveal the changes in the cardiac transcriptome of rainbow trout embryos caused by retene, an AhR-agonistic PAH. Rainbow trout embryos were exposed in a semi-static test to a sublethal concentration of retene, cardiac tissue was collected before, at, and after the onset of BSD signs, and the transcriptome was studied using a microarray.

2 Materials and methods
2.1 Fish embryos and water chemistry
Rainbow trout eyed embryos at 360 degree-days (°D) of development were obtained just prior to hatch from a local fish farm (Hanka-Taimen). They were kept at the University of Jyväskylä research station at Lake Konnevesi until hatch, when they were used immediately for experiments. The characteristics of filtered lake water were: temperature, 10.8 ± 0.4°C; pH 7.0 ± 0.1; conductivity, 41.7 ± 2.5 μS; and oxygen content >95%. The light:dark cycle was 16 h:8 h.
2.2 Experimental design

Newly hatched (<24 h) rainbow trout embryos were carefully transferred with large-bore pipettes to 1.5 L Pyrex glass bowls containing a 1 L solution of either 0.01% DMSO (carrier solvent) or 32 µg/L of retene, a concentration known to cause clear signs of BSD but negligible mortality (< 5%) (Vehniäinen, unpublished). Each bowl contained 15 embryos, and there were 44 bowls per treatment, providing 11 bowls for each of four sampling times: before (day 1 and 3), at (day 7), and after (day 14) the onset of the signs of BSD. At each time, 11 bowls of each treatment were sampled: fish from three bowls (45 hearts) were pooled as one replicate, to give an n=3 for each of the two treatments and four sampling times, for a total of 24 samples. Hearts of the fish in the remaining two samples were pooled to form two extra replicates per treatment, and used when the quality of the RNA extracted from 45 hearts samples was insufficient (one 1d retene sample, one control sample each from days 1, 3, and 7).

All embryos were monitored for signs of BSD (pericardial and yolk sac edema, hemorrhaging, craniofacial deformities, spinal deformities, and fin rot), and BSD indices were calculated according to Villalobos et al. with the modifications of Scott et al. (Scott and Hodson, 2008; Scott, et al., 2009; Villalobos, et al., 2000). All hearts were quickly dissected under the microscope, flash frozen in liquid nitrogen, and stored at -80°C until further analyses.

2.3 Measurements of water retene concentration

The exposure regime was semi-static, with daily replacement of 60% (600 mL) of the retene exposure solution and monitoring of the condition of the embryos. To characterize the expected decline in retene concentration over time, water samples were taken on day 0 and at each sampling event (days 1, 3, 7, and 14) by pooling 300 ml of the exposure solution from three replicate bowls. These samples were taken 24 h after partial solution renewal and represented the lowest exposure concentrations. The
samples were frozen at -20 °C in glass bottles until analysis by gas chromatograph/mass spectrometer (Ratia et al., 2014).

2.4 Microarray analysis
The microarray experiment was designed and carried out according to the “Minimum Information About a Microarray Experiment” (MIAME) guidelines. The microarray platform and data from the experiment have been submitted to NCBI Gene Expression Omnibus (GEO accession number GSE38238). The microarray was an Agilent 8x60K custom array (Design ID 036352, GEO accession number GPL15607) that was designed with Agilent eArray tool (https://earray.chem.agilent.com/earray/) using TC (Tensus Consensus) and EST (Expressed Sequence Tag) sequences obtained from The Institute for Genomic Research rainbow trout database (http://www.tigr.org/tdb/tgi/rtgi). The oligonucleotides on the microarray were 60-mer, and for several genes more than one distinct oligo was included in the array.

Total RNA was extracted with TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions. The quantity of the RNA was measured, and the purity checked using the 260:280 nm optical density ratio (NanoDrop). The quality of the RNA was monitored with Agilent 2100 Bioanalyzer, and samples with Rna Integrity Number (RIN) > 7 used for analyses. After DNase treatment (DNase I Amp Grade, Invitrogen) of 1 µg of total RNA, it was split in two aliquots: 500 ng was reserved for the microarray analysis, and 500 ng for the qPCR verification of the microarray results.

