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Title: Salmo trutta is more sensitive than Oncorhynchus mykiss to early-life stage exposure to retene

Year: 2022

Version: Published version

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Please cite the original version:

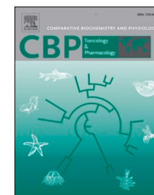
Rigaud, C., Härme, J., & Vehniäinen, E.-R. (2022). Salmo trutta is more sensitive than Oncorhynchus mykiss to early-life stage exposure to retene. Comparative Biochemistry and Physiology C: Toxicology and Pharmacology, 252, Article 109219. https://doi.org/10.1016/j.cbpc.2021.109219

Comparative Biochemistry and Physiology, Part C 252 (2022) 109219

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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



Research article

Salmo trutta is more sensitive than *Oncorhynchus mykiss* to early-life stage exposure to retene

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ARTICLE INFO

Edited by Martin Grosell

Keywords:
PAHs
Retene
Salmonids
CYP1A
Embryotoxicity
AhR

ABSTRACT

Salmonids are known to be among the most sensitive fish to dioxin-like compounds (DLCs), but very little is known about the sensitivity of the brown trout (*Salmo trutta*), which has declined and is endangered in several countries of Europe and Western Asia. We investigated the sensitivity of brown trout larvae to a widespread dioxin-like PAH, retene (3.2 to 320 $\mu g.L^{-1}$), compared to the larvae of a salmonid commonly used in toxicology studies, the rainbow trout (*Oncorhynchus mykiss*). Mortality, growth, *cyp1a* induction and the occurrence of deformities were measured after 15 days of exposure. Brown trout larvae showed a significantly higher mortality at 320 $\mu g.L^{-1}$ compared to rainbow trout larvae. While the occurrence of deformities was only significantly increased at 320 $\mu g.L^{-1}$ for the rainbow trout, brown trout larvae displayed pericardial edemas and hemorrhages already at 10 or 100 $\mu g.L^{-1}$. *cyp1a* induction was increased significantly already at $\geq$3.2 $\mu g.L^{-1}$ for the brown trout, versus $\geq$32 $\mu g.L^{-1}$ for the rainbow trout. Least square regression analysis of the concentration-response relationships suggested that *S. trutta* larvae were at least 2 times more sensitive than *O. mykiss* larvae for *cyp1a* induction. The present study suggests that *S. trutta* larvae are more sensitive than *O. mykiss* larvae to a potent DLC, retene. As it is possible that *S. trutta* populations have declined partly because of pollution by DLCs, we recommend generating more data regarding the sensitivity of threatened fish populations, in order to ensure better risk assessment.

1. Introduction

Dioxin-like compounds (DLCs) are part of a large group of organic pollutants which all have in common to interact with the aryl hydrocarbon receptor (AhR). They include, among others, polychlorinated dibenzo-*p*-dioxins (PCDDs), -furans (PCDFs), non-ortho-substituted polychlorinated biphenyls (PCBs) as well as some polycyclic aromatic hydrocarbons (PAHs). The AhR is a transcription factor, which after activation by DLCs translocates to the nucleus and alters the transcription of numerous genes. Fish are especially sensitive to DLCs: symptoms of DLCs toxicity have been described in various species and include the induction of cytochrome P4501A (CYP1A) detoxification enzymes, developmental arrest, cardiovascular defects, craniofacial deformities, pericardial and yolk sac edemas and hemorrhages (Belair et al., 2001; Henry et al., 1997; Teraoka et al., 2002). These are part of the so-called blue sac disease (BSD) syndrome, which can lead to death when severe enough.

Among AhR agonists, retene (7-isopropyl-1-methylphenantrene) is of particular interest, as it exhibits AhR-dependent toxicity in fish similar to highly potent DLCs (Billiard et al., 1999; Scott et al., 2011). Retene is an alkylated three-ring PAH originating from forest fires as well as from combustion of domestic wood, coal and solid fuel materials (Shen et al., 2012). It has also been indirectly released into the aquatic environment by the pulp and paper industry, where it forms in the sediment following anaerobic degradation of resin acids by microbial communities (Lahdelma and Oikari, 2005). Some interspecies differences among fish in retene developmental toxicity were observed in the past, but they are still largely unexplained (Scott, 2009).

