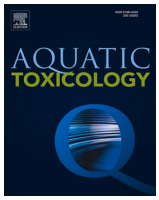
Aquatic Toxicology 244 (2022) 106083

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Exposure to retene, fluoranthene, and their binary mixture causes distinct transcriptomic and apical outcomes in rainbow trout *(Oncorhynchus mykiss)* yolk sac alevins

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ARTICLE INFO

Keywords:
Transcriptome
Mixture
Retene
Fluoranthene
PAH
Rainbow trout
Oncorhynchus mykiss
Early life development

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are widely spread environmental contaminants which affect developing organisms. It is known that improper activation of the aryl hydrocarbon receptor (AhR) by some PAHs contributes to toxicity, while other PAHs can disrupt cellular membrane function. The exact downstream mechanisms of AhR activation remain unresolved, especially with regard to cardiotoxicity. By exposing newly hatched rainbow trout alevins (*Oncorhynchus mykiss*) semi-statically to retene (32 μg l^{-1}; AhR agonist), fluoranthene (50 μg l^{-1}; weak AhR agonist and CYP1a inhibitor) and their binary mixture for 1, 3, 7 and 14 days, we aimed to uncover novel mechanisms of cardiotoxicity using a targeted microarray approach. At the end of the exposure, standard length, yolk area, blue sac disease (BSD) index and PAH body burden were measured, while the hearts were prepared for microarray analysis. Each exposure produced a unique toxicity profile. We observed that retene and the mixture, but not fluoranthene, significantly reduced growth by Day 14 compared to the control, while exposure to the mixture increased the BSD-index significantly from Day 3 onward. Body burden profiles were PAH-specific and correlated well with the exposure-specific upregulations of genes encoding for phase I and II enzymes. Exposure to the mixture over-represented pathways related to growth, amino acid and xenobiotic metabolism and oxidative stress responses. Alevins exposed to the individual PAHs displayed over-represented pathways involved in receptor signaling: retene downregulated genes with a role in G-protein signaling, while fluoranthene upregulated those involved in GABA signaling. Furthermore, exposure to retene and fluoranthene altered the expression of genes encoding for proteins involved in calcium- and potassium ion channels, which suggests affected heart structure and function. This study provides deeper understanding of the complexity of PAH toxicity and the necessity of investigating PAHs as mixtures and not as individual components.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a widespread group of environmental contaminants of either natural or anthropogenic origin. Always occurring as complex mixtures in nature, PAHs are generated in large quantities during combustion or pyrolysis of organic material and are present in petroleum products. The environmental prevalence of PAHs has increased over the last century due to increased anthropogenic activities (Wickström and Tolonen, 1987; Van Metre et al., 2000). The presence of PAHs is particularly problematic in anoxic strata as any degradation of PAHs *in situ* requires aerobic metabolism. Hence, PAH contamination can form legacy deposits that can pose long term environmental risks if remobilized or leached (Haritash and Kaushik, 2009).

Exposure to PAHs during early life development of fish is well known to result in a broad suite of defects at multiple levels of biological organization and consequently, increased mortality. Heart structure and function are especially sensitive to PAHs (Incardona et al., 2011), which has multiple down-stream consequences for the growing fish larvae as

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https://doi.org/10.1016/j.aquatox.2022.106083
Received 14 May 2021; Received in revised form 11 January 2022; Accepted 18 January 2022
Available online 21 January 2022

circulation of nutrients and gas-exchange become restricted. Reduced growth and symptoms referred to as the blue sac disease (BSD; yolk sac and pericardial edemas, craniofacial and skeletal deformities, hemorrhaging and fin rot) are established hallmarks of PAH toxicity (Billiard et al., 1999; Colavecchia et al., 2006). Other developmental defects associated with PAH exposure are genotoxicity and behavioral alterations (Rhodes et al., 2005; Geier et al., 2018).

The exact mechanisms of PAH induced toxicity remain unresolved for most PAHs, although multiple molecular processes are known to contribute to the formation of toxicity in developing fish. What is known is that different PAHs have different modes of action and toxicity potential in different organs and species (Timme-Laragy et al., 2007; Geier et al., 2018). Furthermore, the composition of a PAH mixture affects toxicity as the toxicological potency of certain PAH mixtures has been observed to induce a stronger response than the combined effect of the components (Scott and Hodson, 2008; Brown et al., 2015). However, it must be noted that certain components contribute more to toxicity than others, as exemplified by Geier et al. (2018).

The most studied molecular response in PAH exposed and developing fish is the interaction between certain PAHs and the aryl hydrocarbon receptor 2 (AhR2); a receptor which regulates normal development and metabolism (Billiard et al., 2002). Note that not all PAHs have affinity for AhR2 (Barron et al., 2004), but those PAHs that do trigger its activation as a transcription factor. Activation induces the expression of, among other genes, cytochrome P450 (*cyp1a*) by associating with xenobiotic response elements. The activated form of CYP1a functions as a phase I metabolic enzyme that facilitates metabolism and excretion of a large set of xenobiotics through hydroxylation but also activation of endogenous molecules.

The PAH retene (1-methyl-7-isopropyl phenanthrene) is commonly associated with effluents from pulp- and paper mills as well as microbial metabolism of resin acids (Leppänen and Oikari, 1999) but can also function as a biomarker for forest fires (Gabos et al., 2001). Retene is a known AhR2 agonist with high affinity (Barron et al., 2004). However, the exact mechanisms leading up to cardiotoxicity are not fully understood for retene (Bauder et al., 2005; Hodson et al., 2007), as multiple intertwined processes are likely to be involved; exemplified by the Adverse Outcome Pathway 21 (AOP21) by Doering et al., 2019. From a toxicological perspective, knockdown of *ahr2*, but not *cyp1a*, in retene exposed zebrafish embryos (*Danio rerio*) prevented the formation of cardiac defects (Scott et al., 2011). However, as AhR2 controls a vast number of genes, knockdown does not highlight any underlying mechanism. One suggested downstream gene is cyclooxygenase-2, but the evidence and quantitative understanding for this pathway are currently only moderate, and there are also other pathways that may be involved (AOP21).

Another PAH of interest is fluoranthene, which occurs ubiquitously in any natural PAH mixture (Page et al., 1999). Although a weaker AhR2 agonist than retene (Barron et al., 2004), fluoranthene can impair CYP1a mediated metabolism by blocking the enzymes active site (Willett et al., 1998). The few laboratory studies on the impact of PAH mixtures containing fluoranthene on developing fish larvae report increased toxicity of fluoranthene-containing mixtures compared to the toxicity of the individual components (Wassenberg and Di Giulio, 2004; Geier et al., 2018). Nonetheless, the underlying mechanism(s) for the stronger toxicity of PAH mixtures containing fluoranthene, relative to the sum of toxicity of the individual mixture components, remains elusive. Consequently, it is important to understand how fluoranthene influences and potentiates the toxicity of a simple PAH mixture, especially from the perspective of environmental risk assessment.

Therefore, by exposing newly hatched and developing rainbow trout alevins (*Oncorhynchus mykiss*) to sub-lethal concentrations of retene and fluoranthene (individually or as a binary mixture), mechanisms related to cardiotoxicity were investigated at the transcriptomic level which in turn were related to effects on the whole organism. A targeted microarray approach was employed as per our previous experience with transcriptomic analysis (Rigaud et al., 2020; Vehniäinen et al., 2016). Rainbow trout was selected as test organism due to its (and other salmonid species') ecological, economical and scientific relevance for sub-arctic and boreal aquatic ecosystems. Cardiac tissue was chosen as the endpoint for transcriptomic investigation due to the heart being among the most sensitive organs to the exposure with PAHs. Differently expressed genes and subsequently over-represented terms and pathways were then compared with the effects on the whole organisms (growth and development, body burden and yolk consumption, the latter functioning as a proxy for energy consumption).

2. Materials and methods

2.1. Setup, maintenance, water analysis, animal care and sampling

Newly hatched (< 24 h) and healthy rainbow trout alevins (360°-days; supplied by Hanka-Taimen OY, Hankasalmi, Finland) were randomly selected and semi-statically exposed to dimethyl sulfoxide (DMSO, control), retene, fluoranthene or the binary mixture of the two PAHs (Table 1) and sampled after 1, 3, 7 and 14 days of exposure.