An aliquot of 250 ng total DNase-treated RNA was amplified and Cy3-labeled with Agilent Low Input Quick Amp Labeling kit (one-color, product number 5190-2331). The samples were processed together with Agilent RNA Spike in kit (product number 5188-5282). The RNA and cRNA concentrations were analyzed with NanoDrop ND-2000, and quality monitored with Agilent 2100 Bioanalyzer RNA 6000 Nano kit (product number
An aliquot of 600 ng of Cy3-labeled sample was hybridized to Agilent 8x60K custom array (Design ID 036352) overnight at 65°C using the Agilent Gene Expression Hybridization kit (product number 5188-5242). Agilent Gene Expression Wash Pack (product number 5188-5327) was used for the hybridization washes according to the manufacturer’s instructions. The arrays were scanned with Agilent Technologies Scanner, model G2565CA using the scan profile AgilentG3_GX_1Color, and the numerical data was obtained with Agilent Feature Extraction program (Version 10.7.1). The grid was 036352_D_F_20110902 and the protocol GE1_107_Sep09. The labeling and hybridizations were carried out at the Finnish Microrray and Sequencing Centre.

The gene expression microarray data was analysed using the R (R Development Core Team, 2008) and Bioconductor (Gentleman et al., 2004) software. The data were quantile normalised and quality inspection was performed. Pearson’s correlation values between biological replicates were above 0.91 signifying good reproducibility. All statistical analyses were carried out using the Limma package (Smyth, 2005). Differentially expressed features between the treatments were defined as having an absolute fold-change > 2 and the false-discovery rate < 0.01. As more than one oligo were included on the microarray for some genes, the term “feature” is used here to refer to the signal from one oligo, whereas “gene” stands for annotated genes. Multiple features may thus correspond to one gene.

The differentially-regulated genes were identified with protein (BLASTx) and nucleotide (Megablast) BLAST programmes (Altschul et al., 1997,Zhang et al., 2000), and their gene ontologies (GOs) in salmonids and mammals were investigated using two databases, UniProtKB (http://www.uniprot.org/) and Gene Ontology (http://www.geneontology.org/). Additionally, the sequences constituting the microarray were run through Blast2GO with default settings (Conesa et al., 2005). The annotations were verified manually, comparing the information from all analyses. Most of the annotations that differed between analyses were the result of similar genes or
proteins having different names. For the Blast2GO results, the minimum E-value cutoff was set at $1e^{-4}$ at this point. The annotated differentially-expressed genes were grouped based on GO terms. No GO enrichment analyses were made, because approximately a third of the sequences remained unannotated after the sequence identifications.

2.5 Quantitative real-time PCR
Species- and gene-specific primers were designed using AmplifX (Version 1.5.4). The features of the primers are listed in Table S1. The elongation factor 1 alpha was chosen as the reference gene as it has been shown to have the most stable transcript levels under the experimental conditions used in our study (Vehniäinen, unpublished).

An aliquot of 500 ng of the DNase-treated RNA was reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Bio-Rad, USA) and diluted 1+9. A 25 µl qPCR reaction consisted of 5 µL of the diluted cDNA, 1.5 µL (final conc. of 300 nM) of each of the forward and reverse primer, 4.5 µL water and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad). No template controls (water instead of cDNA) were run for each gene on each plate. The qPCR was run on a CFX96 Real-Time PCR cycler (Bio-Rad). The protocol was 3 min at 95°C; 40 cycles of 10 s at 95°C, 10 s at 58°C and 30 s at 72°C; 10 s at 95°C and melt curve from 65°C to 95°C. A single melting temperature peak was confirmed in the dissociation curves.

3 Results and discussion
3.1 Retene exposure causes developmental defects but no mortality
Retene exposure produced the expected signs of toxicity but did not cause mortality. The onset of signs of BSD occurred by day 7, shown by the BSD index that was on average three times higher in retene than control exposure (Fig 1). The most common sign was fin rot. Up to 40 % of the embryos in retene treatments displayed fin rot at day 7, and at day 14 more than half of the embryos in all retene treatments had fin rot. Other typical signs at days 7 and 14 were hemorrhaging and yolk sac edema.
The developmental defects occurred despite a decline in the measured concentration of retene, from 30 µg/L at day 0 to 14, 4, 3, and 1 µg/L, on days 1, 3, 7, and 14, respectively. A decline in retene concentrations during fish embryo exposures has been observed before (Billiard et al., 1999, Scott and Hodson, 2008, Scott et al., 2009). During the first day retene was most probably adsorbed to vessel walls and accumulated by the embryos. The further decline could be explained by partial water changes, and metabolism of retene by embryos (indicated by cyp1a induction; see 3.2.3) and by natural micro-organisms present in exposure water.

