Comparative Biochemistry and Physiology, Part C 212 (2018) 47–55

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Effects of sequential exposure to water accommodated fraction of crude oil and chlorpyrifos on molecular and biochemical biomarkers in rainbow trout

Julieta S. De Anna[a,*], Leonardo R. Leggieri[a], Luis Arias Darraz[b], Juan G. Cárcamo[b,c], Andrés Venturino[d], Carlos M. Luquet[a,*]

[a] Laboratorio de Ecotoxicología Acuática, INIBIOMA– CONICET– CEAN, Ruta provincial 61, km 3, 8371 Junín de los Andes, Neuquén, Argentina
[b] Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Campus Isla Teja, Valdivia, Chile
[c] Centro FONDAP, Interdisciplinary Center for Aquaculture Research (INCAR), Chile
[d] Centro de Investigaciones en Toxicología Ambiental y Agrobiotecnología del Comahue, CITAAC, UNCo-CONICET, Instituto de Biotecnología Agropecuaria del Comahue, Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, Ruta 151, km 12, 8303 Cinco Saltos, Río Negro, Argentina

ARTICLE INFO

Keywords:
Acetylcholinesterase
Carboxylesterases
CYP1A
Oncorhynchus mykiss
Water-soluble fraction
AhR
ARNT
Chlorpyrifos

ABSTRACT

Fish can be simultaneously or sequentially exposed to various kinds of pollutants, resulting in combined effects. Polycyclic aromatic hydrocarbons induce cytochrome P450 monooxygenase 1A (CYP1A) expression, which catalyzes the conversion of the organophosphorus insecticide chlorpyrifos (CPF) into its most active derivative, CPF-oxon. CPF-oxon inhibits CYP1A and other enzymes, including carboxylesterases (CEs) and acetylcholinesterase (AChE). We studied the effects of an *in vivo* exposure to crude oil water accommodated fraction (WAF) followed by an *ex vivo* exposure of liver tissue to CPF on the expression of *Cyp1a*, *AhR and ARNT* mRNA, CYP1A protein and on the activity of biomarker enzymes in the rainbow trout (*Oncorhynchus mykiss*). Juvenile rainbow trout were exposed to WAF (62 μg L^{-1} TPH) for 48 h. Then, liver was dissected out, sliced and exposed to 20 μg L^{-1} CPF *ex vivo* for 1 h. Liver tissue was analyzed for mRNA and protein expression and for CEs, AChE, glutathione S-transferase (GST) and CYP1A (EROD) activity. WAF induced *Cyp1a* mRNA and CYP1A protein expression by 10-fold and 2.5–8.3-fold, respectively, with no effect of CPF. WAF induced *AhR* expression significantly (4-fold) in control but not in CPF treated liver tissue. *ARNT* mRNA expression was significantly lowered (5-fold) by WAF. CPF significantly reduced liver EROD activity, independently of WAF pre-treatment. CEs activity was significantly inhibited in an additive manner following *in vivo* exposure to WAF (42%) and *ex vivo* exposure to CPF (19%). CPF exposure inhibited AChE activity (37%) and increased GST activity (42%).

1. Introduction

In agricultural areas with growing oil extraction and industry such as those in North Patagonia, Argentina, exposure of aquatic organisms to hydrocarbons can be expected to occur throughout the year, while pesticides may reach dangerous concentrations mainly during the application season (Loewy et al., 2011; Monza et al., 2013). Exposure to a mix of different pollutants can produce combined effects which are challenging to predict due to chemical interactions and effects on detoxification mechanisms (Wassmur et al., 2012).

The North Patagonian region accounts for an important proportion of the Argentine gas and oil production (Monza et al., 2013). Conventional and nonconventional hydrocarbon production activities coincide with the Neuquén River basin (32,450 km^2), which in the lower basin locale includes irrigated areas with fruit production and the main cities of the region. Besides irrigation, the Neuquén River supplies water for nearly 400,000 inhabitants (Monza et al., 2013). Sampled sediments from 17 stations along the Neuquén River, including areas impacted by oil and gas production, agriculture and urban discharges between 2007 and 08 showed low levels of aliphatic hydrocarbons at several stations and almost no polycyclic aromatic hydrocarbons (PAHs) with the exception of naphthalene and pyrene (40 ng/g dw and 50 ng/g dw, respectively) at one site only. However, potentially contaminating activities related to hydrocarbon extraction, transport and processing have been greatly increased since 2010, when a large non-conventional oil and gas reserve (Vaca muerta) was discovered.

PAHs have been extensively studied as contaminants which can affect human and environmental health. These compounds can be present in the environment as a result of oil pollution, petroleum refining, organic material combustion, sewage and industrial discharges,

* Corresponding authors.
E-mail addresses: julideanna@gmail.com (J.S. De Anna), luquetc@comahue-conicet.gob.ar (C.M. Luquet).

https://doi.org/10.1016/j.cbpc.2018.07.003
Received 10 April 2018; Received in revised form 3 July 2018; Accepted 5 July 2018

vehicle exhaust and also from natural sources, such as forest fires, natural petroleum seepage and volcanism (reviewed by Abdel-Shafy and Mansour, 2016). PAHs can cause a variety of effects including interference with cell membrane functions, teratogenesis, carcinogenesis, mutagenesis and immunosuppression (Davila et al., 1996; Pelkonen and Nebert, 1982; Peluso et al., 2008; Uno et al., 2004). Among other chemicals, some PAHs induce the expression of cytochrome P450 oxidases, particularly those of the cytochrome P450 1A subfamily (CYP1A), which play an important role in the Phase I oxidative biotransformation of xenobiotics. These enzymes metabolize PAHs to epoxides which are highly toxic intermediates but can be detoxified by phase II enzymes such as Glutathione S-transferase (GST) and other transferases, and then excreted by phase III transporters (Baird et al., 2005). In fish ecotoxicology, the subfamily CYP1A is by far the most studied CYP isoform and one of the most studied detoxifying enzymes. CYP1A expression is normally low but is highly induced in fish exposed to several PAHs (Goksøyr et al., 1991; Di Giulio and Clark, 2015). Induction of CYP1A is mediated by the aryl hydrocarbon receptor (AhR) which resides in the cytoplasm linked to chaperone proteins. When AhR is activated by a ligand, it is released from the chaperones and translocated into the nucleus, where it dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR-ARNT heterodimer binds to xenobiotic response elements in the promoter of *Cyp1a* (and many other genes), inducing transcription and protein expression (Denison and Nagy, 2003). Increased levels of CYP1A induce phase I biotransformation of PAHs and other xenobiotics (Whyte et al., 2000). In relation to the effects of the exposure to crude oil hydrocarbons on the AhR pathway, a transcriptomic study by Whitehead et al. (2011) reported up-regulated transcription of *Ahr* and several Ahr targets (*e.g. Cyp1a*, UDP-glucuronosyltransferase; *UGT*) in Gulf killifish (*Fundulus grandis*) from a site impacted by the Deep Water Horizon oil spill.

