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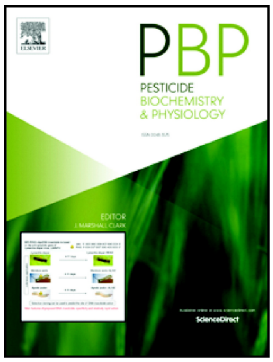

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PII: S0048-3575(21)00151-6

DOI: https://doi.org/10.1016/j.pestbp.2021.104920

Reference: YPEST 104920

To appear in: *Pesticide Biochemistry and Physiology*

Received date: 19 January 2021

Revised date: 1 June 2021

Accepted date: 9 July 2021

Please cite this article as: J.S. De Anna, L.A. Darraz, J.C. Painefilú, et al., The insecticide chlorpyrifos modifies the expression of genes involved in the PXR and AhR pathways in the rainbow trout, Oncorhynchus mykiss, *Pesticide Biochemistry and Physiology* (2018), https://doi.org/10.1016/j.pestbp.2021.104920

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The insecticide Chlorpyrifos modifies the expression of genes involved in the PXR and AhR pathways in the rainbow trout, *Oncorhynchus mykiss*

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Abstract

Chlorpyrifos (CPF) is an organophosphate pesticide, commonly detected in water and food. Despite CPF toxicity on aquatic species has been extensively studied, few studies analyze the effects of CPF on fish transcriptional pathways. The Pregnane X receptor (PXR) is a nuclear receptor that is activated by binding to a wide variety of ligands and regulates the transcription of enzymes involved in the metabolism and transport of many endogenous and exogenous compounds. We evaluated the mRNA expression of PXR-regulated-genes (PXR, CYP3A27, CYP2K1, ABCB1, UGT, and ABCC2) in intestine and liver of the rainbow trout, Oncorhynchus mykiss, exposed in vivo to an environmentally relevant CPF concentration. Our results demonstrate that the expression of PXR and PXR-regulated genes is increased in O. mykiss liver and intestine upon exposure to CPF. Additionally, we evaluated the impact of CPF on other cellular pathway involved in xenobiotic metabolism, the Aryl Hydrocarbon Receptor (AhR) pathway, and on the expression and activity of different biotransformation enzymes (CYP2M1, GST, FMO1, or cholinesterases (ChEs)). In contrast to PXR, the expression of AhR, and its target gene CYP1A, are reduced upon CPF exposure. Furthermore, ChE and CYP1A activities are significantly inhibited by CPF, in both the intestine and the liver.

CPF activates the PXR pathway in O. mykiss in the intestine and liver, with a more profound effect in the intestine. Likewise, our results support regulatory crosstalk between PXR and AhR pathways, where the induction of PXR coincides with the downregulation of AhR-mediated CYP1A mRNA expression and activity in the intestine.

Keywords: Organophosphate, Pregnane X receptor, Liver, Intestine, Aryl hydrocarbon receptor, Fish.

Declaration of interest: none

1. Introduction

The Pregnane X receptor (PXR) is a nuclear receptor that can be activated upon binding to a wide variety of structurally diverse ligands, comprised of both endogenous and exogenous compounds, such as bile salts, drugs, and xenobiotics (Coumoul et al. 2002; Lehmann et al. 1998; Staudinger et al. 2001; Watkins et al. 2001). Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor and binds to the PXR response elements located in the 5'-flanking regions of the PXR target genes, resulting in transcriptional activation. Hence, PXR plays a key role in the response to xenobiotics by regulating the transcription of biotransformation enzymes of phase I such as CYP3A of the cytochrome P450 superfamily (CYP), which is involved in the metabolism of more than 37% of the drugs in humans (Zanger et al. 2008). Mammalian phase II enzymes including UDP-glucuronosyl transferase (UGT), sulfotransferase and glutathione S-transferases (GST) (Xu et al. 2005), as well as phase III transport proteins (e.g., the ATP-binding cassette transporters as ABCC2, ABCB1) (Baldwin and Roling, 2009; Fardel et al. 2005; Jigorel et al. 2006; Schrenk et al. 2001), have been also shown to be transcriptionally regulated by PXR.

Differently from mammals, which have both PXR and Constitutive androstane receptor (CAR), with a partially superimposed spectrum of target genes and ligands, teleost fish possess only PXRs that share some CAR characteristics and differ among species (Krasowski et al. 2011; Maglich et al. 2003). In fish, the PXR pathway impacts the absorption, biotransformation, and excretion of drugs (Bresolin et al. 2005; Gräns et al. 2015 Meucci and Arukwe 2006; Pavlikova et al. 2010). In zebrafish liver, Bressolin et al. 2005 reported an intrinsic association between the induction of CYP3A and ABCB1 (involved in the metabolism and excretion of xenobiotics, respectively) and PXR regulated genes by pregnenolone 16α-carbonitrile (PCN), a prototypical

PXR agonist. Wassmur et al. (2010) have determined that rainbow trout (*Oncorhynchus mykiss*) PXR is predominantly expressed in the liver and intestine. Particularly, in *O. mykiss* hepatocytes, exposure to PCN induces the expression of the CYP3A and ABCB1 mRNA, but no PXR mRNA expression differences could be seen. Furthermore, the exposure of hepatocytes to a high concentration of lithocholic acid results in a concentration-dependent increase in CYP3A and ABCB1 mRNA levels, albeit a decrease in PXR mRNA expression is also observed (Wassmur et al. 2010).

Besides such reports, the role of PXR-dependent gene activation in xenobiotic metabolism in fish is not fully understood (Bainy et al. 2013; Lange et al. 2017). The activation of fish PXR by known PXR agonists shows inter-specific differences in the response to different ligands and the downstream genes activated. For example, it is not yet well established whether PXR regulates CYP3A in *O. mykiss*, as it seems to do so in zebrafish (Bresolin et al. 2005; Wassmur et al. 2010). Therefore, it is important to analyze the response of PXR and putative PXR-dependent genes to xenobiotics that are putative ligands of this signaling pathway in other fish models, like *O. mykiss*, to draw a complete picture of this regulatory pathway.