3.2 Retene causes changes in cardiac transcriptome
Retene caused up- or down-regulation of 270 features, corresponding to 122 annotated genes all together (Table 1, Fig. 2, Supplementary File 1). Approximately one third of the features remained unannotated (Table 1, Supplementary File 1). Whereas the largest number of features and genes were down-regulated on day 1, declining thereafter, the numbers of up-regulated features and genes were less variable among sampling times (Tables 1 and 2).

We identified some GO groups with high numbers of genes showing altered transcription at early time points, but a substantially lower number (less than half of that at early time points) after the onset of the signs of BSD. These “early response” GOs were signal transduction, transcription, apoptosis, cell growth, and cytoskeleton (Table 2). The GOs in which the number of up- or down-regulated genes remained similar across time points were xenobiotic metabolism, lipid metabolism and transport, cell adhesion and mobility, and cardiovascular development (Table 2). The only GO group in which the number of differentially regulated genes went up by time was amino acid metabolism (Table 2).
The differences in differentially-expressed genes between early and late sampling times may arise from the cumulative toxicity of retene, rapid development of the embryo, or the changing retene exposure concentrations. Declining water concentrations and the probable metabolism of retene by the embryos may have lowered the concentration of parent retene in the heart. At longer exposure durations, the cardiac tissue of the embryos may have also been exposed to retene metabolites (Hodson et al., 2007).

It must be noted that GOs of most of the genes are based on human or mouse genes, because no GO information exists for these genes in rainbow trout, or teleosts in general. It is possible that some of the genes have a different function in fish and mammals. However, we believe the use of mammalian GOs is feasible and reliable, because the differentially-regulated genes in this study are homologous to those in mammals, and the cellular processes and signaling routes are usually highly conserved among vertebrates.

There were multiple probes for some genes. The results for different probes for the same gene were, in general, well in line with each other, confirming that the hybridization was reliable. The microarray results were further supported by the qPCR results. All genes that showed up-regulation by microarray showed up-regulation by qPCR, and the same was true for down-regulation. Though the absolute fold-change values were slightly different between methods, the direction and trends of change over time were similar. Up-regulation of genes was greatest on day 1, declining thereafter; for down-regulation, *crabp* showed the greatest response on day 3 by both methods (Table S2).

3.2.1 Signal transduction and transcription
Signal transduction and transcription were the GO groups with the largest numbers of genes affected by retene on day 1. They are groups that may have large and divergent downstream effects, and an early response to AhR agonists is typically seen in genes
belonging to these GO groups (Fletcher et al., 2005, Handley-Goldstone et al., 2005, Carney et al., 2006). Furthermore, the effect of AhR agonists on multiple signaling cascades is consistent with previous transcriptomic studies in the early life stages of zebrafish (Handley-Goldstone et al., 2005, Carney et al., 2006).

Several genes affected by retene belong to the Wnt/β-catenin signaling pathway (Table S3). Disturbance of the Wnt pathway has been observed in zebrafish exposed to TCDD. It is possible that the main factor behind the developmental cardiovascular toxicity and other adverse effects of TCDD is the down-regulated sox9 in epicardium (Mathew et al., 2008, Xiong et al., 2008, Mehta et al., 2008, Hofsteen et al., 2013).

Multiple genes involved in the nuclear factor κB (NF-κB) signaling cascade were differentially regulated in hearts of retene-exposed embryos (Table S4); the major trend seemed to be the activation of the pathway (Table S4). NF-κB promotes cell survival and plays an important role in cardiovascular growth. For example, it is activated in response to cellular stress and cytokines (Hall et al., 2006). NF-κB activation is considered cardioprotective in mammals, but prolonged activation may lead to detrimental cardiac effects (Gordon et al., 2011). Overall, alterations in the NF-κB signaling pathway may have effects on cardiovascular development and function in rainbow trout embryos.

Various transcription-related genes were up- or down-regulated in retene-exposed vs. control hearts. This group was the one that diminished most with time; from 19 genes differentially regulated on day 1 to only 5 on day 14. The ahr gene family is well represented in this group: Several ahr2s were up-regulated on day 1, ahr2a on day 3 as well, and ahr repressor (ahrr) at every time point. The result is in line with previous research showing that ahr2 and ahrr are up-regulated in fish early life stages exposed to AhR agonists (Carney et al., 2006, Goodale et al., 2013). Rainbow trout AHR2α and AHR2β and all members of Atlantic salmon AHR2 protein family are capable of binding TCDD, and may be involved in AhR signaling (Pollenz et al., 2002, Hansson and Hahn,
AhR repressor (AhRR) lowers the activated AhR signal, and also has a developmental role (Jenny et al., 2009).