Besides PAHs, CYP1A can transform xenobiotics, such as organophosphate insecticides (OPs) into more toxic derivatives. For example, in OPs with a thione group (P═S) like Chlorpyrifos (O, O-diethyl-O-3, 5, 6-trichlor-2-pyridyl phosphorothioate; CPF), CYP1A oxidizes the P═S group to the corresponding oxon (P═O); a derivative which is more active and less stable than the parent OP (Fukuto, 1990). Created CPF-oxons inhibit CYP1A activity (Neal, 1980), possibly affecting the metabolism of PAHs and other CYP1A substrates. CPF is a broad spectrum OP, which has caused unintended effects on aquatic organisms by aerial overspray or run-off (Somnuek et al., 2009) and is one of the most widely used insecticides in Argentina (Salgado Costa et al., 2018). CPF is more persistent than other Ops, with a half-life in water ranging from 29 to 74 days (Rivadeneira et al., 2013), which increases the risk of prolonged exposure of aquatic animals. CPF-oxon inhibits β-esterase enzymes, such as acetylcholinesterase and carboxylesterases (AChE, CEs) mostly by stoichiometric binding. AChE is the main target of OPs and other insecticides since its inhibition results in the accumulation of the neurotransmitter acetylcholine in the synaptic space, leading to severe neurotoxicity (Fulton and Key, 2001; Kwong, 2002; Sanchez-Hernandez, 2007; Sogorb and Vilanova, 2002). In general, CEs are more sensitive to OPs than AChE, and protect the organism from anticholinesterase effects by removing OPs through the hydrolysis of ester bonds and by binding to the OP with higher affinity than AChE (Jokanovic, 2001; Maxwell, 1992; Sanchez-Hernandez, 2007; Tang and Chambers, 1999; Wheelock et al., 2005). In addition, CPF and other Ops have been reported to increase oxidative stress and antioxidant responses in various fish tissues (*e.g.* Faria et al., 2015; Ferrari et al., 2007; Guerreño et al., 2016).

There is little information about how previous exposures to PAHs may affect the toxicity of CPF on fish. Clark and Di Giulio (2012) have reported that populations of the Atlantic killifish (*Fundulus heteroclitus*) chronically exposed to PAH down-regulate the AhR pathway and have lower susceptibility to CPF toxicity than reference populations. These authors suggest that, in resistant fish populations, the lack of induction of CYP1A expression reduces the CPF activation exerted by AhR agonists, as observed in the control population. In addition, Clark and Di Giulio (2012) have observed that PAH adapted killifish are more resistant to CPF in the absence of AhR agonists and to other chemicals which are detoxified by CYP1A. Related works reviewed by Di Giulio and Clark (2015) show that, in addition to a recalcitrant AhR, these fish have increased antioxidant capacity and phase II enzyme activity (including GST), and higher expression of the multixenobiotic resistance transporter P glycoprotein (Pgp, ABCB1), which may explain in part the resistance to many unrelated xenobiotics.

The rainbow trout, *Oncorhynchus mykiss*, has been introduced worldwide and is abundant in North Patagonian rivers and lakes where it can be exposed both simultaneously or subsequently to PAHs, inducing CYP1A expression, and to pesticides (such as CPF) which may be activated by CYP1A. The objective of this study is to investigate whether previous exposures to WAFs of crude oil affect *O. mykiss* liver *Cyp1a*, *AhR*, and *ARNT* mRNA expression, and CYP1A protein expression and activity. We also analyze whether the expected augment in CYP1A activity enhances the effects of a subsequent short-term exposure to CPF on CE, AChE, and GST activities in *ex vivo* liver preparations.

2. Materials and methods

2.1. Water-accommodated fraction of crude oil

The WAF was prepared immediately before each experiment, according to Singer et al. (2000), using 4.75 g of crude oil per L of Chimehuin River water (alkalinity 34 mg L^{-1}, conductivity 36 μS cm^{-1}, pH 7.6, 8.37 mg L^{-1} dissolved oxygen at a temperature of 10–12 °C). Crude oil was obtained from the oil spill of an abandoned exploitation, which has not been stopped and continues flowing at present into the La Mina stream, Río Negro Province, Argentina (41°17′21″ S - 71°10′58″ W). The oil sampled from surface seepage was characterized as immature heavy crude oil (Ro = 0.44–0.53%, American Petroleum Institute (API) = 18° and sulfur = 0.45%; Cazau et al., 2005), composed of 33.7% saturated hydrocarbons, 17.8% aromatic hydrocarbons, 5.9% asphaltenes and 42.6% NSOs (compounds with nitrogen, sulfur, oxygen and heavy metals; data provided by YPF S.A. Argentina). Samples were transported on ice to the laboratory of Aquatic Ecotoxicology, Centro de Ecología Aplicada del Neuquén (CEAN, Junín de Los Andes, Argentina) and kept at 4 °C in glass bottles, as recommended for PAH samples before analysis. The obtained WAF was analyzed by the method of the Environmental Protection Agency (USEPA) 3510C- 8015D GC-FID. After liquid-liquid extraction from 1 L, total petroleum hydrocarbons (TPH, C6 to C36, including 16 priority PAHs USEPA, without discrimination, (Supplementary File 1). were determined with a detection limit of 0.002 mg L^{-1}, and a quantitation limit of 0.010 mg L^{-1}. TPH (C6-C36) in WAF were 1.24 mg L^{-1} (CV < 7%). The experimental aquarium water was prepared by diluting WAF at 5% in Chimehuin river water, in order to obtain a nominal TPH concentration of 62 μg L^{-1}. This concentration is similar to the concentrations recorded by Leggieri et al. (2017) from 0 to 1600 m downstream from the oil spill in the La Mina stream, where abundant juvenile rainbow trout can be observed.

2.2. Chlorpyrifos

A standard CPF solution of 20 mg L^{-1} in acetone was prepared by dissolving 1 mg of CPF (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl phosphorothioate], 99% purity, Chem Service, West Chester, Pennsylvania, USA) in 50 mL of chromatographic quality grade acetone (Cicarrelli Reagents S.A, Argentina). The exact concentration of the standard solution was verified by gas chromatography (Agilent 6890 series, Wilmington, USA).

2.3. Animal preparation and experiments

Juvenile diploid *Oncorhynchus mykiss* (17.88 ± 0.87 g; 12.19 ± 0.29 cm; Mean ± SD) were obtained from the CEAN aquaculture facility. Before experimentation, fish were kept in a continuous flow system with Chimehuin river water. The Chimehuin river can be considered pollution free since it is the effluent of the Lake Huechulafquen, a deep glacial lake (surface area 70 km^2) in the Lanín National Park, where agricultural and other economic activities are not allowed (Federal Law 22351, Argentina). Between the lake and the CEAN facility, the river flows along 20 km through a semi-desert area with almost no human population and no agriculture or industrial activity. Fish received 1% body mass ratio of commercial trout feed per day. In the laboratory, fish were individually acclimated for 48 h in cylindrical 10 L containers with continuously aerated water from Chimehuin River in a static system (USEPA, 1996), at a temperature of 16–18 °C, pH 7.4–7.6 and 12 h light: 12 h dark photoperiod. Each experimental group comprised six fish housed in individual containers (n = 6). A factorial design experiment (2 factors × 2 levels) was conducted in 24 individual tanks in order to investigate the effects of *in vivo* pre-treatment with WAF (62 μg L^{-1} TPH concentration) for 48 h and subsequent *ex vivo* exposure of liver slices to 20 μg L^{-1} CPF for 1 h. This concentration, which is about twice the CPF lethal concentration 50 (LC50 96 h) for rainbow trout (9 μg L^{-1}, U. S. Environmental Protection Agency, 1996), was chosen for the *ex vivo* exposure in order to ensure an intracellular CPF concentration high enough to produce biochemical effects upon a short time exposure (1 h).