From an ecotoxicological perspective, several xenobiotics known to be PXR ligands in mammals, including polychlorinated biphenyls, clotrimazole, and chlorpyrifos (CPF), which have an impact on the health of the fish, have been found in bodies of water around the world (Kahle et al. 2008; Marino and Ronco 2005; Ondarza et al. 2012). Specifically, CPF is a broad-spectrum organophosphate pesticide (OP), reported as the second most commonly detected pesticide in food and water (John et al. 2015). The prevalence of OPs in the aquatic environment and their potential for adverse effects makes them good candidates for toxicological studies (Mehler et al. 2008). OPs are widely used for agricultural production in Argentina, particularly in the Pampa húmeda (Alvarez et al. 2019) and the Río Negro and Neuquén Valley (Ondarza et al. 2012; Macchi et al. 2018). CPF has been detected at concentrations ranging from 0.0005 to

10.8 µg/L in water samples from the Pampa húmeda (Alvarez et al. 2019) and at up to 1.02 µg/L in irrigation channels of Río Negro and Neuquén Valley (Macchi et al. 2018). As a typical OP, CPF toxicity results from the inhibition of cholinesterase enzymes (ChE) such as acetylcholinesterase (AChE), which is widely distributed throughout the body and is fundamental for cholinergic synaptic transmission in both the central nervous and the peripheral nervous systems (Giesy and Solomon 2014; Gupta 2019; Van der Wel and Welling 1989). Furthermore, CPF has also been reported to cause oxidative stress in vertebrates (Kutluyer et al. 2017; Verma et al. 2007). It is known that OPs are metabolized by phase I biotransformation enzymes, such as CYPs and flavin-containing monooxygenases (FMOs). For example, in OPs with a thione group (P=S) like CPF, the P=S group can be oxidized to the corresponding oxon (P=O); a derivative that is more active and less stable than the parent compound (Fukuto, 1990). Buratti et al. (2003) have reported that, in human microsomes, CYP1A2 and CYP2B6 are the main enzymes responsible for transforming CPF into CPF-oxon, while CYP3A4 is important at higher CPF concentrations. Additionally, CPF-oxons inhibit CYP1A activity (Neal, 1980), possibly affecting the metabolism of other CYP1A substrates. In *O. mykiss* hepatocytes, CPF is primarily metabolized via CYPs and, to a lower degree, by FMO (Wong, 1989). Besides the CYP1A subfamily that has been widely used as a biomarker in fish toxicology, due to its high susceptibility to contaminants (Goksøyr, 1995; Jönsson et al. 2010; Rees et al. 2003), the CYP2 enzymes represented in *O. mykiss*, CYP2K1 and CYP2M1, are both involved in steroid and fatty acid homeostasis. Particularly, CYP2K1 is the predominant rainbow trout hepatic CYP isoform (Buhler et al. 2000; Buhler and Wang-Buhler 1998; Buhler et al. 1994). The effect of CPF on fish detoxification enzymes activity has been extensively studied (Bonifacio et al. 2017; Kavitha and Rao et al. 2008; Sharbidre et al. 2011; Van der Wel and Welling 1989; Wheelock et al. 2005). In contrast, there are few studies on the transcriptional effects of CPF or other organophosphate insecticides (Bonansea et al. 2017; Topal et al. 2015). CPF has been reported

as a CAR agonist in mouse cell lines and as a PXR agonist in primary human hepatocytes (Baldwin and Roling 2009; Lemaire et al. 2004). To our knowledge, CPF has been studied as a possible PXR ligand only in an *in vitro* study with a zebrafish PXR model (Ekins et al. 2008). Hence, it is important to assess the effects of CPF on PXR and its target genes in other fish models, such as *O. mykiss,* in order to obtain a broader picture of the response of regulatory pathway controlled by PXR to this kind of xenobiotics.

Interestingly, CPF has also been reported as an aryl hydrocarbon receptor (AhR) agonist in mammals (Long et al. 2003; Moyano et al. 2020; Takeuchi et al. 2003), but the studies about the effects of CPF on the AhR pathway in fish are scarce. As far as we know, only two studies have reported the induction of CYP1A expression in Atlantic salmon and hybrid catfish exposed to CPF, likely mediated by AhR (Søfteland et al. 2014; Sommer et al. 2012). The former authors report that CPF exposure induces the expression of CYP3A and CYP1A in primary hepatocytes of Atlantic salmon, presumably via the activation of the PXR and AhR receptors, respectively (Søfteland et al. 2014). Kubota et al. (2015) have reported that pregnenolone, an agonist of mammalian PXR, produces self-induction of PXR in developing zebrafish. Furthermore, these authors have shown that genes of CYP1, CYP2 and CYP3 subfamilies could be regulated by both AhR and PXR, suggesting reciprocal cross-regulation of genes by such receptors. Similarly, Cui et al. (2017) have observed in Hep62 cells (human hepatocytes) that PCN inhibits the expression of AhR target genes, such as CYP1A, CYP1B1, and that PXR activation represses the transcription of AhR-regulated genes.

The rainbow trout is an introduced species particularly abundant in rivers and lakes of North Patagonia, Argentina, including the Río Negro and Neuquén Valley (Macchi et al. 2008; Pascual et al. 2007). Besides its value as a sports fishing catch, the rainbow trout is cultured in reservoirs of this region and constitutes the principal aquaculture resource of the country (FAO 2018). As stated above, the water-courses of the Río Negro and Neuquén Valley can bear

considerable concentrations of CPF together with other pesticides and pollutants from other activities such as oil extraction (Macchi et al., 2018; Monza et al., 2013). Thus, it is relevant to evaluate the effects of CPF on the nuclear receptors PXR and AHR, which modulate numerous cell processes related to the detoxification response against xenobiotics, to help understand the actual CPF impact on fish and aquatic systems' health.

The aims of this work were: i) to study the effects of CPF on the PXR pathway in the liver and intestine of *O. mykiss* exposed *in vivo*, and ii) to explore the possible modulation of the AhR pathway by CPF in *O. mykiss*.

2. Material and Methods

2.1 Fish and Experimental design

Juvenile diploid *Oncorhynchus mykiss* (n = 54. 4.5[illegible] ± 0.28 g; 7.98 ± 0.65 cm; Mean ± SD) from CEAN aquaculture facilitywere kept in a continuous flow system with Chimehuin river water before experimentation. This line has been bred for at least 30 years; thus, these fish have high homozygosity and all individuals share similar genetic information (Valiente et al. 2007). The Chimehuin River, upstream of Junín de los Andes city, is considered pollution-free since it is the main outlet of the Lake Huechulafquen, a deep glacial lake (surface area 70 km^2) located in the Lanín National Park, where no industry or agriculture activity is allowed (Federal Law 22351, Argentina). Fish were fed with 1% body mass ratio of commercial trout feed per day. After 48 h of acclimatization, the experiments were conducted in 12 L cylindrical plastic containers (22-cm diameter, 40-cm height) (3 fish per container) with water from Chimehuín River, continuously aerated (alkalinity 34 mg L^{-1}, conductivity 36 µS cm^{-1}, pH 7.4, 8.37 mg L^{-1} dissolved oxygen at a temperature of 12 ± 2 °C, pH 7.4 ± 0.02), the photoperiod was 12 h light: 12 h dark. No mortality was observed in any of the experimental groups. Each experimental group comprised nine fish distributed in three containers, samples from the three fish in each