### 3.2.2 Development and function of cardiovascular tissues

Several of the most affected GO groups are associated with the development and function of the cardiovascular system. In addition to the cardiovascular development GO group, genes belonging to the group cell adhesion and motility, as well as cytoskeleton, are important in vascular development. Further, genes involved in cell cycle, cell growth and proliferation, and apoptosis have significant roles in both the growth and shaping of tissues.

Many of the differentially-expressed genes in the cell adhesion and motility category were common with the cardiovascular development category (Table 2). The results did not show a clear trend towards increased or decreased angiogenesis, or cell motility (Table S5), however, altered regulation of various genes in this category may be linked to defects in vascular development, which is a typical sign of AhR-mediated toxicity (Guiney et al., 2000, Bello et al., 2004, Bugiak and Weber, 2010). The differential regulation of genes involved in tight junctions, adherens junctions, and focal adhesions suggest modifications of cell-cell and cell-substrate adhesion and communication in cardiac tissues (Table S6). In addition to vascular development, several genes essential for the early development of the heart were differentially regulated by retene (Table S7). It is unclear however, what consequences their differential regulation has later in the ontogeny, at the stages in which the embryos were in the present study.

Retene changed the mRNA abundance of numerous cell growth associated genes. Some of the genes in this category may have both positive and negative impacts on cell proliferation, and there was both up- and down-regulation both among genes that stimulate cell growth and among those that negatively regulate cell growth (Table S8). Thus the direction of change in cell growth cannot be predicted based on the results.
However, the transcriptional changes of genes associated with cell growth and proliferation may have consequences on the structure and function of tissues. TCDD causes reduction in heart size in zebrafish and rainbow trout embryos, and in zebrafish this is at least partly caused by a decrease in the number of cardiomyocytes (Antkiewicz et al., 2005, Hornung et al., 1999).

Transcriptomic studies on the effects of TCDD on cardiac tissue of the zebrafish show an early response (after 12 h of exposure, at 84 hpf) of genes that cause cell cycle arrest and that hamper cell growth and division (Carney et al., 2006, Chen et al., 2008). However, this phenomenon was not found in the present study. In zebrafish this “molecular signature” can be seen when other agents cause heart failure as well, and thus, the absence of this signal in the present study may be associated with the less severe cardiac toxicity (Chen et al., 2008).

Several genes belonging to the GO group apoptotic process were differentially-regulated by retene in the cardiac tissue of rainbow trout embryos. The overall effect of retene on apoptosis cannot be estimated based on the results, as there were pro- and anti-apoptotic genes among both the up- and down-regulated genes (Table S9). However, as ontogeny is dependent on apoptosis, changes in the abundance of apoptosis-related mRNAs may have developmental effects.

Cytoskeleton gives the cells their shape, but it also has a role in cell migration and signal transduction. Therefore, the transcriptomic changes seen in the GO group cytoskeleton strengthen the view that retene has effects on cell motility and signaling routes in the heart (Table 2). Most of the differentially-regulated genes in this GO group coded for proteins that are involved in actin organization (Table S10). In myocardium, actin has an important role as a part of the contractile machinery, and is therefore involved in the function of the heart. Furthermore, there were other differentially-regulated genes coding for proteins that have a role in cardiac function, and in calcium handling and
signaling, which may alter the function of the heart (Tables S11 and S12). The differential regulation of these genes suggests that retene has the potential to modify cardiac function.

3.2.3 Metabolism
The highest induction of all genes was observed for the members of the cytochrome P450 family 1 (Table 2). Though the highest mRNA levels were observed on day 1, the induction was sustained throughout the time course. The high up-regulation of cyp1 genes is in line with previous research (Table S14) (Carney et al., 2006, Goodale et al., 2013, Scott et al., 2011). Also other members of the AhR gene battery were up-regulated (Table S13). Induction of these well-known AhR-regulated genes shows that retene activated AhR in the hearts of developing rainbow trout embryos.