Exposure concentrations were selected in order to provoke toxicological responses, while avoiding exposure-related mortality. Both Billiard et al. (1999) and Vehniäinen et al. (2016) have reported that exposure to 32 μg l^{-1} of retene fulfills the abovementioned requirements while preliminary testing identified 50 μg l^{-1} of fluoranthene as suitable (unpublished data; exposure to 5, 50 and 500 μg l^{-1} of fluoranthene resulted in 0% mortality over a 11 day period; 3 replicates per concentration and 10 alevins per replicate).

Prior to the initiation of exposure, each exposure vessel was presaturated for 24 h with the corresponding chemicals. Stock water, meant for the exposure studies, was delivered from Konnevesi research station (Central Finland) in February and April 2017; the 14 days exposure took place in February, while the 1, 3 and 7 days exposures were performed in April, using different batches of alevins. Lake water was collected from a depth of 6 m and filtered for debris. The concentration of PAHs in the collected exposure water was below the level of detection. Every exposure was conducted in 1.5 L Pyrex glass bowls filled with 1 L of lake water and the corresponding exposure compound (s) while placed in a Latin square sequence (Fig. s1). Exposure was maintained in a semi-static fashion, which meant complete renewal of water and chemicals on a daily basis. Alevins were collected using a 5 ml plastic Pasteur pipet with a broadened tip and temporarily transferred to a 50 ml plastic centrifugation tube along with some exposure water while the exposure medium was renewed; a process that took less than 30 s per replicate.

In order to generate enough cardiac tissue for RNA extraction, 12 replicate bowls per treatment, (each replicate contained 15 alevins) were maintained for exposures lasting 1, 3 and 7 days while the exposure lasting for 14 days required 8 replicates per treatment, with the same number of alevins per replicate. This translated to 180 alevins per treatment lasting 1, 3 and 7 days while the 14 days exposure required 120 alevins per treatment. An additional bowl per treatment but without alevins was maintained and treated in the same fashion as those containing developing alevins to determine loss of PAHs due to microbial degradation, evaporation or adsorption to the glass bowl. Exposure water temperature, measured daily, was maintained at 11.7 ± 0.4 °C and photoperiodicity was set at 16:8 light to darkness. Water samples were collected prior to water and chemical renewal on Days 1, 3, 7, 10 and 14, diluted 50:50 in ethanol (99.5% purity) and stored at 4 °C for later synchronous fluorescence spectroscopy. Water quality, with regards to pH, conductivity and dissolved oxygen content in aerated stock water, was measured after 1, 3, 5, 7, 10 and 14 days of exposure. The Finnish Environment Institute's database Hertta reports that water from lake Konnevesi contains 7 to 14 μg l^{-1} ammonium (as nitrogen), 0.13 to 0.19 mmol l^{-1} alkalinity and has a Ca+Mg hardness of 0.12 mmol l^{-1}.

Table 1
DMSO, retene and fluoranthene stock solution concentrations (μM; dissolved in DMSO), volume of stock solution added per exposure bowl (μL), nominal exposure concentration (nM), chemical purity (%), supplier and CAS-number. Note: 136.6 nM of retene equals 32 μg L^{-1} while 247.2 nM of fluoranthene equals 50 μg L^{-1}. The nominal concentration of DMSO corresponds to 0.002% which is below the threshold of 0.01% solvent recommended by the OECD (2013) and is thus unlikely to contribute to, or increase, the toxicity of PAHs (Christou et al., 2020; Kais et al., 2013; Maes et al., 2012).

Exposure	Stock solution concentration (μM)	Volume added (μL)	Nominal PAH + DMSO exposure concentrations (nM)	Purity (%)	Supplier	CAS Number
DMSO	Pure DMSO	20	256	≥99.9	Sigma Aldrich	67-68-5
Retene +DMSO	13,655	10 + 10	136.6 + 128	98	MP Biomedical	483-65-3
Fluoranthene + DMSO	24,720	10 + 10	247.2 + 128	≥98	Sigma Aldrich	206-44-0
Retene + Fluoranthene	As above	10 + 10	136.6 + 247.2	As above	As above	As above

At sampling, exposed alevins were photographed next to millimeter scale paper, symptoms of BSD were assessed and hearts excised. Hearts from every alevin from 2 (alevins exposed for 14 days; 30 hearts in total per sample) or 3 (exposed for 1, 3 and 7 days; 45 hearts in total per sample) exposure replicates of the same treatment were pooled, snap frozen in liquid nitrogen and stored at −80 °C for later RNA extraction. Utilizing this approach yielded 4 replicates per treatment for transcriptomic analyzes, of which 3 were processed. The remaining alevins carcasses were pooled based upon replicate, snap frozen in liquid nitrogen and stored at −80 °C for later preparation and HPLC analysis.

2.2. Synchronous fluorescence spectroscopy (SFS) and water quality

This SFS protocol has previously been described by Rigaud et al. (2020), and outlines the analytical protocol for phenanthrene, pyrene and retene. The SFS parameters, although slightly adjusted, were obtained elsewhere (Watson et al., 2004; Turcotte et al., 2011) (Table 2). In short, exposure water from 4 replicates per treatment (selected at random) was sampled after 1, 3, 7, 10 and 14 days of exposure, collected in 20 ml scintillation bottles in a 50:50 mixture with ethanol (99.5% purity) and then stored at 4 °C until analysis. The concentration of retene and fluoranthene were measured using a LS55 Luminescence Spectrometer (PerkinElmer Instruments, USA). Every LS55 measurement was performed in quartz cuvettes (Quartz SUPRASIL® High Precision Cell, Hellma Analytics, Germany) as these PAHs have a low fluorescence at investigated exposure concentrations. Standard curves were created for each PAH which were used to calculate concentration regimes, once normalized against their respective control and using the peak area for retene (290–315 nm) and fluoranthene (270–292 nm).

2.3. Morphometric analyzes

Photos were analyzed utilizing ImageJ (v1.51j8, National Institutes of Health, USA). Using the millimeter paper as a known reference, standard length and planar yolk area were measured *in silico* with high accuracy (3 decimals) and resolution (30 pixels per mm). However, temperature and other abiotic factors are known to affect fish development and due to the architecture of the exposure room and the cooling system, the temperature in the exposure bowls varied spatially (1, 3 and 7 days exposure: 99.9–101.7% relative of replicate 1; 14 days exposure: 98.5–103.6% of control replicate 1; see Fig. s1). Therefore, standard length and yolk area were compensated for the influence of temperature by adjusting for the number of degree-days for every control replicate in relation to control replicate 1, as per Eq. (1):

$$Endpoint_{x-adj.} = Endpoint_X \Big/ \frac{DD_1}{DD_x} \quad (1)$$

Where DD_1 is the number of degree days in control replicate 1 and DD_x represents the degree days in the corresponding control $bowl_x$. The measured endpoint was then divided by the degree-day quota from the same $replicate_x$, thereby adjusting the measurements for the spatial temperature variation. The unadjusted results are presented in Fig. s2.

The BSD-index was established per replicate and calculated according to established convention (Eq. (2)) (Villalobos et al., 2000; Scott et al., 2011). Symptoms of pericardial edemas (PE; scored 0 or 1; not present or present), yolk sac edema (YE; scored 0 or 1) and hemorrhages (HM; scored 0 or 1) were assessed upon sampling. This procedure gave a maximum score of 45 per replicate. Additionally, the effect of the mixture on the BSD index, relative to the components (results adjusted for baseline toxicity among DMSO exposed alevins), was assessed as per combination index (Foucquier and Guedj, 2015).

$$\mathrm{BSD} = \frac{\sum \mathrm{PE} + \sum \mathrm{HM} + \sum YE}{\text{Maximum score}} \quad (2)$$

Inconsistencies in the assessment of BSD symptoms occurred during the sampling of alevins exposed. As a consequence, only half of the replicates were included in the calculation of the BSD-indices of alevins exposed for 1, 3 and 7 days. BSD-indices among alevins sampled after 14 days of exposure were completely omitted due to inconsistent scoring.

Alevins sampled after 14 days of exposure were developmentally assessed and scored based upon pigmentation intensity of the dorsal fin (present/not present) and the lateral side low/high intensity) in accordance with Vernier's rainbow trout developmental catalog (Vernier, 1977), as exemplified in Fig. s3. Formation and development of pigmentation is influenced by AhR2 and thus sensitive to the influence of AhR-agonists (Zodrow and Tanguay, 2003).