container were pooled to obtain each independent replica (n = 3). The experimental design consisted of *in vivo e*xposure to a unique concentration of CPF, 1.4 µg L^{-1} (0.004 µM), for 12, 24 and 48 h. This concentration is about 1/6 of the chlorpyrifos median lethal concentration at 96 h (96-h LC50) for rainbow trout (9 µg L^{-1}, USEPA 1986), and is within the range found in freshwater bodies of Argentina (Alvarez et al. 2019; Marino and Ronco 2005). Chlorpyrifos was added to the container water dissolved in acetone. The final acetone concentration was 0.01%. The same acetone concentration was added to control containers (solvent control). After each exposure period, the fish were euthanized by a blow to the head followed by decapitation. The middle intestine and the liver of each fish were quickly removed and cut into halves for mRNA expression and enzymatic activity analysis. Samples for mRNA expression were stored in microcentrifuge tubes with 0.3 mL RNAlater (Life Technologies, Carlsbad, CA) at -20 °C. Samples for enzymatic activities were washed in PBS-Triton X-100 and then stored in liquid nitrogen with PBS 0,1 % Triton X-100 (phosphate-buffered saline; pH 7.4, 30 mmol L^{-1} KCl, 15 mmol L^{-1} KH_2PO_4, 1.4 mol L^{-1} NaCl, 80 mmol L^{-1} Na_2HPO_4, 0.1% Triton X-100) until used. The experimental design and animal handling procedures followed the Canadian Council on Animal Care (CCAC) guidelines (2005), and were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

2.2 Chlorpyrifos

A standard CPF solution of 14 mg L^{-1} was prepared by dissolving 1.4 mg of CPF (O,O- diethyl O-[3,5,6-trichloro-2-pyridyl phosphonothioate], 99% purity, Chem Service, West Chester, PA) in 100 mL of chromatographic quality grade acetone (Cicarelli, Argentina). The CPF concentration in the standard solution was measured by gas chromatography (Agilent 6890 series Wilmington, USA) with a flame photometric detector (GC-FPD), fused silica capillary column HP-5, 5% phenylmethylsiloxane, 30 m × 0.32 mm i.d. and a 0.25 µm film thickness

(Agilent Technologies). The temperature was programmed to increase at 10 ^{o}C min^{-1} from an initial 100 ^{o}C to 200 ^{o}C and then at 4 ^{o}C min^{-1} increase to the final temperature of 220 ^{o}C. The carrier was purified helium at a flow rate of 3.6 mL min^{-1}. The detector temperature was at 250 ºC. The sample solution (1.0 µL) was injected in splitless mode. Quantification of chlorpyrifos was performed, using a chlorpyrifos standard as reference.

2.3 Enzyme activity

2.3.1 Cholinesterase

Tissue homogenates were obtained by grinding using a Teflon manual homogenizer for 30 s on ice in 1 mL of homogenization buffer (20 mmol L^{-1} Tris–HCl, 0.5 mmol L^{-1} EDTA, pH 7.5 and 200 mmol L^{-1} phenylmethylsulphonyl fluoride (PMSF)) per 0.5 g of tissue and then centrifuged at 12,000 x g for 15 min at 4 °C. Supernatants were stored at -20 °C and enzyme activity was measured within 24 h. Cholinesterase (ChE) activity was measured in duplicate, using 150 µL of liver or 200 µL intestine supernatant, 100 mmol L^{-1} phosphate buffer, pH 8.0, with 0.2 mmol L^{-1} 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and 1.5 mmol L^{-1} acetylthiocholine iodide as substrate, according to Ellman et al. (1961). Absorbance at 412 nm was recorded for 1 min every 10 s at 25 °C. Results were normalized to total protein, which was measured by Bradford's method (1976).

2.3.2 EROD

The rate of 7-ethoxyresorufin-O-deethylation (EROD) was determined to estimate the activity of CYP1A according to Kennedy and Jones (1994). Tissue homogenates were obtained by sonication twice for 15-30 s on liquid nitrogen, in CaHBSS (Hank's Balanced Salt Solution with Calcium; Gibco®), pH 7.8, and then centrifuged at 14,000 x g for 5 min at 4 °C. The assay solution (150 µL) contained 30 µL of supernatant, 6.25 µmol L^{-1} 7-ER (Sigma), 10 µmol L^{-1} dicoumarol (Sigma), and 1 mmol L^{-1} NADPH (Sigma) in CaHBSS pH 7.8. The reaction was

performed at 30 °C in a 96-well microplate with a black flat bottom. All samples were analyzed in triplicate. Fluorescence readings were taken every 42 s for 6 min with a fluorescence spectrophotometer (BioTek SynergyTM HT Multi-Mode Microplate Reader), at excitation and emission wavelengths of 530 and 620 nm.

EROD activity was expressed as pmol resorufin mg protein^{-1} min^{-1}, using resorufin standards for calibration. For this assay, the total protein content was determined using the Pierce TM BCA Protein Assay Kit.

2.4 Gene expression by real-time PCR (q-PCR)

We analyzed the mRNA expression of the nuclear receptors PXR and AhR together with possible target genes of either nuclear receptor, including phase I and II detoxification enzymes, and xenobiotics transporters. Total RNA was extracted from the intestine and liver tissues (20 and 30 mg respectively), pooled from the three individuals in each one of the three containers of each experimental group (n = 3 independent replicates from 9 fish), using Trizol reagent (Ambion, Carlsbad, CA), following the manufacturer's instructions. The yield and purity of the extracted total RNA were measured as A260/A280 and A260/A230 ratio. cDNA was synthesized using 2 µg of total RNA and RevertAid reverse transcriptase (Thermo Fisher Scientific, Waltham, MA). For qPCR, each reaction mixture contained 1 µL cDNA template, 0.5 µLof each primer (10 µM), 8 µL water, and 10 µL of 2 × SYBR green q-PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The primer pairs used for qPCR are shown in Table 1. Reactions were performed in a StepOnePlusTM Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). The thermal cycling program consisted of a denaturing step (95 °C, 3 min) followed by 40 cycles of denaturation (95 °C for 10 s), annealing (60 °C for 30 s), and extension (72 °C for 20 s). B-actin was used as a housekeeping gene. Table 1 shows the primer pairs used for qPCR. The target gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and expressed as:

$$\Delta\Delta CT = Ct^{t}_{target} - Ct^{t}_{\beta-actin} - Avg\left(Ct^{c}_{target} - Ct^{c}_{\beta-actin}\right)_{all}$$

Where Ct_{target} and $Ct_{\beta\text{-actin}}$ are the cycle thresholds for target and B-actin genes, ^{t} is the treatment group and ^{c} is the solvent control.

Table 1: Details of primer pairs and their amplicons used in the study.