Salmonids are known to be much more sensitive to DLCs embryotoxicity compared to other fish species (Elonen et al., 1998). The rainbow trout (*Oncorhynchus mykiss*) has been a model fish species in toxicology for several decades, including in studies related to PAHs or DLCs. The embryotoxicity of retene to *O. mykiss* has been studied and described previously (Billiard et al., 1999; Brinkworth et al., 2003; Scott, 2009). The brown trout (*Salmo trutta*) is the most widely distributed freshwater fish species native to the Palearctic region (Bernatchez, 2001). Its stocks are known to have declined in the recent past, and the species is still endangered in several countries (Borsuk et al., 2006;

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https://doi.org/10.1016/j.cbpc.2021.109219
Received 17 September 2021; Received in revised form 11 October 2021; Accepted 20 October 2021
Available online 29 October 2021
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Hyvärinen et al., 2019; Martínez-Páramo et al., 2009; Niksirat and Abdoli, 2009). Several studies linked to AhR-mediated toxicity and involving *S. trutta* have been done in the past, in a context of natural resources management and monitoring, as well as pollution risk assessment (Havelkova et al., 2008; Hylland et al., 2006; Ishaq et al., 1999). To our knowledge, however, the embryotoxicity of DLCs to the brown trout has never been described in detail or compared to other species before in the literature.

In fact, most of the literature linked to salmonids was produced in the early 1990s in North America and with species native to that continent (the rainbow trout and the lake trout, *Salvelinus namaycush*) (Walker and Peterson, 1991; Zabel et al., 1995), in the context of declining fish populations (Cook et al., 2003). Most of that salmonid literature was focused on embryolethality studies following exposure to legacy DLCs such as PCDDs, PCDFs and non-ortho-substituted PCBs. These studies were largely used during the development of the TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) toxic equivalents concentration (TCDD-TEQs) concept, which allows to predict the toxicity of DLCs alone or in mixture (Van den Berg et al., 1998). This concept, however, has been challenged several times as significant interspecies differences have been observed in both DLCs relative potencies and relative sensitivities (Eisner et al., 2016; Elonen et al., 1998; Rigaud et al., 2013; Rigaud et al., 2014). First attempts of developing such a toxic equivalent factor (TEF) scheme for PAHs were mostly focused on their carcinogenic effects in mammals (Nisbet and LaGoy, 1992). More recently, studies have focused on developing TEFs for PAHs specifically for fish, but without taking into consideration possible differences between fish species (Barron et al., 2004; Billiard et al., 2004; Billiard et al., 2002). Very few studies have compared the toxicity of PAHs between different fish species. Jung et al. (2015) found that *Lateolabrax maculates* and *Paralichthys olivaceus* embryos exhibited different sensitivities and bioaccumulation patterns when exposed to heavy crude oil, a complex mixture of PAHs. In another study, zebrafish (*Danio rerio*) and rainbow trout (*O. mykiss*) embryos were both exposed to retene: although the authors did not perform an in-depth comparison between the two species, the latter appeared to be more sensitive to this compound (Billiard et al., 1999).

To help to fill this knowledge gap, we aim to provide new data on the relative sensitivity to retene of two widespread salmonid species, the rainbow trout (*O. mykiss*) and the brown trout (*S. trutta*), following early development exposure. Newly hatched larvae of both species were exposed for 15 days to similar concentrations of retene ranging from 3.2 to 320 μg.L^{-1}. The relative sensitivity of those two salmonids to retene was assessed based on several endpoints: mortality, growth, BSD symptoms as well as the concentration-response relationship for the *cyp1a* gene induction.

2. Materials and methods

2.1. Experimental design

Retene (≥98% purity) was obtained from MP Biomedicals (Illkirch, France). Dimethyl sulfoxide (DMSO, anhydrous, ≥99.9% purity) was from Sigma-Aldrich (St-Louis, MO, USA). A stock solution of retene in DMSO was prepared at a concentration of 32 mg.mL^{-1} and was used to prepare a serial dilution of the following stock solutions concentrations: 0.32, 1.0, 3.2 and 10 mg.mL^{-1}.

Pyrex glass bowls (1.5 L capacity) filled with 1 L of filtered lake water (Lake Konnevesi, Konnevesi, Finland) were used as exposure tanks. Eighteen tanks were used for each species and were divided between 6 different treatments (3 tanks per treatment and per species): 0, 3.2, 10, 32, 100 and 320 μg.L^{-1} of retene into lake water. These exposure concentrations were reached by adding 10 μL of either pure DMSO (for the control group, 0 μg.L^{-1}) or of the stock solution and serial dilutions of retene listed previously. The amount of DMSO (10 μL in 1 L of lake water, or 0.001%) was the same in all tanks and was already shown to be harmless to rainbow trout ELS in previous studies (Rigaud et al., 2020; Vehniäinen et al., 2016). Three additional tanks were set without any fish and were filled with the exposure lake water containing 3.2, 32 or 320 μg.L^{-1} of retene. All tanks were prepared 24 h prior to the start of the exposure to ensure that the glass walls of the Pyrex bowls were saturated with retene. The water in each tank was aerated at all times. Water temperature was measured daily and parameters such as pH, oxygen content and conductivity were monitored on a regular basis. Both species were kept in the same temperature-controlled room. The water temperature in the exposure tanks was equal to 10.23 ± 0.26 °C and 10.18 ± 0.22 °C for *O. mykiss* and *S. trutta*, respectively.