2.4. High-Performance liquid chromatography analysis

The body burden of retene and fluoranthene was assessed using a High-Performance Liquid Chromatography (HPLC) approach. For a detailed description on material preparation, analysis and recovery assessment, see supplementary material s1.1. In short, pooled alevin carcasses (10–13 per replicate) were homogenized in 70% acetonitrile (ACN; Fisher Scientific). The homogenate was centrifuged for 15 min at 14,000 rpm in 4 °C (Centrifuge 5415 R, Eppendorf, Germany) and the supernatant collected. The pellet was resuspended in 70% CAN, centrifuged and the supernatant collected and pooled. The resuspension

Table 2
LS55 luminescence synchronous fluorescence spectroscopy parameters used for the identification of retene and fluoranthene.

PAH	Wavelengths measured (nm)	Delta wavelength (Δλ; nm)	Peak (nm)	Excitation slit (nm)	Emission slit (nm)	Scan speed (nm/min)
Retene	250–350	50	290–315	5	5	300
Fluoranthene	200–500	155	270–292	2.5	5	240

processes of the pellet were repeated twice.

An aliquot of the collected supernatant (100 µl; the remaining supernatant was stored at −20 °C) was transferred to a 250 µL glass insert (Agilent Technologies, German) placed in an amber glass vial (Agilent Technologies, Poland). Ten µl of the aliquot were analysis using a Shimadzu U-HPLC Nexera system connected to a RF-20A xs Prominence fluorescence detector (Shimadzu, Japan) with a 150 mm long ACE C18-AR column with a particle size of 5 µm (Advanced Chromatography Technologies LTD, Scotland, UK). The analytical protocol developed allowed for simultaneous measurement of both PAHs in one run, thereby enabling detection of possible PAH cross-contamination. Retene was measured at excitation 259 nm and emission 370 nm with a retention time of 16.71 ± 0.09 min while fluoranthene was measured at excitation 288 nm and emission 525 nm after 13.5 ± 0.21 min. Chromatogram area under the curve (AUC) was manually adjusted and background- (by subtracting the average AUC from DMSO exposed alevins) and recovery compensated. The concentrations were then calculated according to the standard curves and the average PAH body burden (amount) calculated per fish.

2.5. Transcriptomic analysis

RNA was extracted from the pooled fish hearts with TRI Reagent (Molecular Research Center, USA). RNA was quantified using a NanoDrop 1000 v.3.8.1 and NanoDrop 2000 (Thermo Fisher Scientific, USA) and RNA integrity number (RIN) scored using Bioanalyzer RNA 6000 Nano assay kit (Agilent Technologies) according to manufacturer's instructions; lowest measured RIN was 9.8. Extracted RNA was then divided into two aliquots (one for microarray and one for qPCR validation analysis) and stored at −80 °C.

In brief, qPCR analysis was primarily meant as microarray validation, while methodology was first described by Rigaud et al. (2020). In total, 8 different genes related to xenobiotic, energy and iron metabolism, as well as oxidative stress, were analyzed, using *ndufa8* and *rl17* as references due to their transcript stability. For details, see supplementary material s1.2.

Microarray (Agilent 4 × 44 K, Salgeno Design ID 082,522) preparation and analysis were performed at NOFIMA (Ås, Norway) according to a previously developed protocol (Krasnov et al., 2011). First, 220 ng of RNA was labeled with one-color Cy3 dye (Agilent Low Input Quick Amp Labeling Kit; product number 5190–2305), amplified, and purified using Qiagen RNeasy Mini Kit (Qiagen, Germany). Based upon NanoDrop measurement the cRNA concentration (average 266.3 ± 48.9 ng μl^{-1}) and specific Cy3 activity was calculated (average 12.9 ± 2.5 ρmol per µg cRNA). Samples were hybridized overnight at 65 °C using Agilent Gene Expression Hybridization Kit (product number 5188–5242), and the hybridized microarrays were washed using the Agilent Gene Expression Wash Buffer Kit (product number 5188–5327). The hybridized microarrays were then analyzed using the Agilent SureScan Microarray Scanner and the gene expression readings were processed, analyzed and their intensity levels established using Nofima's bioinformatics package (Krasnov et al., 2011). Average microarray intensity levels were normalized and $\log_2$-transformed compared to controls. The bioinformatics package identified differentially expressed genes relative to control using Student's t-test ($p \leq 0.05$). Microarray raw data output is available at ArrayExpress (E-MTAB-8980).

2.6. Over-representation analyzes

Over-representation analyzes (ORAs) were performed using R (v.3.5.1., R Core Team, USA); extended by the packages Bioconductor v3.7 and clusterProfiler v3.8.1 (Boyle et al., 2004; Yu et al., 2012). Gene annotation in clusterProfiler was established using the AnnotationHub database. However, in accordance with Rigaud et al. (2020), as no gene annotation database were available for rainbow trout in AnnotationHub at the time of data analysis, we used zebrafish RefSeq gene ID and symbols and then attributed these to each feature of the microarray. This was achieved using the NCBI BLAST software 2.7.1 (National Center for Biotechnology Information, Bethesda, MD, USA). From the BLAST output, we generated an ORAs background database containing 19,025 unique zebrafish gene IDs (E-values $\geq 10^{-3}$ were removed). Over-representation- and pathway analysis were performed and include Gene Ontologies terms (GO), and KEGGs pathways. P-value cut-off was set to 0.05 and adjusted for multiple comparisons using Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Once ORAs were performed, corresponding genes in relation to enrichment pathways were sorted based upon the exposure duration as to identify the specific genes involved in term- and pathways over-representations (Supplementary file 1).

The microarray profiles were validated by correlation analysis (Spearman's) with the corresponding qPCR results as well as the microarray results for retene, obtained from Rigaud et al. (2020) (Fig. s4).

2.7. Statistics

Statistical analyzes were performed in R-studio version 3.5.1. (R Core Team, USA). Gaussian distribution was tested using Shapiro-Wilk test and variance comparison was tested using one-way ANOVA with Tukey's post-hoc test (Tukey) if the data was normally distributed, otherwise, data were subjected to Kruskal–Wallis (KW) analysis of variance with Dunnett's post-hoc test (Dunn). Mortality and pigmentation were assessed using Fisher's exact test. Comparing the body burden between alevins exposed to retene and fluoranthene alone and part of the mixture was performed using t-test if the data was normally distributed and Mann-Whitney test (MW) if not. Significance- and cut-off value was set at $p \leq 0.05$. All numerical data are presented as mean ± standard deviation.

3. Results

3.1. Exposure parameters

Measured concentrations of PAHs in exposure water varied with time and treatment (Table 3). The concentrations of the two PAHs in exposure water were higher when co-exposed compared to the individual exposures. After 7 and 14 days of exposure, significantly more retene was measured in the samples from mixture exposure water than those from retene only exposures. The same pattern was observed regarding fluoranthene but only after 1 day of exposure. Water quality was within acceptable limits: conductivity 26.83 ± 4.35 mS m^{-1}; oxygen saturation 103.89 ± 5.32%; and pH 7.16 ± 0.10. The high oxygen saturation was due to constant aeration of stock water.

Table 3
Average measured (nM ± standard deviation) and average percentile of nominal concentration (within parentheses) of retene (Ret) and fluoranthene (Flu) in exposure water measured by synchronous fluorescence spectroscopy after 1, 3, 7, 10 and 14 days of exposure. For comparison, the developing alevins were exposed to 247.21 nM of fluoranthene and 136.55 nM of retene (nominally). Statistical differences between measured concentrations are denoted with ¤, * and # for fluoranthene Day 1, retene Day 7 and 14, respectively (*t*-test).

Concentration in nM, mean ± SD (% of nominal)					
	Day 1	Day 3	Day 7	Day 10	Day 14
Ret alone	35 ± 11 (26)	35 ± 11 (26)	30 ± 10 (22)*	26 ± 20 (19)	19 ± 14 (14)#
Ret mix	32 ± 7 (23)	39 ± 8 (29)	53 ± 28 (39)*	35 ± 12 (26)	47 ± 14 (35)#
Flu alone	37 ± 17 (15)¤	62 ± 32 (25)	88 ± 33 (36)	99 ± 75 (40)	113 ± 36 (46)
Flu mix	74 ± 38 (30)¤	76 ± 22 (31)	103 ± 44 (42)	108 ± 71 (44)	113 ± 42 (46)

3.2. Mortality and morphometrics

Mortality never exceeded 6% in any treatment or replicate. A significant difference was observed between alevins exposed to retene and fluoranthene individually and sampled on Day 7 (Table S2). Exposure to the binary mixture resulted in a significantly increased BSD-index on Day 3 and onward compared to control and the individual compounds on day 3 (retene) and 7 (fluoranthene), respectively (Table 4). The BSD-indices among mixture exposed alevins were, on average, greater than the combined additive effect exerted by the components following exposure for 3 and 7 days, when adjusted for baseline toxicity among DMSO exposed alevins.