At the end of the pre-treatment period, fish were sacrificed by a blow to the head followed by decapitation. The liver of each fish was rapidly removed and rinsed in ice-cold Cortland saline (pH 7.4, 5 mmol L^{-1} $NaHCO_3$, 5.55 mmol L^{-1} glucose); a portion of about 200 mg was cut into thin slices and kept in small glass vessels with 6 mL of the same solution and constant aeration. Liver slices from 6 WAF exposed and 6 pre-treatment control fish were treated with 20 μg CPF L^{-1}. Liver slices from the remaining fish (6 from the WAF exposure and 6 from the pre-treatment control) were treated with 0.1% acetone (solvent control). After 1 h, the liver slices were removed, separated into sub-samples for mRNA expression, protein expression and enzyme activity analysis. Sub-samples were stored in RNA later (Ambion) at −30 °C or in liquid nitrogen with PBS-Triton X-100 (phosphate buffered saline; pH to 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 analysis (within 2 weeks). This experiment allowed the direct exposure of liver tissue from control and WAF exposed individuals to a controlled CPF concentration, in order to analyze possible interactions between both pollutants, avoiding possible CPF metabolization in other organs. Preliminary experiments showed no differences in GST and CEs activity between rainbow trout liver slices treated with either 0.1% acetone in Cortland saline or the Cortland saline control. All the experimental protocols were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

2.4. Cyp1a, AhR and ARNT mRNA expression by real time PCR (q-PCR)

Total RNA was extracted from liver tissue (100 mg) from three individuals randomly selected from each experimental group (n = 3) using Trizol reagent (Ambion), following the manufacturer's instructions. The yield and purity of the extracted total RNA was determined by UV spectrophotometry (A260/A280 and A260/A230 ratio). cDNA was synthesized using 2 μg of total RNA and RevertAid reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, U.S.A.). For qPCR, each reaction mixture contained 1 μL cDNA template, 0.5 μmol L^{-1} of each primer, 8 μL of water, and 10 L 2 × SYBR green q-PCR Master Mix (ThermoFisher Scientific, Waltham, MA, U.S.A.). The primer pairs used for Real-Time qPCR are shown in Table 1. Reactions were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). 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). High resolution melting analysis was performed by collecting data between 60 and 95 °C with a temperature interval of 0.3% (Supplementary File 2). B-actin was used as housekeeping gene. The target gene expression was calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. CYP1A protein expression

Protein extraction was performed as described by Hasselberg et al. (2008). Liver tissue samples (c.a. 50 mg each) from six individuals per treatment (n = 6) were homogenized with a pestle and then sonicated for 5–10 s on ice in 1 mL of RIPA buffer (50 mmol L^{-1} Tris–HCl, pH 7.4; 150 mmol L^{-1} NaCl, 1% Nonidet P-40, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} EGTA) supplemented with Protease Inhibitor Cocktail Set I (Calbiochem, MERCK, Germany) and 200 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF). Samples were frozen in liquid nitrogen, sonicated again and centrifuged at 21,500 × *g* for 30 min at 4 °C. The supernatants were collected and stored at −20 °C. Protein concentration was measured using a bicinchoninic acid (BCA) kit (Pierce Biotechnology, Rockford, IL). Protein samples from 50 μg of liver were separated by 8% SDS-PAGE for 30 min at 70 V and 90 min at 120 V. Electrophoresis was followed by blotting at 1.5 A for 21 min onto a nitrocellulose membrane, using a Trans-Blot Turbo device (BioRad). Non-specific binding sites were blocked for 3 h with 0.1% casein in 1*X* PBS. Anti-CYP1A mouse monoclonal antibody (C10-7, Abcam Inc., Cambridge, USA) and anti-β-actin rabbit antibody (Sigma-Aldrich) were diluted 1:3000 in antibody dilution buffer (0.1% Tween 20 and 0.1% casein in PBS). The nitrocellulose membrane was incubated with primary antibodies overnight at 4 °C with slow agitation. The membrane was then washed three times with PBS, 0.1% Tween 20 and incubated in the dark with IRDye 800CW labeled donkey anti-mouse and IRDye 700DX labeled goat anti-rabbit secondary antibodies (Rockland Immunochemical, Gilbertsville, PA, USA) diluted 1:10,000 in antibody buffer, for 40 min at room temperature. Membranes were rinsed with washing buffer and the signal was detected by infrared emission at 800 and 700 nm using ODYSSEY® CLx Imager (LI-COR Biotechnology, USA). Images were analyzed with Image Studio LiteVer 5.0 software (LI-COR Biotechnology, USA). AccuRuler RGB Prestained protein ladder (Maestrogen) was used as a molecular mass marker. The controls were performed by omitting either the primary or the secondary antibodies (Supplementary File 3). B-actin was used as housekeeping protein and loading control. The presence of a single product of 55 kDa was considered a positive result for CYP1A protein and a single product of 42 kDa was considered a positive result for β-actin. Relative CYP1A expression was calculated as the CYP1A signal/β-actin signal ratio.

2.6. Enzyme activity

2.6.1. EROD

7-ethoxy-resorufin O-deethylation (EROD) activity was measured according to Kennedy and Jones (1994). This method is based on the increase of the fluorescence in the reaction medium due to the transformation of 7-ethoxyresorufin (7-ER, standard substrate) into resorufin. Briefly, liver tissue samples (c.a. 150 mg each) from six individuals per treatment were homogenized in CaHBSS (Hank's Balanced Salt Solution with Calcium; Gibco®) pH 7.8, sonicated twice for 15–30 s on ice, and then centrifuged at 14,000 × *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. Each sample (n = 6) was analyzed in triplicate. Fluorescence was read every 42 s for 6 min with a fluorescence spectrophotometer (BioTek Synergy™ HT Multi-Mode

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

Gene		Primer pair sequence (5′-3′)	Amplicon length (pb)	Reference
AhR	FW	ggatgccactgagttccaaaccaa	147	NM_001124252.1
	RV	aatgcctggtctatgggtagctga		
ARNT	FW	acctgaatgcagagcaatccca	113	NM_001124710.1
	RV	agggtgattgaggaagagctgaga		
Cyp1a	FW	aaccagtggcaggtcaaccatgat	134	NM_001123687.1
	RV	cccatgccgaatacgagcacttt		
B-actina	FW	tgaagtgtgacgtggacatccgta	108	Cárcamo et al., 2011
	RV	aggtgatctccttctgcatcctgt		

Microplate Reader), at excitation and emission wavelengths of 530 and 620 nm, respectively. EROD activity was expressed as pmol of resorufin mg protein^{-1} min^{-1}, using resorufin standards for calibration. Total protein content was determined using the Pierce™ BCA Protein Assay Kit for EROD activity.