Rainbow and brown trout embryos were obtained from a local fish farm (Hanka-Taimen, Hankasalmi, Finland). Newly hatched larvae showing no apparent deformities were randomly distributed into the tanks. Larvae were exposed to retene shortly after hatch, as it has been shown previously that the chorion of rainbow trout embryos provides an efficient protection against retene toxicity (Brinkworth et al., 2003). Brown trout larvae at hatch are larger and heavier compared to rainbow trout (0.100 g versus 0.074 g wet weight approximately, data not shown). In order to have a comparable biomass between the two species, the brown trout tanks contained 12 larvae and those of rainbow trout 15. The exposure of both species lasted for 15 days and was semi-static: the lake water and chemicals were renewed daily for each tank. Larvae were monitored for mortality on a daily basis. The light:dark cycle was set on 16 h:8 h. In one of our previous study, the onset of BSD symptoms after exposure to retene in rainbow trout larvae was around 7 days at 32 μg.L^{-1} (Vehniäinen et al., 2016). In this study, all larvae had to be maintained at a lower temperature than in our previous experiments, since brown trout larvae are less tolerant to high temperatures compared to rainbow trout larvae. Lower temperatures were expected to delay the toxicity of retene (Honkanen et al., 2020), thus an exposure duration of 15 days was decided to ensure that BSD symptoms and mortality were recorded.

At the end of the experiment, the larvae were euthanized with buffered MS-222 (200 mg.L^{-1}, 3-aminobenzoic acid ethyl ester, Sigma-Aldrich, St-Louis, MO, USA), placed in a petri dish and photographed (Canon EOS 100D, Canon Inc., Tokyo, Japan) for morphometric analyses. Larvae length and yolk sac area were measured from the pictures by using the ImageJ software (Rueden et al., 2017). Larvae were scored for any signs of BSD symptoms (pericardial and yolk sac edemas, hemorrhages and craniofacial deformities). Finally, larvae were snap-frozen in liquid nitrogen and individually stored at −80 °C until further analyses.

2.2. Measurements of PAHs concentration in water

The measurements of retene concentrations in water were performed by synchronous fluorescence spectroscopy (SFS) using a LS-55 Fluorescence Spectrometer (PerkinElmer, Waltham, MA, USA), following a method previously described (Rigaud et al., 2020). Water samples were collected after 1, 7, 12 and 15 days of exposure, just before the daily renewal of the exposure water (except for day 15, which was the sampling day). Previous studies with retene showed that concentrations measured by SFS right after the daily renewal are close to the nominal concentrations (Honkanen et al., 2020; unpublished results). In the present study, we chose to measure before the daily renewal (or, in other words, 24 h after the previous renewal) to explore for possible differences between species in retene uptake. Sample collection was performed by pipetting 5 mL of exposure water from two randomly chosen tanks per treatment and per species, as well as from each tank without fish at the beginning of the experiment (see Section 2.1). Before storage at 4 °C, 5 mL of ethanol (99.5% purity) was added in each sample. The SFS curves of each sample were normalized against their respective control before using the peak area of retene (290–315 nm) to calculate its concentration (Rigaud et al., 2020).

2.3. RNA extraction and quantitative real-time PCR

Total RNA was extracted from up to three randomly chosen larvae per exposure tank and per species, depending on the number of larvae remaining alive at the end of the experiment (N = 2–9 per treatment). Extraction was performed with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. RNA concentration and purity (260–280 nm optical density ratio) were assessed using a NanoDrop™ device (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was measured using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions; all samples had a minimum RNA integrity number of 9. RNA samples were then treated with DNase (Thermo Fisher Scientific) and an aliquot of 1 μg was reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA) following the instructions from the manufacturers. The cDNA samples were diluted (1:10) and stored at −20 °C until further analyses.