Gene		Primer pair sequence 5' → 3'	Amplicon length (bp)	GenBank accession
Pregnane X receptor	PXR	FW GGGCATCTGGGAGTGTGGTC RV GTAGGTTCCTCAGGGTGTAGTGG	116	EF517132
Cytochrome P450 3A27	CYP3A27	FW TCTGCTGATGCCCAAACGA RV CGTTGTTGGACTCTTCAGAGTGG[illegible]A	110	U96077
Glutathione-S-transferase omega 1	GST	FW AGCTGCTCCCAGCTGATCC RV CAAACCACGGCCACATCATG[illegible]	246	NM_001160618.1
ATP-binding cassette B1/ P-Glycoprotein	ABCB1/ Pgp	FW ACGTGCGCTCCCTGAACG[illegible]G RV GCGTTGGCCTCCCTAG[illegible]	151	AY863423
UDP-glucuronosyl transferase	UGT	FW ATAAGGACCGTCCCA[illegible] RV ATCCAGTTGAGC[illegible]GTGAGC	113	DY802180
ATP-binding cassette C2/ Multidrug resistant protein 2	ABCC2/ MRP2	FW ATGCTGGGA[illegible]TGGAGTTTG RV GAGAGCTGT[illegible]CTATCCAATGCAGT	142	XM_021604527.1
Aryl hydrocarbon receptor	AhR	FW GGATG[illegible]CTGAGTTCCAAACCAA RV AATGCCTG[illegible]TCTATGGGTAGCTGA	147	NM_001124252.1
Cytochrome P450 1A	CYP1A	FW ACC[illegible]GTGGCAGGTCAACCATGAT RV C[illegible]TGCCGAATACGAGCACTTT	134	XM_021607648.1
AhR nuclear translocator	ARNT	FW [illegible]GAATGCAGAGCAATCCCA RV AG[illegible]GTGATTGAGGAAGAGCTGAGA	113	NM_001124710.1
Flavin Monooxygenase 1	FMO1	FW GGAAGTTCAAGGAAGTTTCTGAGCCC RV CCGCACACTGGTCTGGAAGTGGAT	212	NM_001124664.1
Cytochrome P450 2M1	CYP2M1	FW GCTGTATATCACACTCACCTGCTTTG RV CCCCTAAGTCCTTTGCATGTATAGAT	194	NC_035103.1
Cytochrome P450 2K1	CYP2K1	FW CTCACACCACCAGCCGAGAT RV CTTGACAAATCCTCCCTGCTCAT	164	XM_021558863.1
Beta-actin	β-actin	FW TGAAGTGTGACGTGGACATCCGTA RV AGGTGATCTCCTTCTGCATCCTGT	108	NW_018528586.1

2.5 Data analysis

Statistical analyses were done using the GNU PSPP 1.2.0 software (GNU general public license). Data were presented as mean ± standard error of the mean (SEM), n = 3. Each

independent replicate represents pooled tissue samples from three individuals (nine individuals per treatment group). Comparisons among groups were performed by unpaired Student t-tests. We considered a value of $p < 0.05$ as statistically significant (Zar, 1999).

3. Results

3.1 Impact of CPF exposure on ChE activity in *O. mykiss*

In order to evaluate the effect of CPF in the intestine and liver of juvenile *O. mykiss*, we evaluated ChE enzymatic activity at different exposure times. It is known that ChE is a sensitive biomarker to inhibition by CPF in non-nervous organs, such as liver (Wheelock et al. 2008). Decreased ChE activity was observed in both intestine and liver, upon exposure to CPF, although with slight kinetic differences. Significant inhibition of ChE activity by CPF was detected at 12 h and 48 h of exposure in the intestine, whereas such differences were detected upon 24 h and 48 h of exposure in the liver (Figure 1).

3.2 CPF exposure impact on detoxification pathways in *O. mykiss*

3.2.1 PXR pathway

To evaluate the effects of CPF as a potential modulator of the PXR pathway in the liver and intestine, we exposed fish to 0.004 µM CPF and collected samples at different time points, 12, 24, and 48 h, and expression of several PXR-regulated genes was assessed by qPCR. PXR expression showed a slight but significant upregulation in the intestine of CPF exposed fish after 12 h, when compared to solvent control fish, and the same tendency was observed at 24 h, with a nearly two-fold increase, although not statistically significant. No significant effect was detected at 48 h. Consistently, two PXR-regulated genes, ABCC2 and CYP2K1, were highly induced upon exposure to CPF for 12 h and 48 h respectively but were not affected at 24 h. In

contrast, the expression of three genes expected to be regulated by PXR, CYP3A27, ABCB1, and UGT, did not show any significant change in this organ (Figure 2a).

Following the tendency observed in the intestine, liver PXR expression was 2.5-fold upregulated upon 12 h of exposure to CPF, while no differences between CPF and control fish were detected at later time-points. UGT expression was ~3-fold upregulated at 24 h but showed no change at 12 h or 48 h of exposure. No effects of CPF were recorded for CYP3A27, CYP2K1 ABCB1, or ABCC2 expression in the liver (Figure 2b).

3.2.2 AhR pathway

The expression of AhR was significantly downregulated in the intestine of fish exposed to CPF at 12 h of exposure, whereas no significant changes were detected at longer exposure times. Consistently, the expression of the prototypical AhR-regulated gene CYP1A was downregulated in fish exposed to CPF for 12 hours, with respect to the solvent control. On the contrary, exposure to CPF led to higher expression of the aryl hydrocarbon receptor nuclear translocator (ARNT) at 12 h (Figure 3a).

Concerning the impact of CPF on the AhR pathway in the liver, AhR expression was significantly downregulated upon 24 h of exposure to CPF, while ARNT and CYP1A remained unchanged throughout the experiment (Figure 3b).

We also evaluated EROD activity due to the role of CYP1A in the bioactivation of CPF (Tang et al. 2001). Intestinal EROD activity was significantly inhibited by CPF at 12 h of exposure, but no significant differences were detected at longer exposure times. There was a tendency to the inhibition of liver EROD activity by CPF at 12 and 24 h of exposure but a significant effect was detected only at 24 h. Inhibition was almost totally reversed at 48 h (Figure 3c).

3.3 Phase I and II enzymes

In addition to known PXR and AhR regulated genes, commonly associated with these pathways and with xenobiotics metabolism, we have tested the expression of additional molecules also implicated in xenobiotic metabolism (Cárcamo et al. 2011; Hayes et al. 2005; Wong 1989). As shown in Figure 4a, the expression of FMO1 and GSTo in the intestine was upregulated upon 12 h of exposure to CPF. In contrast, CYP2M1 expression was not induced upon exposure to CPF at any of the time-point. In the liver, although there was a tendency to the increase in FMO1 expression at 12 h, we detected no significant change in the expression of FMO1, CYP2M1, or GSTo at any time of exposure to CPF (Figure 4b).