Furthermore, alevins exposed to retene (for 14 days) and mixture (Days 3 and 14) were significantly shorter compared to control alevins (Fig. 1a). Fluoranthene exposed alevins were significantly shorter after 3 days of exposure compared to control. Retene-exposed alevins had significantly smaller planar yolk area than those exposed to mixture on Day 14 (Fig. 1b). Furthermore, no differences with regards to significances between the different treatments were observed between temperature adjusted and un-adjusted standard lengths (Fig. s2a) and planar yolk areas (Fig. s2b). Alevins exposed for 14 days to fluoranthene and mixture had reduced pigmentation intensity on their lateral side, and all PAH exposures caused hypopigmentation of the dorsal fin (Table 5; Fig. s3).

3.3. Body burden

Each PAH treatment produced exposure specific body burden profiles as exposure to the mixture altered the accumulation pattern of the PAHs compared to alevins exposed to the individual components. In the case of retene, the body burden was significantly increased by 353% after 1 day of exposure, which reached 364% by Day 14, compared to alevins exposed to retene alone (Fig. 2a). The body burden of fluoranthene by contrast was reduced significantly by 60% after 1 day of exposure and by Day 14, the reduction was 94% compared to alevins exposed to fluoranthene alone (Fig. 2b).

Low recovery rates of the two PAHs highlight that the absolute quantification should be considered carefully. Yet, the chromatograms yielded clear exposure specific peaks, which were 122 and 602 times greater for retene and fluoranthene, respectively, than background (control), already after 1 day of exposure.

3.4. Transcriptomic responses

Altogether, 1896 differently expressed genes (DEGs) were identified in rainbow trout heart tissue, independent of exposure duration and across all PAH treatments. Irrespective of exposure duration, exposure to fluoranthene, retene and the mixture results in 344, 937 and 615 DEGs, respectively. Few DEGs were shared among the different exposures and those that were shared were primarily between retene and mixture exposed alevins (Fig. 3a–d). The cardiac microarray results were validated by qPCR analysis as well as by comparing the shared DEGs reported for retene by Rigaud et al. (2020) with ours. In their study, rainbow trout alevins were exposed to retene under nearly identical conditions as those reported in this present study; the only difference being the additional 10 µl of DMSO added to the exposure replicate as per this study. Correlation analysis (Spearman) of the DEGs identified by microarray analysis provided a reliable approach for validation ($R^2 = 0.79$; $p < 0.0001$; Fig. s4) compared to qPCR analysis, which suffered from poorer matching ($R^2 = 0.39$, −0.05, 0.65 and 0.37 on Day 1, 3, 7 and 14, respectively; Table s3). Poorer correlation between the microarray and qPCR could stem from microarray probe sensitivity and specificity in relation to the qPCR primers but also possible degradation of the frozen material used for qPCR validation.

Of the 1896 DEGs identified by the microarray approach, only 6 DEGs were shared among the treatments; *cyp1a* being the only gene consistently upregulated by every treatment. The remaining 5 DEGs shared by all treatments were nebulette (*nebl*, downregulated, day 3), slow myosin heavy chain b (*smyhc2*, downregulated, Day 3), cholesteryl ester transfer protein (*cetp*, upregulated, Day 7), zinc finger protein (*Danio rerio* gene id: 103,910,593; downregulated by retene and upregulated by fluoranthene and mixture, Day 14) and C1q and TNF-like domains (*cbln11*, downregulated, Day 14).

Over-representation analysis revealed that exposure to fluoranthene altered the fewest number of pathways (Figs. 4, 5 and s5). Retene over-represented a vast number of GOs and KEGGs, although several were of low gene count and with overlapping functions. An interesting observation was that retene and fluoranthene over-represented different signaling pathways in a unique fashion and in opposite directions; retene over-represented G-protein signaling (downregulation) whereas fluoranthene affected GABA-signaling (upregulation). Another noteworthy effect of exposure to the individual components was that both exposures resulted in altered expression of genes encoding for components related to cardiac potassium- (*kcnq*) and calcium ion channels (*cacna*).

Exposure to the binary mixture did not over-represent any functions related to cardiac ion channels or signaling. However, the mixture over-represented a wide repertoire of terms and pathways related to metabolism of xenobiotics, amino acid metabolism and biosynthesis, oxidoreductase activity as well as growth and development (details are presented in Supplementary file 1). The most pronounced effect of exposure to the mixture was the stronger and broader upregulation of genes related to phase I and II metabolism compared to the individual PAHs: exposure to retene upregulated *cyp1a*, cytosolic sulfotransferase (*sult1*), UDP-glucuronosyltransferase (*ugtp1a1*) and carbonyl reductase (*cbr1l*) while fluoranthene only upregulated *cyp1a* and glutathione S-transferase P (*gstp*). Consequently, the mixture, but not the components, upregulated processes aimed at counteracting oxidative stress which

Table 4
Average blue sac disease index (BSD; ±SD) per treatment (control = DMSO, retene = Ret, Fluoranthene = Flu and mixture = Mix) sampled after 1, 3 and 7 days of exposure. Occurrence of BSD symptoms (pericardial and yolk sac edemas and hemorrhages) were scored during sampling (Villalobos et al., 2000; Scott et al., 2011). Assessment of BSD among alevins sampled on Day 14 are omitted due to inconsistent scoring during sampling. Significant differences between treatments are denoted with lowercase (Day 3) and uppercase letter (Day 7) ($p < 0.05$; KW + Dunn). Baseline adjusted BSD indices (average BSD index among control alevins subtracted from the average index among PAH exposed alevins) were utilized to assess the effect of the mixture relative to the components (combination index < 1; denoted + (Foucquier and Guedj, 2015)). Note, due to inconsistencies between the samplers, only half of the replicates are included in the calculation of BSD-index.

Exposure duration	BSD indices				Baseline adjustment			
	DMSO	Flu	Ret	Mix	Flu	Ret	Mix	Number of alevins
Day 1	0.03 ± 0.03	0.08 ± 0.10	0.07 ± 0.09	0.10 ± 0.06	0.05	0.04	0.07	90
Day 3	0.20 ± 0.08^{a}	0.34 ± 0.17^{ab}	0.25 ± 0.08^{a}	0.49 ± 0.09^{b}	0.14	0.05	0.29^{+}	90*
Day 7	0.23 ± 0.11^{A}	0.25 ± 0.06^{A}	0.29 ± 0.16^{AB}	0.43 ± 0.10^{B}	0.02	0.06	0.20^{+}	80–90**

*) Flu: $n = 89$.
**) Ret: $n = 80$; DMSO and Mix: $n = 87$; Flu: $n = 90$.
+) Mixture induced a BSD index greater than the additive effect of the components; combination index < 1.