For the quantitative real-time PCR analyses (qPCR), *rl17* and *rl2* were both used as reference genes as they showed the highest stability in transcript levels in the condition of our study (data not shown). The parameters (sequences and efficiency) for the *cyp1a* and *rl17* primers for *O. mykiss* were obtained from Rigaud et al. (2020). The same primer pairs were used for *S. trutta*, and their efficiencies were 90.2% and 100.4% for *cyp1a* and *rl17*, respectively. For *rl2*, the primers were designed using the online tool Primer3web (Untergasser et al., 2012) and its parameters were as follow: accession number NM_001165173.1, forward primer sequence TTGAGACATGCATGGCCACAGT, reverse primer sequence CGGTTCCTGCCAGCTTTACCAAT, product length 82 bp (for both species), efficiency 106.0% and 99.6% for *S. trutta* and *O. mykiss*, respectively. Each qPCR reaction was done in a final volume of 25 μL: 5 μL of the diluted cDNA, 1.5 μL of each of the forward and reverse primers (300 nM concentration), 4.5 μL of sterile water and 12.5 μL of iQ SYBR Green Supermix (Bio-Rad). No-template controls with sterile water instead of cDNA were run on each plate for each gene. 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 observed in the dissociation curves for each target gene. For each sample, the *cyp1a* gene expression (efficiency corrected) was calculated using the CFX Maestro™ software (Bio-Rad) according to the methods described by Pfaffl (2001) and Vandesompele et al. (2002).

2.4. Statistical analyses

Statistical analyses were achieved using R 3.5.1 (The R Foundation for Statistical Computing) with the significant level set at $\alpha = 0.05$. Body length, yolk sac area and *cyp1a* gene expression data were expressed as percentage relative to their respective DMSO controls (for each species) and log-transformed prior to analyses. The normal distribution of each dataset was tested with the Shapiro-Wilk test. Differences among treatments were investigated using a one-way ANOVA for normally distributed data and a non-parametric Kruskal-Wallis test (KW) in other cases. If any significant differences were found, it was followed by a multiple comparisons Tukey's HSD test for an ANOVA or a Dunn's post hoc test if a KW was used. Differences among treatments were tested using the Fisher's exact test (FE) for the datasets expressed as proportions (%) of individuals, i.e. for mortality and BSD symptoms.

Concentration-response relationships of *cyp1a* gene expression and body length were investigated by least square regression following a procedure similar to the one described in Rigaud et al. (2013). The DMSO control group was excluded from the regression analyses for both species. The nominal concentrations of retene were log-transformed before performing regression analyses. Outliers were detected using the Bonferroni's test for outliers and removed from the datasets. Regression analyses were validated by examining the normal distribution (Shapiro-Wilk statistic) and the homoscedasticity of the residuals. Those two conditions were respected in the case of the *cyp1a* gene expression dataset, but not for the body length dataset. After confirmation of the homogeneity of the slopes, the intercepts of the *cyp1a* gene expression concentration-response relationships for *O. mykiss* and *S. trutta* were compared by using an analysis of covariance (ANCOVA). The relative sensitivity of *O. mykiss* and *S. trutta* to retene was then estimated by comparing the concentration of retene required to induce an arbitrary level of *cyp1a* gene expression. These concentrations were calculated using the ANCOVA-adjusted regression lines for each species. The relative sensitivity (ReS) of *S. trutta* to retene compared to *O. mykiss* was defined as ReS = $\text{Concentration}_{O.mykiss}/\text{Concentration}_{S.trutta}$.

3. Results

3.1. Mortality, deformities and morphometry

The mortality was significantly induced only at the highest concentration of retene (320 $\mu g.L^{-1}$) for both species, and was significantly higher for *S. trutta* compared to *O. mykiss* (FE, $p \leq 0.05$). It reached 75.56% (61.18–85.92) and 94.44% (80.91–99.41) after 15 days of exposure for *O. mykiss* and *S. trutta*, respectively (% mortality and 95% confidence interval). No mortality was observed in the DMSO control groups for either of the species. The mortality of all other treatment groups was below 5% for both species. Regarding BSD deformities, the prevalence of pericardial edemas, yolk sac edemas, hemorrhages and craniofacial deformities were all significantly increased compared to controls at the highest concentration of retene for both species (FE, $p \leq 0.05$) (Table 1). In the case of *S. trutta*, significant effects were also detected for pericardial edemas at concentrations as low as 10 $\mu g.L^{-1}$ (but not at 32 $\mu g.L^{-1}$) and for hemorrhages at 100 $\mu g.L^{-1}$ (FE, $p \leq 0.05$) (Table 1).