2.6.2. Glutathione S-transferase

For measuring glutathione S-transferase (GST) activity, 0.5 g of liver tissues were homogenized in 1 mL of homogenization buffer (20 mmol L^{-1} Tris–HCl, pH 7.5, 0.5 mmol L^{-1} EDTA and PMSF) and then centrifuged at 11,000 × *g* for 15 min at 4 °C. Supernatants were stored at −20 °C and enzyme activity was measured within 24 h. GST activity was measured at 340 nm following the methodology described by Habig et al. (1974). Each sample was measured in triplicate, using 15 µL of supernatant per tube, at 25 °C, using 1-chloro-2, 4-dinitrobenzene (CDNB) 100 mmol L^{-1} dissolved in ethanol as the substrate). Specific GST activity was calculated using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1} and referred to total protein, which was measured by Bradford's method (1976).

2.6.3. Acetylcholinesterase

Acetylcholinesterase (AChE) activity was measured in duplicate, using 200 µL of liver 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 the substrate, according to Ellman et al. (1961). Absorbance at 412 nm was recorded for 1 min every 10 s at 25 °C. Results were referred to total protein, which was measured by Bradford's method (1976).

2.6.4. Carboxylesterase

CE activity was determined in duplicate using p-nitro-phenyl butyrate (p-NPB) as the substrate, according to Ferrari et al. (2007). Reactions were performed in 2.5 mL of 100 mmol L^{-1} phosphate buffer, pH 8.0, containing 5% acetone and 1 mmol L^{-1} p-NPB (molar extinction coefficient, 18.6 mM^{-1} cm^{-1}). Absorbance was measured at 400 nm for 1 min every 10 s at 25 °C. Results were referred to total protein, measured by Bradford's method (1976).

2.7. Data analysis

Statistical analyses were performed using SPSS 11.5 (Inc., Chicago, Illinois, USA), under the License from National University of Luján, Argentina. All data were expressed as the mean ± standard deviation. Experimental data were checked for normality and homogeneity of variance using the Kolmogorov–Smirnov one-sample test and Levene's test, respectively. Protein expression data were normalized by log (x + 1) transformation. Enzyme activities, mRNA expression and CYP1A protein expression (CYP1A/β-actin) data were analyzed by two-way ANOVA. The explanatory variables in the two-way ANOVA were WAF *in vivo* exposure (pre-treatment control *vs*. WAF) and CPF *ex vivo* exposure (Solvent control *vs*. CPF). Enzyme activity data were also analyzed by *ad hoc* orthogonal contrasts. When the interaction term of the ANOVA was significant; Tukey HSD posttest comparisons were performed.

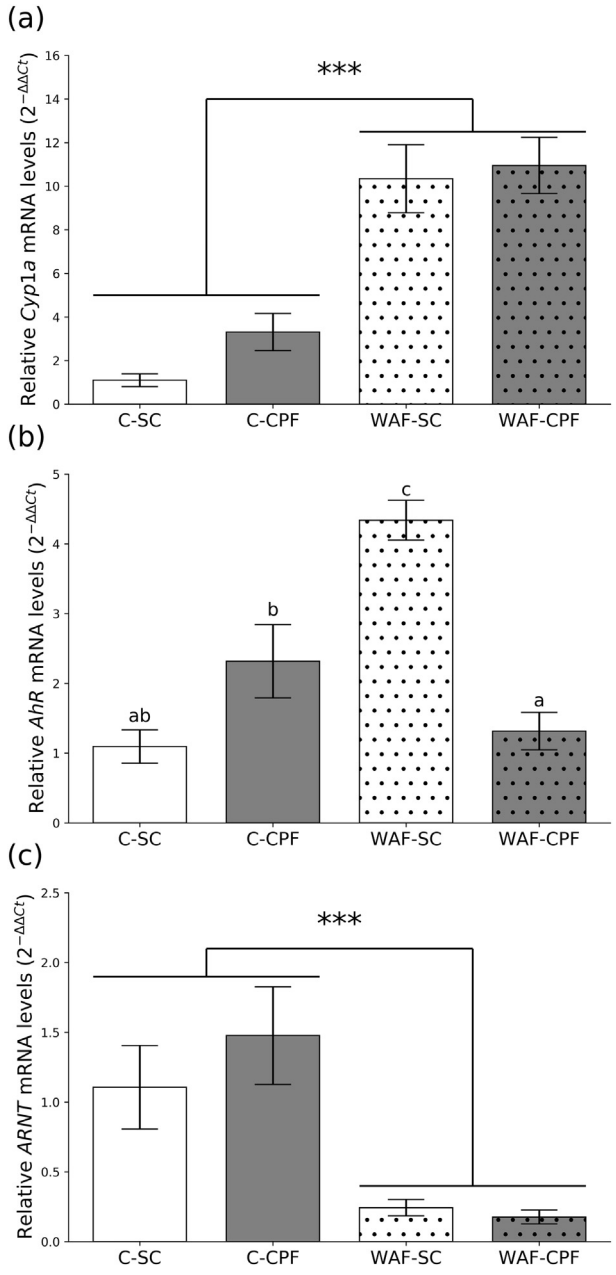

Fig. 1. Relative mRNA levels. ($2^{-\Delta\Delta Ct}$) of *Cyp1a* (a), *AhR* (b) and *ARNT* (c) in liver samples from rainbow trout (*Oncorhynchus mykiss*) exposed to 62 µg L^{-1} water accommodated fraction (WAF) or to control medium (C) and subsequently exposed *ex vivo* (liver slices) to chlorpyrifos (CPF) or to solvent control (acetone, SC) Two-way ANOVA, $^{***}P < 0.001$ for WAF effects. Different letters indicate significant differences between group means (*post hoc* Tukey's multiple comparisons). Values are expressed as mean ± SEM (n = 3).

3. Results

3.1. Cyp1a, AhR and ARNT mRNA expression

Cyp1a mRNA expression was significantly induced in liver slices from fish pre-treated with WAF *in vivo* compared with non-pre-treated fish (control, C) (two-away ANOVA $P < 0.001$, $F = 38.69$ for WAF effect). There were no significant interaction or CPF effects. Liver slices from WAF pre-treated fish and subsequently exposed to solvent control

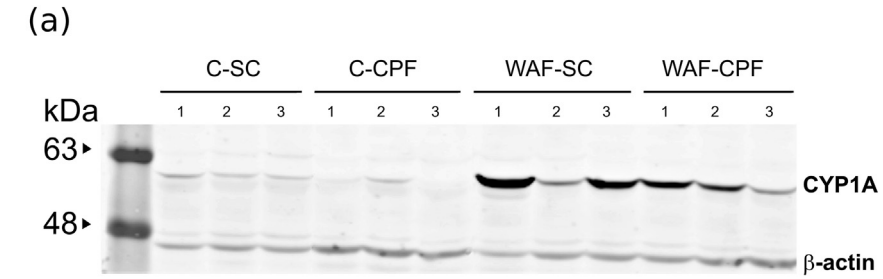

Fig. 2. (a) Scan of a representative Western blot membrane (showing three out of six replicates) for CYP1A protein in liver samples from rainbow trout (*Oncorhynchus mykiss*) exposed to 62 µg L^{-1} water accommodated fraction (WAF) or to control medium (C) and subsequently exposed *ex vivo* (liver slices) to chlorpyrifos (CPF) or to solvent control (acetone, SC) (b) Relative expression of CYP1A protein (CYP1A/β-actin ratio). Two-way ANOVA: ***P < 0.001 for WAF effects. Values are expressed as mean ± SEM (n = 6).