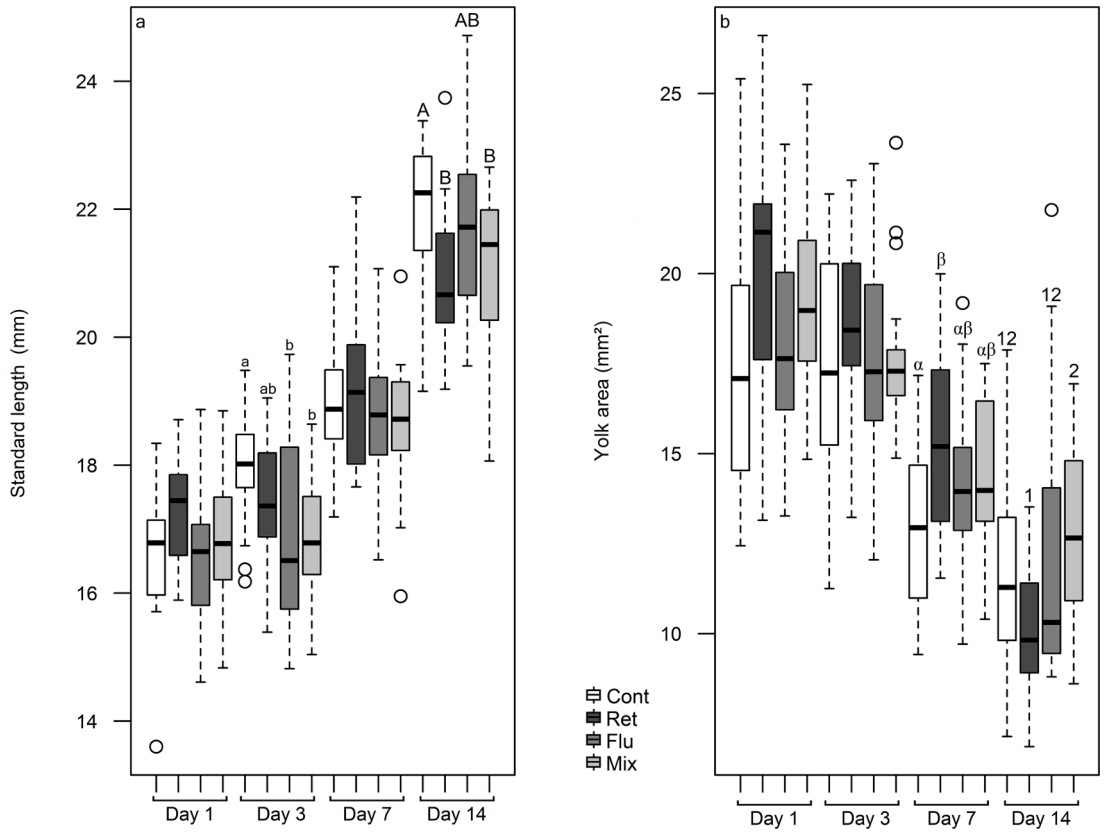

Fig. 1. Boxplot of rainbow trout alevin standard length (growth) (a) and planar yolk area (b) after 1, 3, 7 and 14 days of exposure to DMSO (Cont), retene (Ret), fluoranthene (Flu) and the binary mixture of the two PAHs (Mix). Significant differences ($p < 0.05$; depending on normality, either KW + Dunn or ANOVA + Tukey) are denoted using: (1) different lowercase Latin letters for standard length at Day 3; (2) uppercase Latin letters for standard length at Day 14; (3) Greek letters for yolk area at Day 7; and (4) numbers for yolk area at Day 14. A total of 18 alevins (3 alevins sampled from un-evenly numbered replicates) were analyzed per treatment and sampled after 1, 3 and 7 of exposure while 24 alevins (6 alevins sampled from un-evenly numbered replicates) exposed for 14 days were measured per treatment.

Table 5
Developmental observations (% ± SD) measured as pigmentation intensity of the lateral side (low - high) and the dorsal fin (yes - no) at sampling after 14 days of exposure to control (DMSO), fluoranthene (Flu), retene (Ret) and the binary mixture of the two PAHs (Mix). Significant differences are denoted using lower (lateral side) and uppercase letters (dorsal fin), as per Fisher's exact test. N per treatment = 24; 8 replicates and 3 alevins per replicate.

Tissue type	Outcome	DMSO	Flu	Ret	Mix
Pigmentation intensity of the lateral side	High (%)	83.3 ± 38.1^{a}	66.6 ± 48.2^{b}	70.8 ± 46.4^{ab}	62.5 ± 45.5^{b}
Pigmentation of the dorsal fin	Yes (%)	100^{A}	87.5 ± 33.8^{B}	41.7 ± 50.4^{C}	29.2 ± 46.4^{C}

included both antioxidative- and heat shock processes. Furthermore, exposure to the mixture also over-presented several terms related to growth and development which shifted from embryonic morphogenesis (GO:0048598) by Day 1 to formation- and development of extracellular structures (GO:0005615) and growth and development (GO:0040007 and GO:0048589) by Day 3.

4. Discussion

Exposure to retene and fluoranthene, alone or as a binary mixture, produced exposure specific toxicity profiles; both at the whole organism level and at the transcriptomic level. As expected per previous research (Billiard et al., 2008; Van Tiem and Di Giulio, 2011), the binary mixture produced a stronger toxicity response and a modulated body burden profile compared to exposure to the individual components (while retene being stronger than fluoranthene). Subsequent over-representation analysis of the cardiac transcriptome provided insights on multiple potential molecular responses that can explain the body burden profiles and contribute to the understanding on how PAH induced cardiotoxicity as well as how exposure impacts growth and development. The changes in gene expression are not, however, necessarily specific to the cardiac tissue, and may therefore suggest other types of toxicity as well.

4.1. Effect of exposure on genes involved in heart function and development

Exposure to the PAHs alone, but not the mixture, produced several DEGs that are known to be involved in maintaining heart function and development. Throughout the exposure duration, fluoranthene downregulated *kcnq5b* (Day 3; a component of the slow potassium ion channel which stabilizes the membrane potential (Jentsch, 2000)) and upregulated *cacna1* (Day 7; linked to calcium ion channel function (Grant, 2009)). Exposure to retene resulted in downregulation of 6 types of *kcnq* genes and 4 types of *cacna* genes by Day 14. These alterations suggest a delayed disruption of the action potential and heart function compared to fluoranthene. It is known that PAHs interact with cardiac ion channels which results in alterations of the action potential in cardiomyocytes (Incardona et al., 2011). Ventricular cardiomyocytes (from juvenile rainbow trout) exposed to retene, *in vitro*, resulted in shorter and altered action potential duration while exposure to phenanthrene had less impact (Vehniäinen et al., 2019). It is possible that altered expression pattern of *cacna*- and *kcnq* genes are compensatory mechanisms as to maintain normal heart functions. This assumption is supported by previous research where mice were administrated with the drug ivabradine

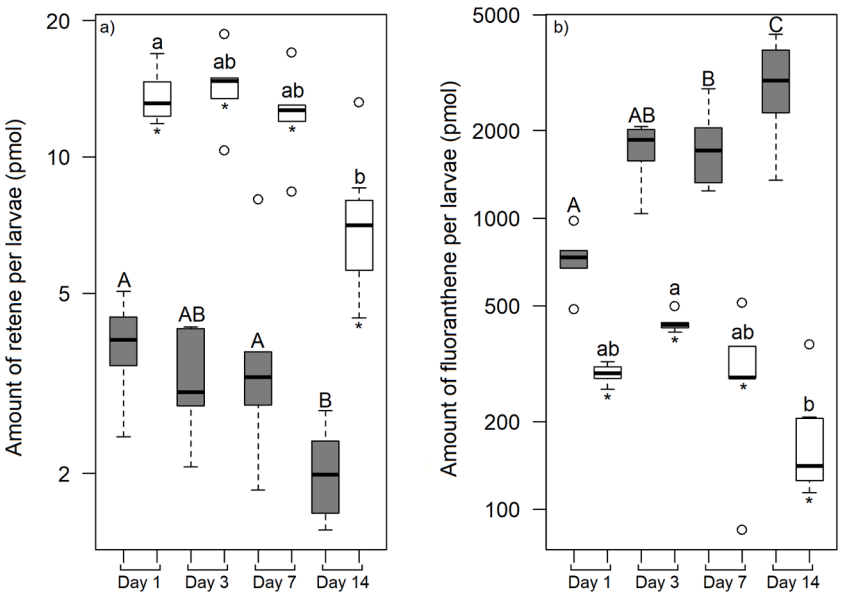

Fig. 2. Boxplot of retene (a; Ret; pMol fish^{-1}) and fluoranthene (b; Flu; pMol fish^{-1}) body burden in whole body rainbow trout alevin carcasses (background compensated) exposed for 1, 3, 7 and 14 days to the PAHs individually (filled boxes) or the binary mixture of the two (Mix, unfilled boxes). Significant differences ($p < 0.05$) in the body burden of retene and fluoranthene among mixture exposed alevins are denoted with lowercase letters, while uppercase letters denote significant differences in body burden among alevins exposed to the individual PAHs (Fluoranthene alone and in mixture: KW + Dunn; Retene alone and in Mix: ANOVA + Tukey). Significant differences in PAH body burden between single and mixture exposed alevins are denoted with a * underneath mixture box plots (Ret: *t*-test; Flu: MW). Note, alevins were exposed to 136.56 nM of retene and 247.21 nM of fluoranthene.