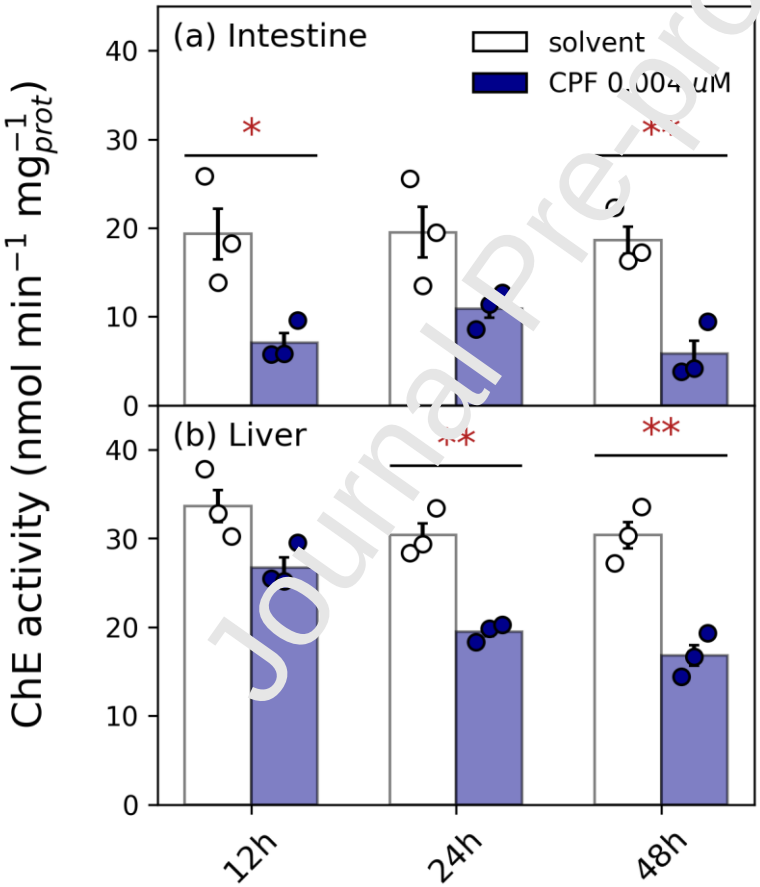

Figure 1. Cholinesterase (ChE) activity in the intestine (a) and liver (b) of *Oncorhynchus mykiss* exposed *in vivo* to 0.004 µM Chlorpyrifos (CPF) for 12, 24, and 48 h. * $P < 0.05$ and ** $P < 0.01$ for CPF effects. Data are shown as mean ± standard error of the mean (SEM), n = 3. Each independent replicate represents pooled tissue samples from three individuals (nine individuals per treatment group).

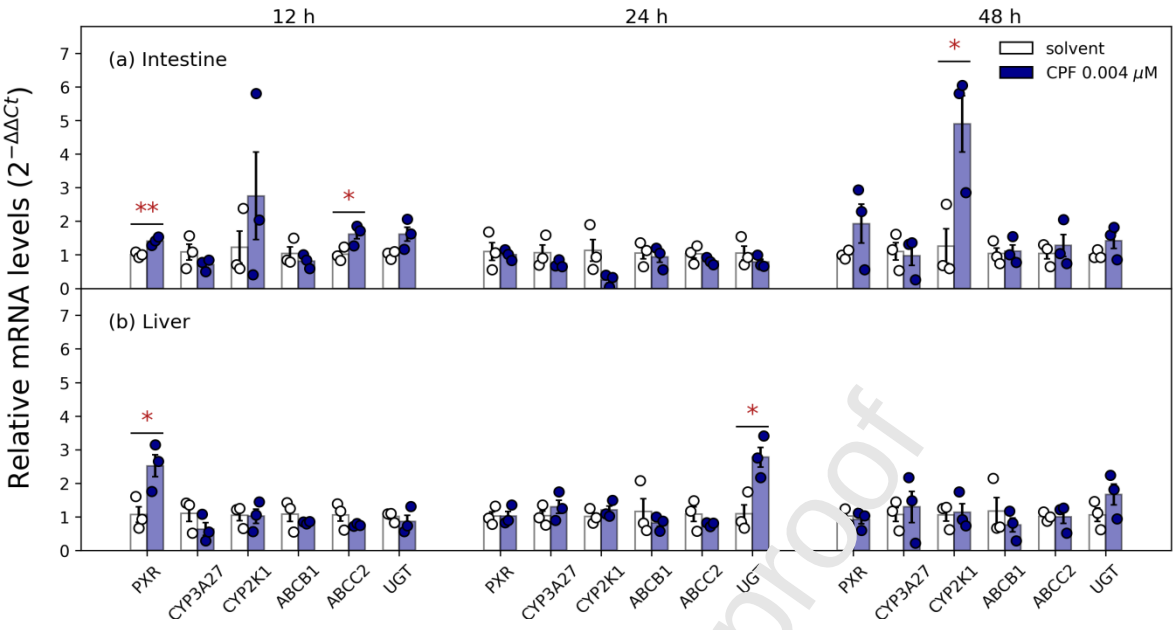

Figure 2.The impact of *in vivo* exposure to Chlorpyrifos (CPF) on the PXR pathway, in *Oncorhynchus mykiss.* Relative mRNA expression ($2^{-\Delta\Delta Ct}$) of genes of the PXR pathway, PXR, cytochrome P4503A27 (CYP3A27) and 2K1 (CYP2K1), ATP-binding cassette transporters B1 (ABCB1) and C2 (ABCC2), and UDP-glucuronosyl transferase (UGT), in the intestine (a) and liver (b) of fish exposed to 0.004 µM Chlorpyrifos (CPF) for 12, 24 and 48 h. * $P < 0.05$ and ** $P < 0.01$ for CPF effects. Data are shown as mean ± standard error of the mean (SEM), n = 3. Each independent replicate represents pooled tissue samples from three individuals (nine individuals per treatment group).

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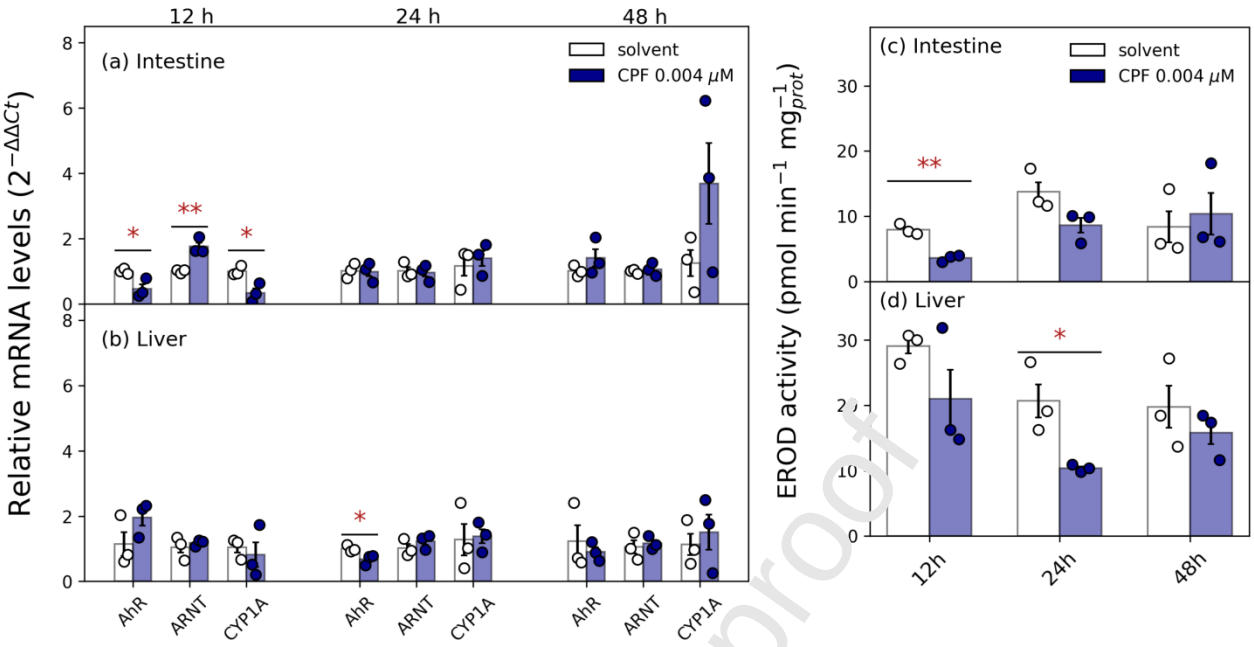