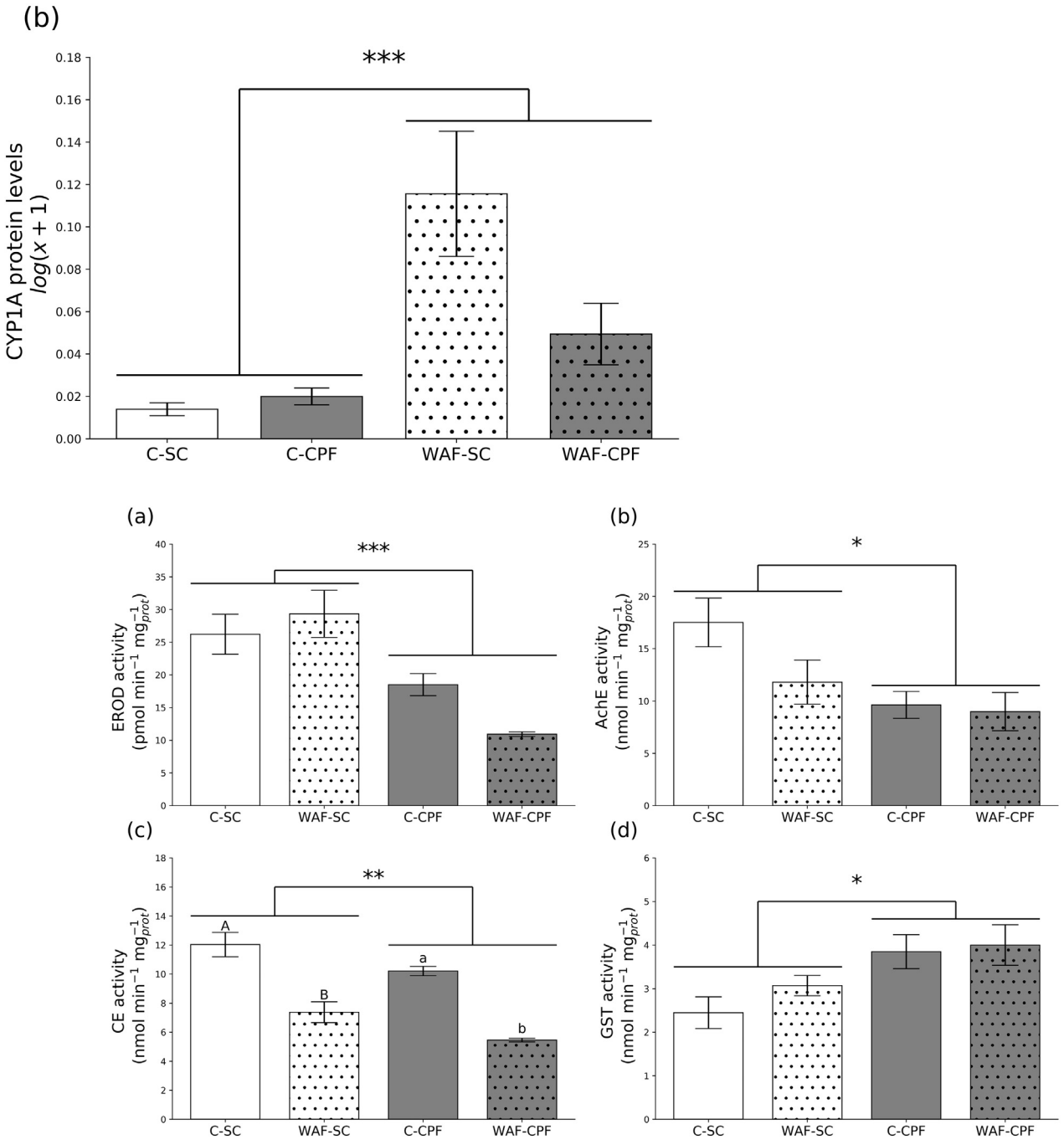

Fig. 3. Effects of water accommodated fraction (WAF) and Chlorpyrifos (CPF) on liver EROD activity (*a*), AChE (*b*), CEs (*c*) and GST (d). The experimental groups include control fish (C) and fish pre-treated with 62 µg L^{-1} WAF for 48 h (WAF), which were then sacrificed and their livers sliced and exposed *ex vivo* to a solvent control of treatment (C-SC and WAF-SC) or to 20 µg L^{-1} Chlorpyrifos (C-CPF and WAF-CPF). Two-way ANOVA: ***P < 0.001; **P < 0.01 and *P < 0.05 for CPF effects. In (*c*), different uppercase and lowercase letters indicate significant effects of WAF in the orthogonal contrasts C-SC *vs.* WAF-SC and C-CPF *vs.* WAF-CPF, respectively. Results are shown as mean ± SEM (n = 6).

(WAF-SC) or to CPF (WAF-CPF) showed similar increments (about 10-fold) in *Cyp1a* mRNA expression with respect to C-SC (Fig. 1a). Liver slices from WAF pre-treated fish showed significantly higher *AhR* mRNA expression than those from control fish (two-away ANOVA $P < 0.05$, $F = 6.896$ for WAF effect). Interaction was also significant ($F = 24.7$, $P < 0.005$) and there was no significant effect of CPF. Tukey's multiple comparisons showed that only in the WAF-SC group, *AhR* mRNA expression was significantly induced (about 4-fold with respect to C-SC, $P < 0.01$) (Fig. 1b). There was no induction by WAF in the WAF-CPF group, which showed an *AhR* expression even lower than that of C-CPF ($P < 0.05$). In contrast, mRNA expression of *ARNT* in liver slices from fish pre-treated with WAF was reduced to < 20% of those from control fish (two-away ANOVA $P < 0.001$, $F = 14.34$ for WAF effect). There were no significant interaction or CPF effects (Fig. 1c).

3.2. CYP1A protein expression

CYP1A protein levels in liver slices from fish pre-treated with WAF *in vivo* were significantly higher than in those from control fish. Liver slices from WAF-SC and WAF-CPF showed 8.3-fold and 2.5-fold higher CYP1A protein levels than their respective controls (C-SC and C-CPF) (Fig. 2b). No significant effects of subsequent *ex vivo* exposure to CPF or interaction were detected (Two-way ANOVA: $F = 12.4$, $P < 0.001$, for WAF effects; $F = 2.62$, $P = 0.125$, for CPF; and $F = 3.77$, $P = 0.070$ for interaction).