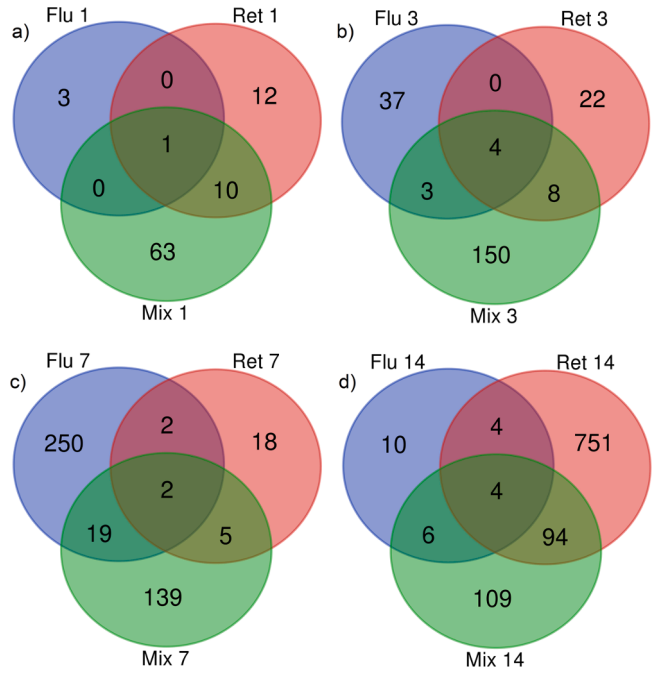

Fig. 3. Venn-diagrams representing the number of differently expressed cardiac genes affected by exposure to retene (Ret; red), fluoranthene (Flu; blue) and the mixture of the two PAHs (Mix; green) following 1 (a), 3 (b), 7 (c) and 14 (d) days of exposure. The Venn-diagram was created using the online platform provided by Bioinformatics & Evolutionary Genomics.

(reduces the heart rate by affecting the cardiac pacemaker cells (Bois et al., 1996)), which resulted in altered expression of a number of genes encoding for ion channels in the sinoatrial node of mouse heart and to a lesser extent, the ventricle (Leoni et al., 2006). In contrast to the individual PAHs, exposure to the mixture, independent of exposure duration, did not over-represent or alter the expression of any gene related to cardiac ion channels. However, it cannot be ruled out that exposure to the mixture affected heart function through other mechanisms without giving rise to a transcriptomic response(s).

Similar cardiac transcriptomic alterations have previously been reported in fish exposed to retene or fluoranthene. Jayasundara et al. (2014) exposed newly hatched zebrafish to a combination of benzo[*a*]pyrene and fluoranthene (albeit at much higher concentrations than used in this study) and identified a number of exposure-specific changes in the cardiac over-representation profiles and DEGs related to calcium ion homeostasis, embryonic development and cardiovascular system development and function. Both Vehniäinen et al. (2016) and Rigaud et al. (2020) exposed newly hatched rainbow trout alevins to retene (using the same setup as presented in this study) and assessed the cardiac transcriptome (the results reported by the latter correlated well with those presented here). Among the numerous over-representations related to cardiac function and development, the former reported over-representation of signaling transduction, growth, and cardiovascular development, while the latter reported over-representation of calcium, sodium, and potassium ion channel function, homeostasis and muscle contraction. Therefore, their results, when considered along those presented here, provide ample support to the notion that PAHs affect heart function through multiple pathways and that there is a transcriptomic component involved in the development of PAH induced cardiotoxicity. However, it is unknown if these DEGs are compensatory with regards to exposure or a direct effect of exposure, nor if they are specific to the heart only or differently expressed in a similar fashion in other tissues.

A striking difference in gene expression and over-representation was found in alevins exposed to fluoranthene or retene alone. Both exposures over-represented several terms and pathways related to cellular- and transmembrane signaling (high gene count and significance), including signaling receptor activity (GO:0038023) and neuroactive ligand receptor interactions (dre04080). The direction of expression of the involved DEGs constituting these terms and pathways were both exposure and signaling pathway specific, as exposure to fluoranthene (Day 7) resulted in the upregulation of involved DEGs involved in GABA receptor complex signaling (GO:1902710), while exposure to retene resulted in downregulation of G protein-coupled receptor signaling (GO:0007186; Day 14). These exposure specific impacts highlight potential exposure specific signaling disruption. Over-representation of GABA receptor complex and upregulation of the involved DEGs, following exposure to fluoranthene, suggests an increase in GABA receptor activation and consequently less parasympathetic influence on blood pressure and heart function through the vagus nerve (Leite et al., 2009; Bentzen and Grunnet, 2011). By contrast, prolonged saturation and activation of G-protein receptors can lead to downregulation of genes encoding for the receptors (Tsao and von Zastrow, 2000). Why

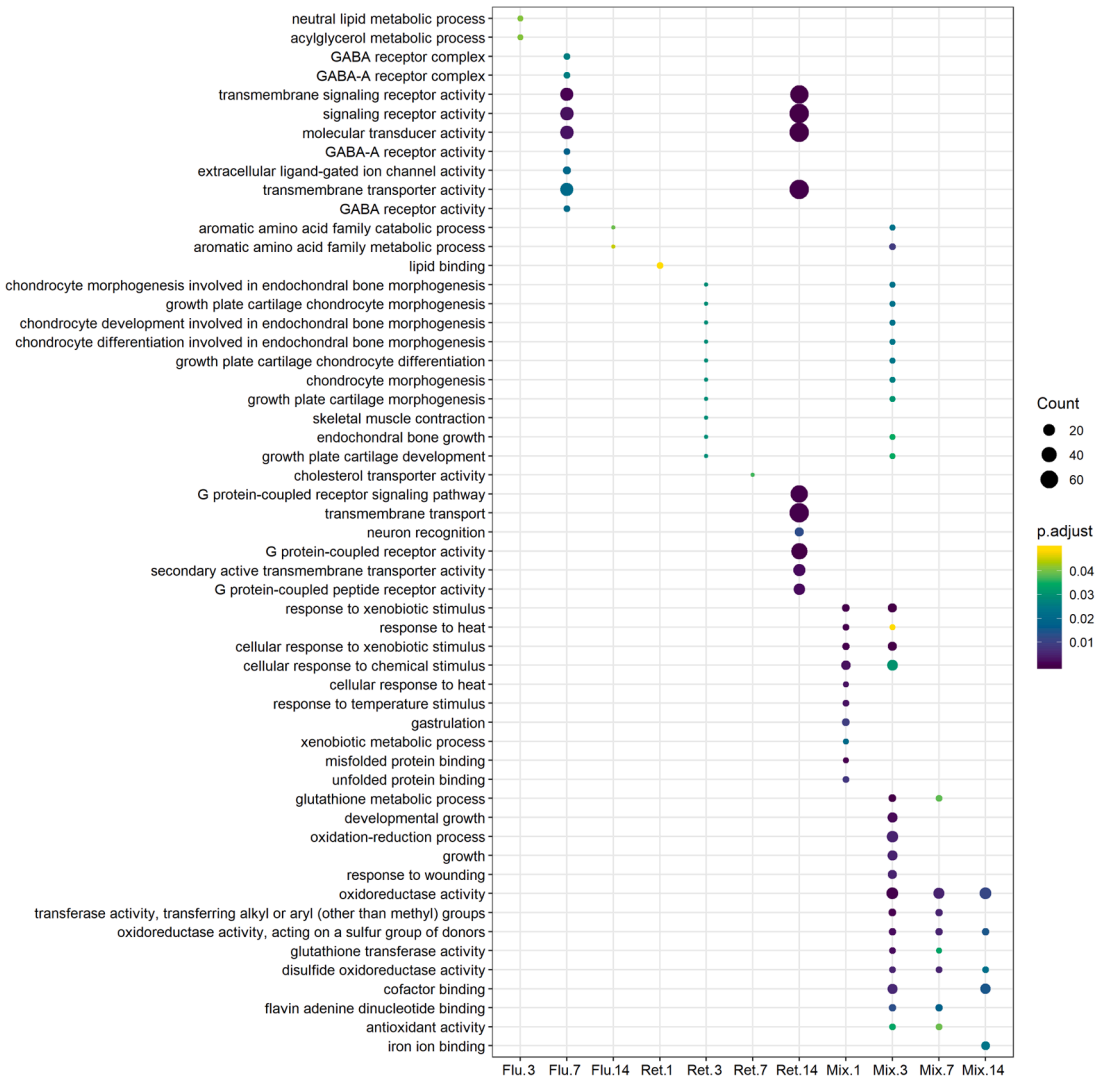

Fig. 4. Over-representation analysis of gene ontology (GO) groups following exposure to retene (Ret), fluoranthene (Flu) and the binary mixture (Mix) lasting for 1, 3, 7 and 14 days. Data was analyzed, p-value adjusted and plotted using clusterProfiler. The size of each dot equals the number of genes involved in each GO-term. Note: due to figure size limitations, Fig. 3 contains 54 out a total of 135 over-represented GO-term (whole data is presented in Fig. s5).

exposure to retene and fluoranthene over-represented signaling pathways so specifically is currently unknown but requires further investigation. However, it may be that the specificity is linked to how the PAHs interact with cardiomyocyte ion-channels and subsequently alter the repolarization of cardiac action potential, thus making these over-representations compensatory rather than a direct consequence of exposure. Yet, it is unknown if this phenomenon is heart-specific or if it could take place in other tissues as well. Additionally, the fact that these two over-representations only occurred among alevins exposed for 7 and 14 days supports the view that certain endpoints require extended exposure duration (or have to take place at a specific developmental stage) before the compensatory events become manifested in the developing organism (Price and Mager, 2020).