Newly hatched rainbow trout yolk sac embryos were also able to induce genes for glucuronidation (UDP glucuronosyltransferase 1) and sulfate conjugation (sulfotransferase family 1, cytosolic sulfotransferase 3) of phase I metabolites. Previous microarray studies have shown that AhR agonists induce these genes in fish embryos (Table S14). Whereas previous research has shown that retene is metabolized in trout embryos of a similar age (Hodson et al., 2007), enzyme activity measurements or metabolite analyses are still needed to confirm whether both of these conjugation processes are active, and which one is preferred by the embryos.

In addition to the gene products that have a role in phase I and II metabolism, two genes coding for phase III proteins, membrane transporters, were identified (Table 2). These proteins can transfer organic compounds (such as PAHs) and their metabolites out of cells, keeping the cellular concentrations low (Klaassen and Aleksunes, 2010).

Retene altered the transcription of several genes coding for proteins that have a role in proteolysis or protein processing (Table 2). The genes in this category code for
peptidases or proteases, protease inhibitors, or proteins involved in protein folding and stabilization or ubiquitylation (Table S15). These changes may point to enhanced turnover of (certain) proteins, and the need for chaperones assisting in correct folding of newly synthetized or damaged proteins. Genes in this category are often upregulated by AhR agonists (Hook et al., 2006, Li et al., 2013). There is proof that at least some of the changes in this group are manifested at the protein level as well: HSP/HSC70 protein is induced in salmonid embryos (whitefish) upon retene exposure (Vehniainen et al., 2003).

Retene up- or down-regulated several genes that have a role in lipid metabolism and transport (Table 2). Alteration of lipid metabolism and transport may hamper the energy metabolism of the heart, and ultimately its function, as the cardiac tissue of fishes preferentially uses lipids as the source of energy (Moyes, 1996).

Fatty acid binding protein 3 (FABP3) regulates the uptake and intracellular transport of fatty acids. It has been shown earlier that AhR agonists such as TCDD and pyrene cause down-regulation of FABP3 mRNA and protein in fishes (Zhang et al., 2013, Bain, 2002). Further, in mouse embryos, FABP3 affects growth and differentiation of myocardial cells, and fapb3 knockout in zebrafish causes cardiac developmental defects (Zhu et al., 2011, Tang et al., 2004). Thus, down-regulation of fabp3 in rainbow trout embryos is very likely to exert similar negative effects on cardiac development.

Cellular retinoic acid binding protein 1 (CRABP1) sequesters all-trans retinoic acid and regulates its metabolism. Retinoic acid is an important signaling molecule during cardiovascular development, and altered retinoid levels cause cardiovascular defects (D'Ambrosio et al., 2011). In addition to the down-regulated crabp1, there were also other transcriptomic changes in retene-exposed embryos that point to possibly altered retinoic acid metabolism: one of the most up-regulated genes codes for CYP1B1 that
may break down retinoids and may be involved in their synthesis, and FABP3, down-regulated by retene, is able to bind retinoids (Table S16)(Chambers et al., 2007).

Several genes involved in cholesterol metabolism were differentially regulated in hearts of retene-exposed embryos (Table S16). The finding is well in line with earlier studies that have shown that TCDD alters cholesterol metabolism in rat and mouse liver (Fletcher et al., 2005, Sato et al., 2008). As maintaining cholesterol homeostasis is important for proper function of cardiomyocytes, up-regulation of genes involved in cholesterol metabolism may have functional consequences in the heart (Reboulleau et al., 2012).

Amino acid metabolism was the only GO group that became larger during the exposure (Table 2). A shift in the energy metabolism from lipids to amino acids may have taken place in the hearts of retene-exposed embryos, as several of these genes are involved in catabolism of amino acids (Table S17). Multiple genes involved in tyrosine and phenylalanine metabolism were up-regulated by retene (Table S17). This may indicate a more specific change towards catabolism of tyrosine and phenylalanine. The finding is in line with the observation that TCDD decreases the concentration of phenylalanine in the hippocampus of rat (Lin et al., 2011).

3.3 Conclusions and future aspects
The transcriptomic changes suggest that retene affects multiple signaling cascades in the heart of rainbow trout yolk sac embryos, and potentially disturbs several processes related to cardiovascular development and function. The results indicate possibly disturbed Wnt and NF-κB signaling that may cause cardiovascular toxicity, potential alterations in cell adhesion and motility that may lead to vascular defects, and plausible changes in cell growth, cytoskeleton and apoptosis that may have effects on growth and shaping of the heart. The changes in genes with a role in cardiac function, calcium
signaling, and actin organization, as well as those involved in lipid and amino acid metabolism, may cause defects to cardiac function.