3.3. Enzyme activity

3.3.1. EROD

EROD activity was lower in liver slices exposed to CPF than in solvent control ones, with an average reduction of 47% (ANOVA $F = 21.51$, $P < 0.001$ for CPF effect). There were no significant effects of WAF pre-treatment or interaction between treatments (Fig. 3a).

3.3.2. Acetylcholinesterase

Liver acetylcholinesterase activity was inhibited by CPF ($F = 6.45$, $P < 0.05$), irrespective of the pre-treatment (control or WAF). AChE activity was 37% lower in liver slices treated with CPF than in solvent controls. No statistically significant effects of WAF or interaction between treatments were detected (Fig. 3b).

3.3.3. Carboxylesterase

Liver CE activity was significantly reduced by WAF as well as by CPF, without significant interaction effects. This enzyme's activity was reduced by CPF (19% in average, $F = 8.74$, $P < 0.01$). In addition WAF inhibited CE activity by 42% in average ($F = 55.25$, $P < 10^{-6,}$ different small letter indicate significant effect of WAF according to two-way ANOVA, Fig. 3c). The effects of both treatments were additive, resulting in 55% lower liver CE activity in WAF-CPF than in C-SC (Fig. 3c).

3.3.4. Glutathione S-transferase

Glutathione S-transferase activity was similarly increased by CPF in liver slices from WAF-pre-treated and control groups (42%, ANOVA $F = 8.14$, $P < 0.01$). There were no significant effects of WAF or interaction between WAF and CPF (Fig. 3d).

4. Discussion

This paper reports the effects of sequential exposure of juvenile rainbow trout to WAF (*in vivo* for 48 h) and CPF (*ex vivo* liver preparations) on key hepatic enzymes involved in xenobiotic detoxification and/or in PAH and CPF toxicity in the rainbow trout. The activation of AHR signaling pathway by WAF exposure was analyzed by qPCR.

4.1. Cyp1a, AhR and ARNT mRNA and CYP1A protein expression

WAF prepared from the La Mina stream oil spill induced expression of *Cyp1a*/CYP1A mRNA and protein in rainbow trout liver by 9- and 3–8-fold, respectively. These results are of comparable magnitude as those seen in primary cultured rainbow trout hepatocytes treated with β-naphthoflavone (βNF) for 24 h (3.5- and 5-fold induction of *Cyp1a* mRNA and CYP1A protein expression, respectively, Wiseman and Vijayan, 2007). However this is a modest induction compared to those obtained in the liver of rainbow trout and other fish species exposed to other AhR agonists, including a 110–200-fold increase in rainbow trout *Cyp1a* mRNA expression after exposure to 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) for 24 h (Jönsson et al., 2010); a 62.5-fold increase in rainbow trout injected with the PAH B[*a*]Pyrene (B[*a*]P) (Woźny et al., 2010) and a 418-fold increase in CYP1A protein expression in Atlantic killifish injected with the PAH 3-methylcholanthrene (Van Veld and Westbrook, 1995).

AhR mRNA expression was also induced (4–fold) by WAF exposure while *ARNT* expression was reduced to < 20% of the control. Induction of *AhR* upon treatment with AhR agonists has been reported for several fish species including rainbow trout (Abnet et al., 1999; Andreasen et al., 2002; Hanno et al., 2010; Tanguay et al., 1999; Wiseman and Vijayan, 2007; Woźny et al., 2010). While other fish species have two *AhR* genes (*AhR1* and *AhR2*), the rainbow trout lacks *AhR1* but possess three *AhR2* forms (*AhR2α AhR2β* and *AhR2δ*). The former two have been well characterized and show induction by different Ahr agonists (Abnet et al., 1999; Wiseman and Vijayan, 2007; Woźny et al., 2010). The latter authors have reported induction of *Ahr2α* and *Ahr2β* mRNA by B[*a*]P in the same order as the obtained in our experiment (5.9- and 6.9-fold, respectively *vs*. 4-fold). Recently, Bak et al. (2017) have identified three potential xenobiotic responsive elements (XREs) in the red seabream (*Pagrus major*) *rsAhR1* and rs*AhR2* genes. Besides, these authors showed that the XREs found in the rs*AhR2* gene sequence were able to mediate TCDD-induced *Ahr2* transcription and suggested that AhR auto-induction could amplify the signal for regulating downstream genes. Our results suggest that the PAHs present in WAF promote rainbow trout *Ahr2* auto-induction, which results in increased CYP1A expression although the lack of *AhR* induction in WAF-CPF treated fish is difficult to explain. The down-regulation of *ARNT* mRNA would reduce the formation of the AhR-ARNT heterodimer, which would reduce the induction of downstream genes, if the abundance of ARNT were a limiting factor. This ARNT down-regulation could also affect the crosstalk of the AhR pathway with other pathways such as the hypoxia induced factor (HIF-1α), which also depend on dimerization with ARNT (Fleming et al., 2009: Mandl et al., 2016). In a study performed with liver samples from wild Baikal seals chronically exposed to polychlorinated dibenzo-p-dioxins, dibenzofurans and PCBs pollution, Kim et al. (2005) reported positive and negative correlation of *AhR* and *ARNT* mRNA expression, respectively, with CYP1A and CYP1B protein expression. These authors suggested that CYP1A/B metabolites could downregulate ARNT. Although ARNT has been described for several fishes including the rainbow trout (Powell et al., 1999; Tanguay et al., 1999), little is known about the effects of AHR agonists on its expression. We have detected a clear down-regulation of *ARNT* mRNA in the same experimental groups in which the AHR pathway was activated. Since we did not detect any induction of EROD activity, the down-regulation of *ARNT* by CYP1A metabolites seems unlikely. *ARNT* has been considered as constitutively expressed although this notion has recently been revised for cancer cell lines (reviewed by Mandl and Depping, 2014). These authors have posteriorly demonstrated that the HIF-1α-ARNT heterodimer binds to specific sites in the *ARNT* promoter region inducing its transcription, in human hepatocellular carcinoma Hep3B cells (Mandl and Depping, 2017). In contrast, there is little or no information about the regulation of fish *ARNT* transcription. Calò et al. (2014) have reported that *in vivo* exposure to PCB126 induces both, AhR and ARNT protein expression in liver of the seabream (*Spaurus*

aurata). This is contrasting with the downregulation of *ARNT* mRNA observed in the present work. However, these studies are not totally comparable since increased protein level does not necessary imply increased transcription. Based on our results and on the literature on cancer cell lines (*e.g.* Mandl and Depping, 2014, 2017), we can speculate that rainbow trout *ARNT* transcription could be downregulated by WAF exposure, either through the AhR signaling pathway or through other pathways which involve ARNT interactions. Additionally, changes in RNA stability cannot be disregarded.