Retene significantly reduced the body length of *O. mykiss* larvae at concentrations $\geq$10 $\mu g.L^{-1}$ (KW, $p \leq 0.05$) (Fig. 1). For *S. trutta* larvae, a significant effect on body length was only observed at 100 $\mu g.L^{-1}$ (KW, $p \leq 0.05$) (Fig. 1): the *p*-values of the Dunn's post hoc test were however very close to the significant level for 32 and 320 $\mu g.L^{-1}$ ($p = 0.06$). Retene had no significant effect on the yolk sac area of the larvae of either species at any concentration (data not shown, KW, $p > 0.05$). The assumption of the normal distribution of the residuals was not met when performing linear regressions with the body length datasets (Shapiro-Wilk, $p \leq 0.05$). Consequently, the concentration-response relationships of retene for *O. mykiss* and *S. trutta* were not investigated for this parameter.

3.2. Retene concentration in water

The concentrations of retene in the exposure water measured by SFS after 1, 7, 12 and 15 days are displayed in the Table S1 (Supplementary materials). Overall, the measured concentrations of retene in water were comparable between species for any time point or treatment, except for the lowest nominal concentration where the measured concentrations were slightly higher in the case of *S. trutta*. For the nominal concentrations up to 32 $\mu g.L^{-1}$, the concentrations measured in water after 1 and 7 days were lower compared to when measured after 12 and 15 days. Retene was in most cases not detected in the water after 1 and 7 days of exposure at the two lowest nominal concentrations (3.2 and 10 $\mu g.L^{-1}$). When retene was detected consistently (at 32 $\mu g.L^{-1}$ and higher), the measured concentrations were on average equal to 24.17%, 36.91% and 58.73% of the nominal concentrations for *O. mykiss* at 32, 100 and 320 $\mu g.L^{-1}$, respectively. For *S. trutta*, these percentages were very similar and respectively equal to 23.47%, 37.15% and 62.29%.

Table 1
Percentage (%) of *O. mykiss* and *S. trutta* larvae presenting pericardial edemas (PE), yolk sac edemas (YSE), hemorrhages (HEM) and craniofacial deformities (CD) after 15 days of exposure to either pure DMSO (0.001%, nominal concentration of retene 0 μg.L^{-1}) or increasing concentrations of retene (3.2, 10, 32, 100 and 320 μg.L^{-1}). Numbers between brackets represents the 95% confidence interval of each percentage value. *Indicates a significant difference compared to the corresponding DMSO control group (FE, $p \leq 0.05$).

Nominal concentration (μg.L^{-1})	*Oncorhynchus mykiss*					*Salmo trutta*				
	N	PE	YSE	HEM	CD	N	PE	YSE	HEM	CD
0	45	0.00 (0.00–9.38)	0.00 (0.00–9.38)	0.00 (0.00–9.38)	0.00 (0.00–9.38)	36	0.00 (0.00–11.47)	0.00 (0.00–11.47)	2.78 (0.00–15.42)	0.00 (0.00–11.47)
3.2	44	4.55 (0.42–15.97)	2.27 (0.00–12.89)	0.00 (0.00–9.58)	2.27 (0.00–12.89)	35	0.00 (0.00–11.76)	2.86 (0.00–15.81)	0.00 (0.00–11.76)	0.00 (0.00–11.76)
10	44	6.82 (1.68–18.89)	2.27 (0.00–12.89)	4.55 (0.42–15.97)	0.00 (0.00–9.58)	36	16.67* (7.79–32.27)	2.78 (0.00–15.42)	5.56 (0.59–19.09)	2.78 (0.00–15.42)
32	44	0.00 (0.00–9.58)	0.00 (0.00–9.58)	0.00 (0.00–9.58)	0.00 (0.00–9.58)	35	8.57 (2.21–23.13)	0.00 (0.00–11.76)	14.29 (5.78–29.85)	0.00 (0.00–11.76)
100	42	7.14 (1.77–19.70)	0.00 (0.00–9.99)	4.76 (0.46–16.65)	4.76 (0.46–16.65)	35	20.00* (9.74–36.19)	5.71 (0.62–19.57)	25.71* (13.98–42.25)	5.71 (0.62–19.57)
320	11	81.82* (51.15–96.01)	18.18* (3.99–48-85)	18.18* (3.99–48-85)	81.82* (51.15–96.01)	3	100.00* (38.25–100.00)	66.67* (20.24–94.37)	100.00* (38.25–100.00)	100.00* (38.25–100.00)