Figure 3.The impact of *in vivo* exposure to Chlorpyrifos (CPF) on the aryl hydrocarbon receptor (AhR) pathway in *Oncorhynchus mykiss*. Relative mRNA expression ($2^{-\Delta\Delta Ct}$) AhR, AhR nuclear translocator (ARNT) and the cytochrome P4501A (CYP1A), (a, b); and 7-ethoxyresorufin-O-deethylation (EROD) activity, (c, d), in the intestine and liver of fish exposed to 0.004 µM Chlorpyrifos (CPF) at 12, 24 and 48 h. * P < 0.05 and ** P < 0.01 for CPF effects. Data are shown as mean ± standard error of the mean (SEM), n = 3. Each independent replicate represents pooled tissue samples from three individuals (nine individuals per treatment group).

Journal Pre-proof

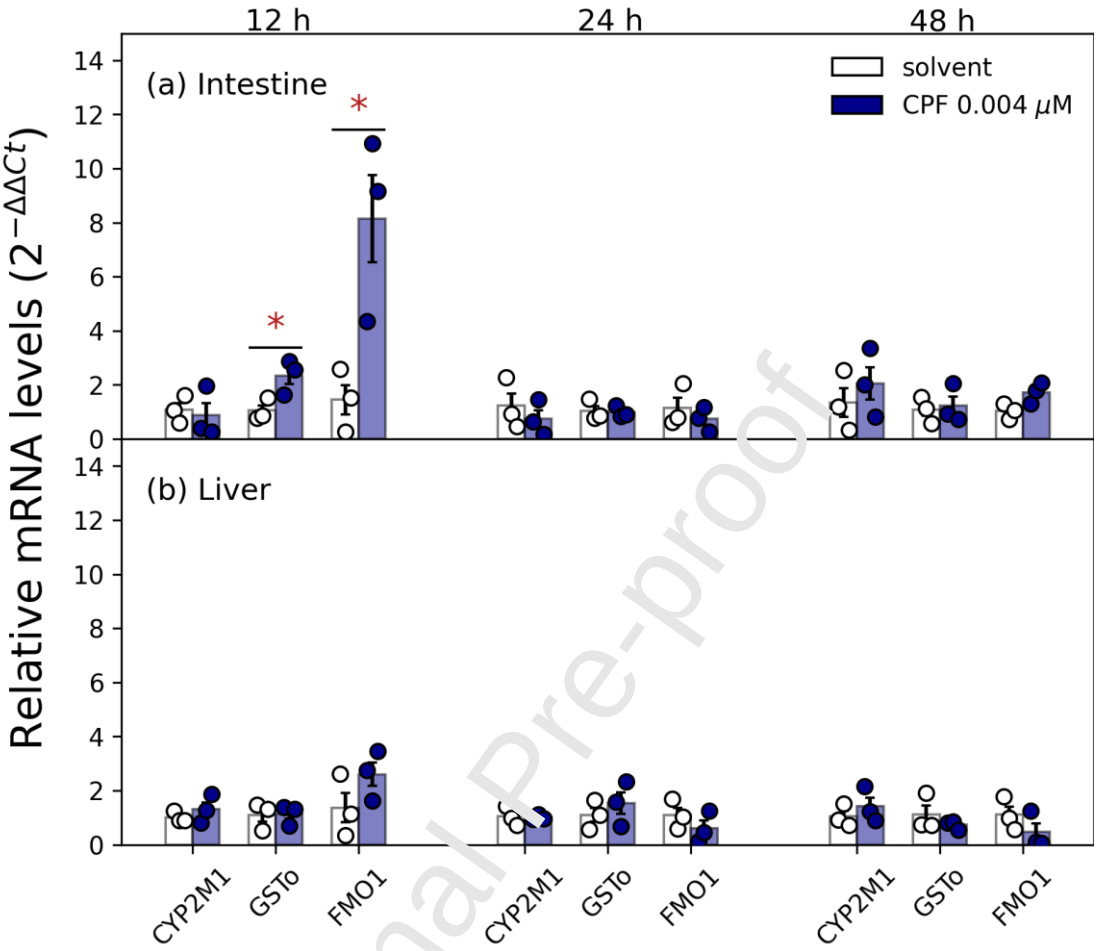

Figure 4.The impact of *in vivo* exposure to Chlorpyrifos (CPF) on *Oncorhynchus mykiss* phase I and II detoxifying enzymes. Relative mRNA expression ($2^{-\Delta\Delta Ct}$) of cytochrome P4502M1 (CYP2M1), glutathione S-transferase omega (GSTo) and flavin-containing monooxygenase 1 (FMO1) in the intestine (a) and liver (b) of fish exposed to 0.004 µM CPF at 12, 24 and 48 h. * $P < 0.05$ and ** $P < 0.01$ for CPF effects. Data are shown as mean ± standard error of the mean (SEM), n = 3. Each independent replicate represents pooled tissue samples from three individuals (nine individuals per treatment group).

4. Discussion

Organophosphate insecticides are of great ecotoxicological concern and their toxic effects have been extensively studied. However, the capacity of these compounds to modulate gene transcription pathways has received far less attention. Here, we evaluated the effects of CPF

exposure in *O. mykiss in vivo*, at an environmentally relevant concentration (Alvarez et al. 2019) and about one-fifth of the species acute lethal concentration-50 (Buckler et al. 2003), on the PXR and AhR transcriptional pathways. Upon exposure, we assessed the mRNA expression of a set of prototypical target genes (PXR, CYP3A27, CYP2K1, ABCB1, UGT, ABCC2, for the PXR pathway, and AhR, CYP1A and ARNT, for the AhR pathway), and CYP1A activity (as EROD) in the liver and intestine. In addition, we have studied the expression of other genes that are involved in xenobiotic biotransformation, such as FMO1, CYP2M1, and GSTo, and whose regulatory pathway has not yet been elucidated for fish. We detected a marked inactivation of the known target of the organophosphate pesticides ChE. This inactivation occurred earlier and was more pronounced in the intestine than the liver, which suggests that the OP was, at least in part, absorbed in the intestine.