4.2. Body burden and xenobiotic metabolism

Unique and exposure specific body burden profiles were obtained already after 1 day of exposure and throughout the exposure duration, suggesting exposure specific phase I and II metabolic profiles. The body burden of retene was significantly reduced by Day 14, compared to Day 1, following exposure to retene alone or as a mixture (albeit significantly more abundant in mixture exposed alevins). By contrast, exposure to fluoranthene alone resulted in a significantly increased body burden by Day 7 and onward, which can be attributed to partial inhibition of the catalytic function of CYP1a by fluoranthene, alongside activation of a narrow suite of phase II metabolic processes. When co-administered with retene, the body burden of fluoranthene increased non-significantly from Day 1 to Day 3 before decreasing significantly with time. Similar changes in the body burden profiles of PAHs have previously been observed in rainbow trout exposed to a binary mixture of alpha-naphthoflavone (ANF; a CYP1a inhibitor) and retene (Hodson et al., 2007). Their specific binary mixture resulted in increased body burden of retene with increased dose of ANF. Changes in the body burden profiles of specific PAHs do not seem to be species specific as per previous observations in zebrafish exposed to a complex mixture of PAHs and a binary mixture of the AhR2 agonist beta-naphthoflavone and ANF (Timme-Laragy et al., 2007; Geier et al., 2018). Hence, the body burden of PAHs is both PAH and mixture specific while the underlying molecular processes resulting in the subsequent body burden profiles are related to accumulation in relation to metabolism. However, due to low recovery rates of the PAHs, the quantification of body burden should be considered carefully. Nonetheless, the low recovery rates appear justifiable in light of the similar recovery rates of retene (14.3%) and fluoranthene (14.6%) as well as the ratio between the subsequent

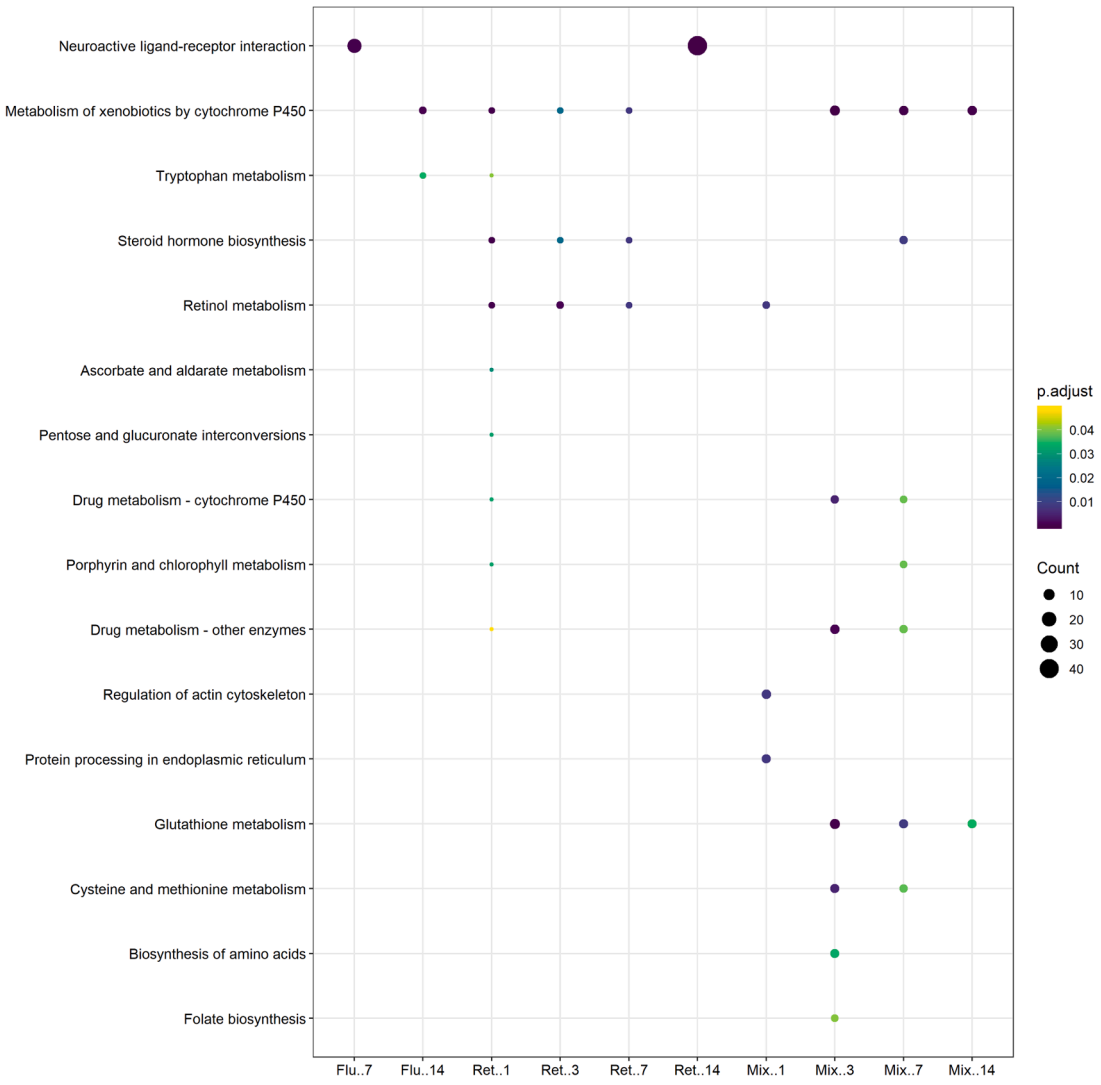

Fig. 5. Over-representation analysis of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways following exposure to retene (Ret), fluoranthene (Flu) and the binary mixture (Mix) lasting for 1, 3, 7 and 14 days. Data was analyzed, p-value adjusted and plotted using clusterProfiler. Dot size equals the number of genes involved in each KEGG-term.

body burden profiles (alevins exposed to the individual PAH compared to the mixture) and the exposure specific temporal accumulation patterns.

Possible molecular explanations to the exposure specific body burden profiles were present in the cardiac transcriptome. Through over-representation analysis, we found that the GO-term: cellular response to chemical stimulus (GO:0070887; mixture); and the KEGG: metabolism of xenobiotics by cytochrome P450 (dre00980; retene and mixture) best explained the body burden profiles. It must be noted that several uncertainties are involved when drawing conclusions on metabolism-related functions based upon a cardiac transcriptome, primarily that heart tissue is less metabolically active compared to the liver. Secondly, a differently expressed gene does not necessarily result in an equally enriched protein. However, the genes involved in above-mentioned GO-terms and KEGG pathways were upregulated in an exposure specific pattern, which suggests a strong relationship between the body burden profiles and expression of genes encoding for metabolic enzymes.

Fish exposed to certain PAHs are known to induce *cyp1a* (Nebert and Gonzalez, 1987; Shankar et al., 2019). Exposure to retene resulted in upregulation of *cyp1a* alongside cytosolic sulfotransferase (*sult1*), carbonyl reductase (*cbr1l*) and UDP-glucuronosyltransferase (*ugt1a1*), which is in accordance with the metabolism of retene as described by Huang et al. (2017). Exposure to fluoranthene over-represented no metabolism-related pathways but upregulated two genes related to xenobiotic metabolism: *cyp1a* (phase I) and glutathione s-transferase (*gst*; phase II), indicating AhR-activation and metabolism through oxidation, and conjugation by gst, which suggests that electrophiles are formed during phase I metabolism. However, as the body burden of fluoranthene constantly increased throughout the exposure period, it can be concluded that the capacity of the activated metabolic processes is rather low. These findings comply with the ability of fluoranthene to induce *cyp1a*-expression through AhR-activation alongside inhibition of the catalytic function of the corresponding enzyme, which together with a lower rate of metabolism would force fluoranthene to accumulate with time, as observed previously by Willet et al. (1998). Co-exposure upregulated abovementioned phase I and II metabolic genes plus a wider battery of genes involved in the metabolism of xenobiotics. This broader activation of phase I and II genes facilitated metabolism of both retene and fluoranthene as per the altered body burden profiles compared to those yielded from alevins exposed to the individual components.