4.2. EROD activity

It was expected that the increased CYP1A protein expression described above would be associated with an increase in enzyme activity. However, EROD activity was not significantly increased by WAF exposure. According to the supplier, the antibody used in this work (Abcam 10-7) detects both CYP1A1 and CYP1A2 mouse proteins and also reacts with rainbow trout CYP1A. Since mammalian CYP1A2 displays 7-methoxy-resorufin *O*-demethylation (MROD) rather than EROD activity, the induction of protein expression by WAF with no increase in EROD activity recorded in this work could be explained by the induction of a rainbow trout CYP1A protein with little or no EROD activity. In this sense, Gooneratne et al. (1997) have characterized two rainbow trout *Cyp1* genes (*Cyp1a1* and *Cyp1a3*) and Jönsson et al. (2010) have reported *Cyp1a3* but not *Cyp1a1* induction in rainbow trout caged at a polluted site. In addition, Liu et al. (2013) have reported that rainbow trout CYP1A3 is about 10-fold more sensitive to TCDD than CYP1A1. However, since Gooneratne et al. (1997) have shown that the protein products of rainbow trout *Cyp1a1* and *Cyp1a3* genes display similar EROD and MROD activities, the lack of EROD activity induction in our experiment cannot be explained by differential expression of a particular *Cyp1a* gene. Thus, the most likely explanation for this result is that, besides PAHs, which induce AHR-mediated CYP1A expression, the utilized WAF could also contain other compounds, such as high molecular weight PAHs and alkylphenols, which can inhibit EROD activity (Melbye et al., 2009).

An increase in CYP1A activity would result in higher rate of conversion of CPF into its active form, CPF-oxon (Tang et al., 2001), as it was reported for humans (Croom et al., 2010; Sams et al., 2004). In turn, the sulfur ion released in CPF activation is highly reactive and is believed to bind immediately to the heme iron of CYP molecule, inhibiting its activity (Neal, 1980; Flammarion et al., 1998; Tang et al., 2002). In rainbow trout liver slices, we have found that 1 h of *ex vivo* exposure to CPF inhibits EROD activity in preparations from both control and WAF pre-treated individuals. Although there is a tendency to a higher percentage inhibition of EROD activity by CPF in liver preparations from WAF pretreated fish (63% and 29% for WAF-CPF and C-CPF, respectively), although this effect is not statistically significant.

4.3. AChE, CE, and GST activity

Acetylcholinesterases are considered as the specific targets of OPs and other anticholinesterase agents (Sanchez-Hernandez, 2007). For CPF and other OPs, this effect is enhanced by the formation of OP-oxon catalyzed by CYP1A (Chambers and Carr, 1996). *O. mykiss* liver slices exposed *ex vivo* to CPF for 1 h showed significantly reduced AChE activity with respect to liver slices exposed to the solvent control. This effect was similar in preparations obtained from control and WAF pretreated individuals and the ANOVA showed no interaction effects. Thus, there is no evidence in this work about additive or synergistic effects of CPF and WAF on AChE activity. These results agree with the lack of change recorded in EROD activity and allow us to speculate that the CYP1A protein induced in our experiments displays low or no EROD or CPF-oxon formation activity.

In many species CEs have higher affinity for OPs than AChEs. Therefore CEs would be preferentially inhibited over AChE following exposure to OPs (Gupta and Dettbarn, 1993; Wogram et al., 2001; Wheelock et al., 2005). The lack of interaction effects between WAF and CPF indicates no synergistic or antagonistic effects of both toxicants. This agrees with the lack of induction of EROD activity and coincides with the results observed for AChE. However, WAF and CPF have shown additive inhibitory actions on CEs activity, as evidenced by the significant effects of both treatments and by the significantly lower CEs activity recorded in the WAF-CPF group with respect to C-CPF. These effects are probably mediated by different mechanisms, taking into account the differential time and modes of exposure to both toxicants. Moreover, the additive effect of WAF and CPF on CEs inhibition highlights the fitness of CEs activity as a biomarker, reflecting in this case reduced detoxification capacity upon exposure to a mixture of contaminants.

Environmental toxicants detoxified by GST include PAH, pesticides, and reactive intermediates produced by phase I biotransformation and other biochemical reactions. In general terms, there are abundant reports on increased GST activity in aquatic organisms in response to a range of xenobiotics (Ferrari et al., 2007; Guerreño et al., 2016; Hayes et al., 2005), indicating an evolutionarily conserved response. GST actively detoxifies OP pesticides as well as their oxon metabolites (Testai et al., 2010). However, reports on CPF effects on fish GST activity are scarce and show mostly no significant effects in short-term studies (6 to 96 h) (Botté et al., 2012; Bonifacio et al., 2017; Kavitha and Rao, 2008), and induction of GST in *Cyprinus carpio* liver after 40 d CPF exposure (Xing et al., 2012). Our results indicate that liver GST activity in *O. mykiss* is not affected by 48 h *in vivo* exposure to WAF but is induced by 1 h CPF exposure independently of the pre-treatment, which suggests a rapid detoxifying response against this toxicant.

We have performed a sequential exposure design in order to study the possible interaction effects of WAF and CPF on crucial detoxifying and target enzymes. Clark and Di Giulio (2012) have found that a PAH-adapted population of the killifish *F. heteroclitus* which shows no induction of CYP1A by AHR agonists, has increased resistance to CPF, probably due to reduced rate of CPF-oxon formation. Additionally, they suggested that other defense molecules, namely other CYPs, phase II enzymes such as GST, and ATP-binding cassette proteins, would be involved in the resistance against a variety of pesticides and other xenobiotics. In contrast, our results show an induction of the AHR pathway in *O. mykiss* liver by 48 h-exposure to WAF does neither increase EROD activity nor the sensitivity to CPF.

In conclusion, exposure to WAF activates the AHR-CYP1A pathway by inducing the transcription of *AhR* and *Cyp1a* and the expression of CYP1A protein in *O. mykiss* liver but also reducing the expression of *ARNT*. This activation is not accompanied by an increase in EROD activity; and therefore CPF effects on acetylcholinesterase or carboxylesterases are not reinforced by WAF exposure. On the other hand, WAF and CPF produce additive inhibitory effects on carboxylesterase activity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2018.07.003.

Acknowledgements

We acknowledge material support from CEAN (Neuquén Province, Argentina), and thank Walter Torres for his kind help with fish and aquaria. In addition, we would like to thanks to Federico Elias of Instituto Balseiro for performing graphics. We appreciate the strong support from the staff of the Institute of Biochemistry and Microbiology, Austral University of Chile (Valdivia, Chile), especially to Alejandra Mancilla Nova and Tamara Vera for their kind help in the laboratory work. We also acknowledge the detailed and constructive comments by two anonymous reviewers. This work was supported by CONICET, Argentina [PIP 11220130100529 CO]; ANPCYT, Argentina [PICT 2013-1415]; FONDAP, Chile [15110027] and FONDECYT, Chile [1150934].

References

Abdel-Shafy, H.I., Mansour, M.S.M., 2016. A review on polycyclic aromatic hydrocarbons: source, environmental impact, effect on human health and remediation. Egypt. J. Pet. 25, 107–123. https://doi.org/10.1016/j.ejpe.2015.03.011.

Abnet, C.C., Tanguay, R.L., Hahn, M.E., Heideman, W., Peterson, R.E., 1999. Two forms of aryl hydrocarbon receptor type 2 in rainbow trout (*Oncorhynchus mykiss*). Evidence for differential expression and enhancer specificity. J. Biol. Chem. 274, 15159–15166.