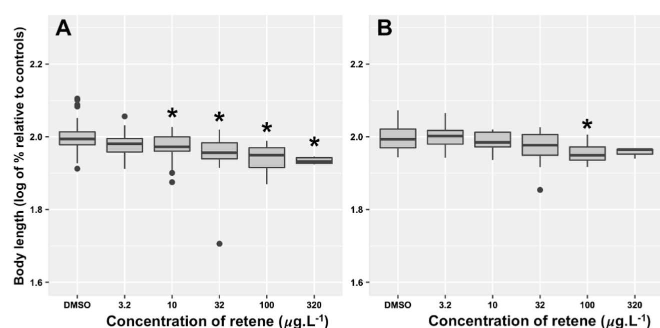

Fig. 1. Effect of 15 days of exposure to increasing concentrations of retene (3.2, 10, 32, 100 and 320 μg.L^{-1}) on the body length of (A) *O. mykiss* or (B) *S. trutta* larvae. The body length is expressed as percentage relative to the respective DMSO control group (for each species) and was log-transformed prior to plotting and analyses. N = 45 for *O. mykiss* and N = 36 for *S. trutta*. *Indicates a significant difference compared to the corresponding DMSO control group (KW, $p \leq 0.05$).

3.3. *cyp1a* gene expression

The level of expression of the *cyp1a* gene increased with the concentration of retene for both *O. mykiss* and *S. trutta*, with significant induction at ≥32 and ≥3.2 μg.L^{-1}, respectively (Fig. 2). Comparison of the concentration-response relationships for the *cyp1a* gene expression for *O. mykiss* and *S. trutta* (Fig. 3) revealed that the slopes of the linear regressions were not significantly different (ANCOVA, $p = 0.09$). Homogeneous slopes allowed the comparison of the intercepts of the two linear regressions: the ANCOVA revealed a significant difference between the intercepts ($p \leq 0.05$). The non-adjusted and ANCOVA-adjusted (with the common recalculated slope value) regression parameters are displayed in the Table 2. The ANCOVA-adjusted regression parameters were used to calculate the concentration of retene required to induce an arbitrary chosen *cyp1a* gene expression level equal to 2.5 (value expressed as % relative to controls and log-transformed). The corresponding concentrations of retene for *O. mykiss* and *S. trutta* were equal to 14.95 and 6.45 μg.L^{-1}, respectively. Thus, the relative sensitivity (ReS) of *S. trutta* to retene compared to *O. mykiss* was estimated at ReS = 2.32.

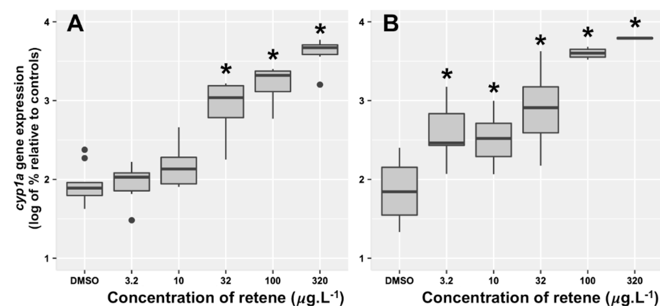

Fig. 2. Effect of 15 days of exposure to increasing concentrations of retene (3.2, 10, 32, 100 and 320 μg.L^{-1}) on the *cyp1a* gene expression level of (A) *O. mykiss* or (B) *S. trutta* larvae. The *cyp1a* gene expression level is expressed as percentage relative to the respective DMSO control group (for each species) and was log-transformed prior to plotting and analyses. N = 2–9. *Indicates a significant difference compared to the corresponding DMSO control group (ANOVA, $p \leq 0.05$).

4. Discussion

To our knowledge, the present study is the first to show that the fry of the two salmonids *O. mykiss* and *S. trutta* show a difference in sensitivity to an AhR agonist. Our data did not allow us to compare effectively the