It has been reported that CPF induces the PXR pathway in mammalian models (Baldwin and Roling 2009; Lemaire et al. 2004). In fish, Søfteland et al. (2014) have reported that CPF induces a prototypical PXR target, CYP3A in primary hepatocytes of the Atlantic salmon, and Ekins et al. (2008) have shown the activation of zebrafish PXR by CPF in an *in vitro* model. Coinciding with the effects on ChE activity, CPF induces the mRNA expression of *O. mykiss* PXR and downstream genes earlier and more consistently in the intestine than in the liver. In the intestine, we report significant upregulation of two genes at 12 h, PXR and its putative target ABCC2, plus a tendency to increase CYP2K (2.5-fold), although not significant. In contrast, in the liver, PXR is the only gene induced at 12 h, followed by the upregulation of UGT at 24 h. The significant (5-fold) induction of CYP2K expression at 48 h seems to respond to the 2-fold increase in PXR expression at this same time, although the change in PXR expression is not significant. On the other hand, there is no evident CPF effect on the expression of other prototypical PXR targets, namely CYP3A27 and ABCB1, nor in the intestine or the liver.

In contrast, Søfteland et al. (2014) have reported the induction of CYP3A by CPF in *Salmo salar in vitro* hepatocytes, and Bonansea et al. (2017) report that CPF induces liver ABCB1 in *Jenynsia multidentata*. Similar discrepancies have been reported for the effects of mammalian PXR agonists in different fish species. Wassmur et al. (2010) report that, in *O. mykiss's* primary hepatocytes, the mammalian PXR agonist PCN induces CYP3A and ABCB1 expression without changing the expression of PXR. In the common carp, *Cyprinus carpio*, the azole fungicide clotrimazole transactivates a PXR gene reporter *in vitro*, at near environmental concentrations (Lange et al. 2017), but two studies of the same group have reported that clotrimazole either does not induce or even downregulates CYP3A and other PXR-regulated genes (Corcoran et al. 2012), or induces CYP3A and ABCB1 but at a concentration 100 times higher than the needed to transactivate PXR *in vitro* (Corcoran et al. 2014). Also, the mammalian PXR agonist, rifampicin fails to induce *C. carpio* PXR *in vitro* (Lange et al. 2017) but consistently induces CYP3A, CYP2K, GSTa, GSTp, ABCB1, and ABCC2 (Corcoran et al. 2012). Other species respond to PXR agonists in the same way as mammals, e.g. Bresolin et al. (2005) have reported an intrinsic association between the induction of CYP3A, ABCB1, and PXR in the liver of zebrafish exposed to PCN, and Bao et al. (2018) report the induction of PXR, CYP3A, ABCC2 and UGT in the mosquito fish (*Gambusia affinis*) by the hypocholesterolemic drug simvastatin. The fact that we observe the induction of PXR and three PXR targets, at an environmentally relevant concentration (in the nanomolar range), strongly suggests CPF as a PXR agonist of rainbow trout PXR, although the lack of CYP3A and ABCB1 induction deserves further attention. Lange et al. (2017) have discussed that some putative PXR target genes, such as CYP3A and CYP2K could be induced by a non-PXR pathway in some fish species or that there is a concentration threshold for the agonist to activate the whole transcriptional pathway *in vivo*. The differences in the responses to PXR agonists among fish species and with respect to mammals have also been discussed in terms of inter-specific

variability in the sequence or structure of the DNA-binding (DBD) and/or the ligand-binding domain (LBD) (Bainy et al. 2013; Corcoran et al. 2012; Gräns et al. 2015; Krasowski et al. 2011; Lange et al. 2017; Moore et al. 2002; Wassmur et al. 2010).

The lack of induction of CYP3A in *O. mykiss* intestine and liver and of CYP2K in the liver could also be related to CPF activation of other regulatory pathways that crosstalk with PXR. We have recently reported that CPF increases the expression of IL-6 and TNFα in *O. mykiss* liver (De Anna et al. 2020), both of which induce NF-κB. In turn, IL-6 and NF-κB impair the binding of the PXR/retinoid X receptor α heterodimer and its binding to PXR-response elements in CYP3A4, CYP2, and ABCB1 genes (Ning et al. 2017; Pascussi et al. 2008, Pavek et al. 2016, for reviews).

AhR is another nuclear receptor that plays a major role in xenobiotic metabolism by regulating the expression of diverse enzymes involved in detoxification and metabolic processes, including CYP1A. Studies in mammalian cell lines have reported dose-dependent agonistic effects of CPF on AhR (Long et al. 2003; Takeuchi et al. 2008). In fish, the available information relates to the effects of CPF on the expression of AhR target genes of the family CYP1A, but no study addresses the modulation of the AhR pathway by this pesticide. Søfteland et al. (2014) have reported that the AhR target gene, CYP1A, and the PXR target CYP3A are upregulated in *S. salar* primary hepatocytes by different concentrations of CPF at, 10 µM and 0.1µM, respectively. Similarly, CYP1A, CYP1B, and CYP1C are upregulated by 0.03 to 0.33 µM CPF in the gills of *C. carpio* exposed *in vivo* (Fu et al. 2013). On the other hand, liver CYP1A protein was downregulated in *Oncorhynchus tshawytscha* after *in vivo* exposure for 96 h to 0.021 µM CPF (Wheelock et al. 2005). We find in this work that both intestinal AhR and CYP1A expression are downregulated at 12 h of exposure to CPF, with a consequent decrease in EROD activity. This effect is coincident with a marked effect of CPF on PXR targets. Interestingly, the downregulation of AhR expression at 12 h is accompanied by an augment in

the mRNA expression of ARNT, the molecule that heterodimerizes with ligand-bound-AhR to activate downstream genes transcription (Denison and Nagy, 2003). In a previous study (De Anna et al. 2018), we have reported that the exposure to a water-soluble fraction of crude oil for 48 h upregulates AhR and CYP1A, and downregulates ARNT mRNA expression in *O. mykiss* liver; thus, suggesting a negative feedback regulation of this pathway. In the present study, we observe a trend to the increase of liver AhR expression by CPF at 12 h, but not accompanied by any change in ARNT or CYP1A expression, or EROD activity. Besides, there is a slight but significant decrease (30%) in liver AhR expression at 24 h together with a significant decrease in liver EROD activity. Liver PXR expression is upregulated at 12 h of CPF exposure, while its downstream target gene UGT is also upregulated at 24 h. These results suggest that the induction of the PXR pathway by CPF might downregulate the AhR and/or its downstream genes, mainly in the intestine, whereas the results obtained for the liver are not clear. In this sense, Cui et al. (2017) have reported an inhibitory crosstalk between PXR and AhR in mouse liver cells, in which PXR activation decreases the transcriptional activity of the AhR pathway by blocking the binding of the AhR ARNT complex to the promoter region of downstream genes, e.g., CYP1A. On the contrary, Kubota et al. (2015) report reciprocal stimulatory crosstalk between the PXR and AhR pathways in zebrafish, using pregnenolone and PCB 126 as PXR and AhR agonists, respectively. Thus, further studies with diverse agonists and model species are needed to establish the characteristics of a possible AhR-PXR crosstalk in fish and to know its implications in the response of fish to pollutants mixtures.