An interesting aspect of *cyp1a* is how the expression varied with exposure and over time. Following exposure to retene and the mixture, the expression of *cyp1a* decreased with time and reached similar

expression levels by Day 14. In the case of retene the expression (log_2 fold change) decreased from 3.91 by Day 1 to 1.35 by Day 14, while the expression in mixture exposed alevins decreased from 5.35 to 1.30 over the same exposure duration. These trends of *cyp1a* expression correspond well with the decreasing body burden of retene with time (and fluoranthene when co-administrated with retene), yet it is plausible that the shift in metabolic capability is related to a more developed liver. Such correlation pattern was not present in alevins exposed to fluoranthene alone. Among those alevins, the expression of *cyp1a* remained stable over time, from an upregulation of 2.65 by Day 1 to 2.68 by Day 14, while the body burden of fluoranthene constantly increased. It is unclear if the hepatic expression of *cyp1a* would follow similar trends or increase throughout the exposure duration; an aspect that should be investigated further.

Activation of xenobiotic metabolism is associated with increased oxidative stress due to the formation of radical intermediates (Halliwell and Gutteridge, 1985). Exposure to the mixture (Day 3 and onward) resulted in the over-representation of the GO-term oxidoreductase activity (GO:0016491). In addition to metabolism-related genes, this specific GO-term highlighted DEGs encoding for proteins involved in oxidative stress response (*gsr, gsto1, nqo1, prdx1, txn*, and *txnrd3*). Said over-representation and upregulations suggest that exposure to the mixture resulted in increased oxidative stress. The underlying cause(s) can plausibly be linked to the metabolism of the xenobiotics, partial CYP1a-inhibition, accumulation of parental and metabolized PAHs and the formation of endogenous metabolites (Rannug and Rannug, 2018). In addition to these anti-oxidative responses, there is transcriptomic evidence that exposure to the mixture caused cellular stress as per increased expression of genes encoding for heat shock proteins: *hsp90A1,* (also among retene exposed alevins), *hsp70L* and *-A8*. Upregulation of *hsp*-genes and enrichment of the corresponding proteins are linked to increased oxidative stress and protein damage (Sanders, 1993). Similar upregulation of *hsp*-genes and proteins have been reported in different species of fish exposed to retene (Räsänen et al., 2012; Vehniäinen et al., 2016) and other PAHs (Song et al., 2019). Combined, upregulation of the abovementioned genes suggests that retene alone or combined with fluoranthene potentially increases cellular stress and protein damage during the first few days of exposure, before the liver reached a certain level of maturation.

4.3. Growth and development

Reduction of standard length was not consistent over time as significant impacts were observed after 3 days of exposure (fluoranthene and mixture exposed alevins relative to control), but not after 7 days. Interestingly, exposure to the mixture for 3 and 7 days, but not the components, resulted in a significantly increased BSD-index compared to control, which were also greater than additive effect of the components (adjusted for baseline toxicity of control). However, a greater BSD-index among mixture exposed alevins was expected and consistent with previous observations on PAH exposed and developing fish (Geier et al., 2018). By Day 14, alevins exposed to retene and the binary mixture were significantly shorter than control alevins, while retene exposed alevins had consumed significantly more yolk than alevins exposed to the mixture. This combination of reduced standard length but differently sized planar yolk areas highlight that growth and development were impacted in an exposure-specific fashion. These differences are plausibly related to the development and formation of sub-intestinal venous plexus which, with time, encases the yolk sac (Goi and Childs, 2016). Furthermore, based upon patterns and intensity of pigmentation, alevins exposed to any of the PAH treatments appeared to be at an earlier developmental stage by Day 14 compared to control, irrespective of how the exposure affected standard length and yolk consumption. This could have implications for the toxicological and transcriptional assessment; are the exposed alevins just shorter or at an earlier developmental stage. What caused hypopigmentation is unknown. However, considering that hyperpigmentation has been observed in zebrafish larvae exposed to the dioxin TCDD (Zodrow and Tanguay, 2003), it is plausible that the hypopigmentation observed in our study equates to an earlier developmental stage.

Central to maintaining normal development post hatching in fish larvae is the consumption of yolk, which is the sole source of energy during early life development before the larvae can actively feed. Impaired absorption of yolk would therefore affect the whole developing organism negatively (Laurel et al., 2019). Additional stress is exerted upon the developing organism due to increased energy requirements set by constantly maintained phase I and II metabolic activity, while at the same time counteracting increased oxidative stress. Over-representation analysis identified several impacted pathways that are involved in growth and development in mixture exposed alevins, but not following exposure to the individual components. After 1 day of exposure, embryonic morphogenesis (GO:0048598) was over-represented which shifted to developmental growth (GO:0048589), growth (GO:0040007) and extracellular region, parts and space (GO:0005615; GO:0005576; GO:0044421) by Day 3. These findings imply potential defects in cardiac development and growth, which could affect cardiac function and thereby the growth of the alevins. However, inferring that the identified alterations in the cardiac transcriptome serves as the principal origin for the impact on growth and development of the organism has several limitations: primarily due to the organ specificity, but also due to the fact that cross-communication between several organs and tissues, often along endocrine axes, is required to facilitate growth and development (Dickhoff et al., 1997).

Impaired heart function may restrict the circulation of nutrients and energy, which in turn could force amino acids catabolism as an alternative energy source. Restriction(s) in the availability of nutrients are suggested by upregulation of tyrosine aminotransferase (*tat*; mixture) and fumarylacetoacetate hydrolase (*fah*; mixture and fluoranthene). Upregulation of these genes indicate increased transformation rate of phenylalanine to tyrosine by Tat, which in turn is converted to fumarate or pyruvate by Fah. The latter two compounds are components that can fuel the citric acid cycle and thus provide energy. It is possible that impaired and decreased absorption of yolk, reduced availability of nutrients, and energy being reallocated away from growth to fuel metabolism could be part of the underlying rationale on why exposure affected development and growth.

5.1. Conclusive remarks

The cardiac transcriptome of rainbow trout alevins exposed jointly to retene and fluoranthene had few overlaps compared with alevins exposed to the individual compounds. This was especially evident in the expression of genes related to metabolism of xenobiotics (phase I and II), which in turn corresponded with, and explained the exposure-specific body burden profiles. The reduced standard length and slower development among retene and mixture exposed alevins could, partly, be caused by common mechanisms such as reallocation of energy to fuel xenobiotic metabolism and counteract increased oxidative stress. All in all, the combination of retene and fluoranthene increased the overall toxicity when compared to the individual components at several levels of biological organization, highlighting the fact that the toxicity of the mixture cannot be assessed from its individual components alone.

Ethical approval

Under the European Union Directive 2010/63/EU, Chapter 1, Article 1, point 3: no ethical approval is required for *in vivo* experiments on non-human vertebrates who fulfills their nutritional requirements through the consumption of yolk.

Funding sources

Academy of Finland project number: 285296, 294066 and 319284 granted to Eeva-Riikka Vehniäinen.

CRediT authorship contribution statement

Andreas N.M. Eriksson: Visualization, Writing – review & editing, Writing – original draft, Data curation, Resources, Investigation, Formal analysis, Conceptualization, Methodology, Validation. **Cyril Rigaud:** Writing – review & editing, Data curation, Resources, Investigation, Formal analysis, Conceptualization, Methodology, Validation. **Aleksei Krasnov:** Writing – review & editing, Data curation, Resources, Validation. **Emma Wincent:** Writing – review & editing, Methodology, Validation. **Eeva-Riikka Vehniäinen:** Supervision, Project administration, Funding acquisition, Writing – review & editing, Data curation, Resources, Validation.

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.

Acknowledgment

We acknowledge the contribution of laboratory technicians Mervi Koistinen, Emma Pajunen, Hannu Pakkanen and the laboratory personnel at Konnevesi research station for technical support as well as the master student Terhi Rahkonen for assessing fluoranthene lethality. We also like to thank Hanka-Taimen OY fish farm for supplying us with rainbow trout alevins for scientific purposes and research. Finally, we thank the anonymous peer reviewers who provided valuable feedback.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106083.

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