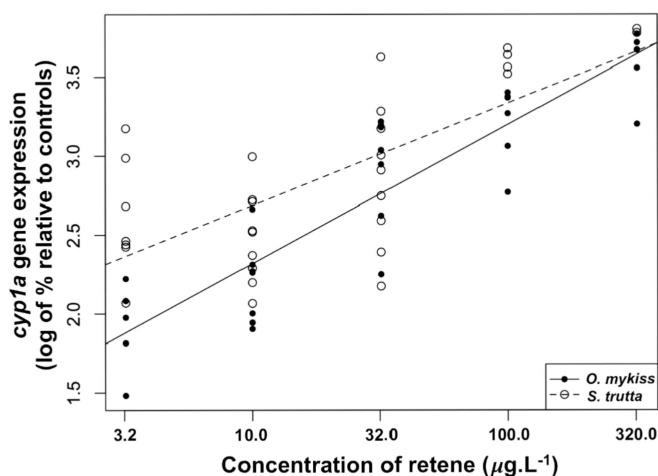

Fig. 3. Concentration-response relationships (non-adjusted by ANCOVA) for *cyp1a* gene expression for *O. mykiss* and *S. trutta* following 15 days of exposure to increasing concentrations of retene (3.2, 10, 32, 100 and 320 μg.L^{-1}). The *cyp1a* gene expression level is expressed as percentage relative to the respective DMSO control group (for each species) and was log-transformed prior to plotting and analyses. Concentrations of retene were log-transformed as well before performing the regression analyses, but the x-axis labels were replaced by the nominal concentrations for clarity purpose. N = 2–9.

Table 2
Non-adjusted and ANCOVA-adjusted regression parameters relating the *cyp1a* gene expression (expressed as percentage relative to controls and log-transformed) and the log-transformed exposure concentration of retene for *O. mykiss* and *S. trutta* larvae.

	Regression parameters[a]					
	a	*b*	*F-statistic*	DF	R^2	*p*
Non-adjusted regression lines						
Oncorhynchus mykiss	0.88 ± 0.07	1.44 ± 0.12	146.9	31	0.82	<0.001
Salmo trutta	0.65 ± 0.12	2.04 ± 0.17	28.67	29	0.48	<0.001
ANCOVA-adjusted regression lines						
Oncorhynchus mykiss	0.78 ± 0.07	1.57 ± 0.12	68.02	61	0.68	<0.001
Salmo trutta	0.78 ± 0.07	1.86 ± 0.09	68.02	61	0.68	<0.001

[a] Linear model formula is: $Log(y) = a*Log(x) + b$, where *a* is the slope, *b* is the intercept, *x* is the concentration of retene and *y* is the *cyp1a* gene expression.

sensitivity of the two species to retene in regard to mortality: it was significantly increased only at the highest tested concentration (320 μg.L^{-1}) and below 5% for all other concentrations for both species, making the calculation of LC50 (median lethal concentrations) impossible. However, the significantly higher mortality at 320 μg.L^{-1} for *S. trutta* suggests it is more sensitive than *O. mykiss* for that endpoint. Consistent with that observation, several potentially lethal BSD symptoms (pericardial edemas and hemorrhages) were significantly induced at concentrations lower than 320 μg.L^{-1} for *S. trutta* only. In a recent study performed by Honkanen et al. (2020), the authors exposed rainbow trout larvae to a similar range of waterborne (temperature set at 11 °C) retene concentrations (10–320 μg.L^{-1}) and estimated the LC50 at 122 μg.L^{-1} after 18 days. However, the mortality at 100 μg.L^{-1} was below 20% during the whole duration of the exposure (Honkanen et al., 2020). A slightly longer exposure time or intermediate exposure concentrations between 100 and 320 μg.L^{-1} would probably have allowed us to calculate LC50 for the present experiment. Interspecies differences of one order of magnitude for LC50 in fish have been observed for benzo[*a*]pyrene (BaP), a PAH known to be a highly potent AhR agonist (Wu et al., 2016).

Significant induction of *cyp1a* (the gene coding for the detoxification enzyme cytochrome P4501A) was observed already at the lowest concentration tested for *S. trutta* (3.2 μg.L^{-1}), but only at a concentration ten times higher for *O. mykiss*. Comparison of the concentration-response relationships for *cyp1a* between the two species followed that trend, suggesting that the brown trout larval stage is at least two times more sensitive to *cyp1a* induction by retene compared to the rainbow trout larval stage. To our knowledge, very few studies have explored or reported interspecies differences in *cyp1a* induction following exposure to PAHs in fish. Two *Lepomis* species (adult stage) exposed by intraperitoneal injection to BaP (50 mg.kg^{-1}) showed large differences in terms of CYP1A protein activity (15-fold versus 38-fold induction compared to controls) (Brammell et al., 2010). Experiments conducted in vitro demonstrated that such interspecies differences also exist for birds and mammals (Head et al., 2015; Vondrácek et al., 2017), but none of these studies assessed the effect of retene. For legacy DLCs (dioxins, furans and PCBs), several studies have shown that interspecies differences exist in fish (including salmonids) in terms of sensitivity for both *cyp1a* induction and embryo mortality (both predicted in vitro) (Doering et al., 2018; Doering et al., 2020; Doering et al., 2013; Eisner et al., 2016). From these studies, it is known that the lake trout (*Salvelinus namaycush*) and the brook trout (*Salvelinus fontinalis*) are noticeably (2 to 4-times) more sensitive to TCDD for embryo mortality compared to the rainbow trout.