We have also studied the mRNA expression of two biotransformation enzymes that could be important in the effects of and the response to CPF, GSTo, and FMO1. The significant induction of GSTo in *O. mykiss* intestine upon CPF exposure could be part of the organ's antioxidant/detoxifying response. It has been reported that human GSTo1 has thiol transference and reductase activities (e.g. reduction of methylated arsenic), and seems to be induced through

Nrf2 signaling in mouse hippocampal HT22 cells (Sukprasansap et al. 2020). The response of GSTo to pesticides has been studied so far only in insects. Balakrishnan et al. (2018) report that several insecticides, including the phosphorothioate malathion, induce the expression of GSTo among other GSTs in the aphid *Rhopalosiphum padi*. FMO1 is an important phase I biotransformation enzyme, which is induced in fish by high salinity and cortisol, and has been reported to be involved in the activation of several phosphorothioate pesticides in *O. mykiss* (El-Alfy et al. 2002; Lavado et al. 2012). We have recorded a significant 8-fold induction of FMO1 expression in *O. mykiss* intestine at 12 h, coinciding with significant induction of the PXR pathway and the downregulation of CYP1A and EROD activity. This suggests that FMO1 can also contribute to the biotransformation of CPF but its role in this process remains to be investigated. Since both GSTo and FMO1 are induced at 12 h, their regulation by the AhR is not likely but the involvement of PXR is just one out of several possibilities, including NRF2.

In general terms, the effects of CPF on gene expression were more prominent in the intestine than in the liver. Regarding this, Rao et al. (2005) reported that fish accumulate CPF mostly in the visceral organs (intestine, liver, heart, kidneys) followed by head and body. For example, in *J. multidentata* exposed to 0.0011 µM CPF, in the same order of magnitude as the concentration used in this study, the CPF accumulated was intestine > liver > gills (Bonansea et al. 2017). The fact that the intestinal mucosa is in direct contact with the CPF in the gut content suggests that the pesticide enters the enterocytes earlier than the hepatocytes. In addition, the CPF absorbed at the intestine or gills surface can be at least partially biotransformed before it reaches the liver.

5. Conclusions

Altogether, we have demonstrated that CPF induces the PXR pathway in *O. mykiss*, upregulating the expression of PXR, CYP2K, UGT and ABCC2 in the intestine and liver, at a nanomolar concentration, which is not only environmentally relevant, but also in the

concentration range of the potent nuclear receptor inducers. Our results support a potential regulatory crosstalk between PXR and AhR pathways, where the induction of the PXR pathway coincides with the downregulation of AhR-mediated CYP mRNA expression and EROD activity in the intestine.

All these effects are more significant in the intestine than in the liver. Besides, CPF inhibits ChE enzymatic activity, which may be serving as "suicide" buffer protein to decrease OP bioavailability in both tissues, and upregulates the expression of enzymes involved in biotransformation, such as FMO1 and GSTo.

Further *in vivo* and *in vitro* work is needed to establish CPF as a PXR agonist and to know the effects of the CPF-induced PXR pathway activation on the health of *O. mykiss* and its capacity to respond to other environmental challenges. It would also be interesting to investigate the mechanism involved in the crosstalk between AhR and PXR pathways and the downstream genes involved.

CRediT author statement

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Acknowledgments

This research was supported by CONICET, Argentina [PIP 11220130100529CO]; ANPCYT, Argentina [PICT-2018 2653]; FONDAP, Chile [15110027] and FONDECYT, Chile [1150934]. We thank our colleagues from the Institute of Biochemistry and Microbiology, Austral University of Chile (Valdivia, Chile) who provided technical expertise that greatly assisted the

research. We also thank material support from CEAN (Neuquén, Argentina) and thanks to Walter Torres for his help in keeping fish in good health condition.

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Table 1: Details of primer pairs and their amplicons used in the study.

Gene		Primer pair sequence 5' → 3'	Amplicon length (bp)	GenBankK accession
Pregnane X receptor	PXR	FW GGGCATCTGGGAGTGTGGTC RV GTAGGTTCCTCAGGGTGTAGTGG	116	EF517132
Cytochrome P450 3A27	CYP3A27	FW TCTGCTGATGCCCAAACGA RV CGTTGTTGGACTCTTCAGAGTGGTA	110	U96077
Glutathione-S-transferase omega 1	GST	FW AGCTGCTCCCAGCTCATCC RV CAAACCACGGCCACATCATGTAATC	246	NM_001160618.1
ATP-binding cassette B1/ P-Glycoprotein	ABCB1/Pgp	FW ACGTGCGCTCCCTGAACGTG RV GCGTTGGCCTCCCTAGCAGC	151	AY863423
UDP-glucuronosyl transferase	UGT	FW ATAAGGACCGTCCCATCGAG RV ATCCAGTTCAGGTCGTGAGC	113	DY802180
ATP-binding cassette C2/ Multidrug resistant protein 2	ABCC2/ MRP2	FW ATGCTGGGAAGATCGTGGAGTTTG RV GAGAGCTGTGCTATCCAATGCAGT	142	XM_021604527.1
Aryl hydrocarbon receptor	AhR	FW CGATGCCACTGAGTTCCAAACCAA RV AATGCCTGGTCTATGGGTAGCTGA	147	NM_001124252.1
Cytochrome P450 1A	CYP1A	FW ACCCAGTGGCAGGTCAACCATGAT RV CCCATGCCGAATACGAGCACTTT	134	XM_021607648.1
AhR nuclear translocator	ARNT	FW ACCTGAATGCAGAGCAATCCCA RV AGGGTGATTGAGGAAGAGCTGAGA	113	NM_001124710.1
Flavin Monooxygenase 1	FMO1	FW GGAAGTTCAAGGAAGTTTCTGAGCCC RV CCGCACACTGGTCTGGAAGTGGAT	212	NM_001124664.1
Cytochrome P450 2M1	CYP2M1	FW GCTGTATATCACACTCACCTGCTTTG RV CCCCTAAGTCCTTTGCATGTATAGAT	194	NC_035103.1
Cytochrome P450 2K1	CYP2K1	FW CTCACACCACCAGCCGAGAT RV CTTGACAAATCCTCCCTGCTCAT	164	XM_021558863.1
Beta-actin	β-actin	FW TGAAGTGTGACGTGGACATCCGTA RV AGGTGATCTCCTTCTGCATCCTGT	108	NW_018528586.1

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Highlights

- Chlorpyrifos induces the PXR pathway in *Oncorhynchus mykiss* liver and intestine
- Induction of PXR-related genes coincides with downregulation of AhR-related genes
- Chlorpyrifos induces a detoxification response at a nanomolar concentration

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