The changes in mRNA abundance, especially at the early sampling times, may point to initial mechanisms of toxicity causing the cardiovascular defects observed in salmonid embryos exposed to AhR-agonists. Identification of the unknown genes may reveal additional players in the process of cardiovascular toxicity caused by activated AhR. As the amount of mRNA does not necessarily correlate with the amount or activity of the protein, the transcriptomic results still need to be verified at the level of protein abundance or activity. Immunological methods (e.g. whole-mount immunofluorescence of the heart) or enzyme activity measurements could be used for this. Metabolomic profile of the embryo hearts could be analyzed to examine if the changes suggested by this study are actually taking place in cardiac metabolism.

More research is also needed to connect the transcriptomics with physiological and morphological endpoints, in particular the separate pathologies of BSD. The results in this study indicate changes in genes involved in cell-cell and cell-substrate adhesion, which may cause vascular leaking leading to hemorrhage and pericardial edema, both typical signs of BSD. Another sign of BSD is cardiovascular deformities, and the transcriptomic results suggest that retene affects the structure and function of the heart in rainbow trout embryos. Retene at a higher concentration than used in this study (nominal 100 µg/L) lowered the heart rate, reduced the size of the heart, and increased the fragility of cardiac tissue, as indicated by damage during dissection (Vehniäinen, personal observations). On-going studies on cell and tissue morphology of the hearts of retene-exposed embryos will reveal if retene has effects on the cardiac structure and ultrastructure of the heart.
The results obtained in this study form a basis for future work, where the roles of proteins or cellular processes in the cardiovascular toxicity of AhR-agonistic PAHs can be elucidated.

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Figure legends:
Figure 1. Blue sac disease (BSD) severity index in rainbow trout embryos exposed to vehicle (DMSO 0.01%) or retene (nominal concentration of 32 µg/L) for 7 and 14 days. The data are shown as means ± SD. Asterisks denote statistical differences from control (P < 0.05).

Figure 2. Heatmap displaying genes differentially expressed between treatments at any sampling point (|fold-change| > 2 and FDR < 0.01). Data show means of log2-transformed intensity triplicates for each sampling point-by-treatment combination and have been mean-centered across combinations per gene. Dendrogram groups genes with similar differences between treatments and across sampling points.
Table 1. Numbers of the features and genes up- or down-regulated by retene (nominal concentration 32 µg/L) in the cardiac tissue of rainbow trout yolk sac fry after different exposure durations. The total numbers of up- and down-regulated genes were 89 and 33, respectively. Differentially expressed features between the treatments were defined as having a \(|\text{fold-change}| > 2\) and a false-discovery rate < 0.01.

<table>
<thead>
<tr>
<th>Duration of retene exposure (d)</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
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<td>Unidentified up-regulated features</td>
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<td>Down-regulated features</td>
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<td>8</td>
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Table 2. Gene Ontology (GO) groups with the largest number of genes up-regulated (regular font) or down-regulated (bold) in the hearts of rainbow trout yolk sac fry after different retene (nominal concentration 32 µg/L) exposure durations.

<table>
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<tr>
<th>Duration of retene exposure (days)</th>
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<th>14</th>
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<td>ahr2a, ahr, cck, ccr9, cclel1, cnpy1, col1a1, csk, cxc4, gsn, hsp70, hsp90a, ncam1a, ppap2b, rassf10, rassf8, timp2, vsnl1; <em>csk</em>, <em>crabp1</em>, <em>draxin</em>, <em>hes5</em>, <em>lgi1</em>, <em>plk2</em></td>
<td>ahr, cck, ccr9, col1a1, cclel1, csk, cxc4, gem, gsn, hsp90a, ncam1a, nkx3-2, ppap2b, rassf10, virus-induced CXC chemokine, vsg1; <em>crabp1</em>, <em>cd59</em>, <em>plk2</em></td>
<td>ahr, col1a1, cclel1, csk, cxc4, gsn, ifitm2, lpar6, ppap2b, rassf10, tpp1, vsg1; <em>crabp1</em>, <em>tgfb1</em>, <em>prkcq</em></td>
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<td>ahr, col1a1; <em>crabp1</em>, <em>prkcq</em>, <em>tgfb1</em></td>
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