Persistent DLCs such as polychlorinated dibenzo-*p*-dioxins or non-ortho-substituted polychlorinated biphenyls are very slowly metabolized by most organisms, including fish. PAHs, however, are metabolized much more efficiently compared to more persistent DLCs. Interspecies differences among fish species have been observed in terms of bioaccumulation and metabolism of complex mixtures of PAHs, and these differences appear to play a role in their sensitivity to these mixtures (Jung et al., 2015; Sørensen et al., 2017). The existence of comparable toxicokinetic differences between fish species exposed to individual PAHs cannot be excluded. Thus, it is possible that the higher *cyp1a* induction that we observe in *S. trutta* larvae is due to a higher uptake and/or from a slower metabolism compared to *O. mykiss*. As retene is a lipophilic compound and since *S. trutta* larvae have a larger yolk sac compared to *O. mykiss* larvae, the hypothesis of a higher uptake is plausible. The measured concentrations of retene in the exposure water were comparable between the two species, suggesting similar retene uptake. We did not evaluate retene concentrations in the tissues of the exposed larvae, as it is well established that fish metabolize and clear PAHs rapidly from their body. This has been observed before in rainbow trout larvae or juveniles exposed to retene (Brinkworth et al., 2003; Hodson et al., 2007; Rigaud et al., 2020). However, analyzing PAH metabolites in the tissues of the exposed larvae may have provided insights into whether toxicokinetic differences following retene exposure exists between the two species.

The rainbow trout larvae appeared to be more sensitive to retene exposure for growth. Significant effects of retene on body length were observed already at 10 μg.L^{-1} for *O. mykiss* versus 100 μg.L^{-1} for *S. trutta*. This observation appears to be contradictory with the *cyp1a* induction data. As the *cyp1a* gene induction was higher for *S. trutta*, one would except that it would translate into higher energetic costs associated with higher demand for the metabolism of xenobiotic compounds (retene and its metabolites) (Bains and Kennedy, 2004), resulting in less energy being allocated for growth. This contradictory observation can be partially explained by the lower number of larvae used for *S. trutta* compared to *O. mykiss*, reducing the statistical power in the case of the body length datasets. This is supported by the fact that the *p*-values were close to the significant level for 32 and 320 μg.L^{-1} ($p = 0.06$, see Section 3.1), and thus would probably have been significant with a slightly higher N. Moreover, larval growth occurs faster in *O. mykiss* compared to *S. trutta* (Kizak et al., 2011). It is possible that a slightly longer exposure duration would have elicited larger and significant differences in body length at the lower concentrations compared to control for the brown trout larvae.

The present study suggests that *S. trutta* larvae are more sensitive than *O. mykiss* larvae to a potent AhR agonist, retene. Brown trout stocks have been historically exposed to pulp and paper mill effluents in Finland (and thus to retene) or are still exposed to it nowadays (Könönen, 2018; Meriläinen et al., 2007). The same can be said about legacy DLCs such as polychlorinated dibenzo-p-dioxins, -furans and polychlorinated biphenyls, in different European countries (Havelkova et al., 2008; Hylland et al., 2006). Similar to what happened to several salmonid species in the North American Great Lakes, it cannot be ruled out that *S. trutta* populations in Europe have declined partly because of pollution by AhR agonists, and are still pressured by it. Using environmental guidelines designed following experiments conducted with traditional laboratory fish species such as zebrafish or rainbow trout, which are often more resistant to anthropogenic pollution compared to threatened fish species, can possibly lead to an underestimated risk. To ensure more accurate risk assessment, more data regarding the sensitivity of threatened fish populations to key AhR agonists is needed.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2021.109219.

CRediT authorship contribution statement

Cyril Rigaud: Conceptualization, Methodology, Data Acquisition, Data Analysis, Writing – Original Draft. **Julia Härme:** Methodology,

Data Acquisition, Writing – Review & Editing. **Eeva-Riikka Vehniäinen:** Conceptualization, Methodology, Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing.

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.

Acknowledgements

The present study was funded by the Academy of Finland (projects 285296, 294066 and 319284 granted to Eeva-Riikka Vehniäinen). The authors thanks Mervi Koistinen and Iina Könönen as well as the personnel of the Konnevesi Research Station for their precious technical assistance, and Hanka-Taimen for providing the fish larvae.

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