Andreasen, E.A., Hahn, M.E., Heideman, W., Peterson, R.E., Tanguay, R.L., 2002. The zebrafish (*Danio rerio*) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor. Mol. Pharmacol. 62, 234–249. https://doi.org/10.1124/mol.62.2.234.

Baird, W.M., Hooven, L.A., Mahadevan, B., 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ. Mol. Mutagen. 45, 106–114. https://doi.org/10.1002/em.20095.

Bak, S.M., Iida, M., Soshilov, A.A., Denison, M.S., Iwata, H., Kim, E.Y., 2017. Auto-induction mechanism of aryl hydrocarbon receptor 2 (AHR2) gene by TCDD-activated AHR1 and AHR2 in the red seabream (*Pagrus major*). Arch. Toxicol. 91 (1), 301–312. https://doi.org/10.1007/s00204-016-1732-9.

Bonifacio, A.F., Ballesteros, M.L., Bonansea, R.I., Filippi, I., Amé, V.M., Hued, A.C., 2017. Environmental relevant concentrations of a chlorpyrifos commercial formulation affect two neotropical fish species, *Cheirodon interruptus* and *Cnesterodon decemmaculatus*. Chemosphere 188, 486–493. https://doi.org/10.1016/j.chemosphere.2017.08.156.

Botté, E.S., Jerry, D.R., Codi King, S., Smith-Keune, C., Negri, A.P., 2012. Effects of chlorpyrifos on cholinesterase activity and stress markers in the tropical reef fish *Acanthochromis polyacanthus*. Mar. Pollut. Bull. 65, 384–393. https://doi.org/10.1016/j.marpolbul.2011.08.020.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Calò, M., Licata, P., Bitto, A., Lo Cascio, P., Interdonato, M., Altavilla, D., 2014. Role of AHR, AHRR and ARNT in response to dioxin-like PCBs in *Spaurus aurata*. Environ. Sci. Pollut. Res. Int. 21, 14226–14231. https://doi.org/10.1007/s11356-014-3321-x.

Cárcamo, J.G., Aguilar, M.N., Barrientos, C.A., Carreño, C.F., Quezada, C.A., Bustos, C., Manríquez, R.A., Avendaño-Herrera, R., Yañez, A.J., 2011. Effect of emamectin benzoate on transcriptional expression of cytochromes P450 and the multidrug transporters (Pgp and MRP1) in rainbow trout (*Oncorhynchus mykiss*) and the sea lice *Caligus rogercresseyi*. Aquaculture 321, 207–215. https://doi.org/10.1016/j.aquaculture.2011.09.012.

Cazau, L., Cortiñas, J., Reinante, S., Asensio, M., Bechis, F., Apreda, D., 2005. Cuenca de Ñirihuau. In: Chebli, G.A., Cortiñas, J., Spalletti, L.A., Legarreta, L., Vallejo y, E.L. (Eds.), Frontera Exploratoria de la Argentina. 6° Congreso de Exploración y Desarrollo de Hidrocarburos Actas, Mar del Plata, pp. 251–273.

Chambers, R.L., Carr, J.E., 1996. Kinetic analysis of the in vitro inhibition, aging, and reactivation of brain acetylcholinesterase from rat and channel catfish by paraoxon and chlorpyrifos-oxon. Toxicol. Appl. Pharmacol. 139, 365–373. https://doi.org/10.1006/taap.1996.0177.

Clark, B.W., Di Giulio, R.T., 2012. *Fundulus heteroclitus* adapted to PAHs are cross-resistant to multiple insecticides. Ecotoxicology 21, 465–474. https://doi.org/10.1007/s10646-011-0807-x.

Croom, E.L., Wallace, A.D., Hodgson, E., 2010. Human variation in CYP-specific chlorpyrifos metabolism. Toxicology 276, 184–191. https://doi.org/10.1016/j.tox.2010.08.005.

Davila, D.R., Romero, D.L., Burchiel, S.W., 1996. Human T cells are highly sensitive to suppression of mitogenesis by polycyclic aromatic hydrocarbons and this effect is differentially reversed by alpha-naphthoflavone. Toxicol. Appl. Pharmacol. 139, 333–341. https://doi.org/10.1006/taap.1996.0173.

Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309–334. https://doi.org/10.1146/annurev.pharmtox.43.100901.135828.

Di Giulio, R.T., Clark, B.W., 2015. The Elizabeth river story: a case study in evolutionary toxicology. J. Toxicol. Environ. Health B Crit. Rev. 18 (6), 259–298. https://doi.org/10.1080/15320383.2015.1074841.

Ellman, L., Courtney, K.D., Andreas Jr., V., Featherstone, R.M., 1961. A new rapid colorimetric determination of cholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Faria, M., Garcia-Reyero, N., Padrós, F., Babin, P.J., Sebastián, D., Cachot, J., Prats, E., Arick Ii, M., Rial, E., Knoll-Gellida, A., Mathieu, G., Le Bihanic, F., Escalon, B.L., Zorzano, A., Soares, A.M., Raldúa, D., 2015. Zebrafish models for human acute organophosphorus poisoning. Sci. Rep. 5, 1559. https://doi.org/10.1038/srep15591.

Ferrari, A., Venturino, A., Pechén De Angelo, A.M., 2007. Effects of carbaryl and azinphos methylon juveniles rainbow trout (*Oncorhynchus mykiss*) detoxifying enzymes. Pestic. Biochem. Physiol. 88, 134–142. https://doi.org/10.1016/j.pestbp.2006.10.005.

Flammarion, P., Migeon, B., Urios, S.B., Morfin, P., Garric, J., 1998. Effect of methidathion on the cytochrome P-450 1A in the cyprinid fish gudgeon (*Gobio gobio*). Aquat. Toxicol. 42, 93–102. https://doi.org/10.1016/S0166-445X(98)00046-0.

Fleming, C.R., Billiard, S.M., Di Giulio, R.T., 2009. Hypoxia inhibits induction of aryl hydrocarbon receptor activity in topminnow hepatocarcinoma cells in an ARNT-dependent manner. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 150 (3), 383–389. https://doi.org/10.1016/j.cbpc.2009.06.003.

Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspect. 87, 245–254. https://doi.org/10.1289/ehp.9087245.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ. Toxicol. Chem. 20, 37–45. https://doi.org/10.1002/etc.5620200104.

Goksøyr, A., Andersson, T., Buhler, D.R., Stegeman, J.J., Williams, D.E., Forlin, L., 1991. Immunochemical cross-reactivity of i-naphthoflavone-inducible cytochrome P450 (P4501A) in liver microsomes from different fish species and rat. Fish Physiol. Biochem. 9, 1–13. https://doi.org/10.1007/BF01987606-.

Gooneratne, R., Miranda, C.L., Henderson, M.C., Buhler, D.R., 1997. β-Naphthoflavone induced CYP1A1 and 1A3 proteins in the liver of rainbow trout (*Oncorhynchus mykiss*). Xenobiotica 27, 175–187. https://doi.org/10.1080/004982597240677.

Guerreño, M., López Armengol, M.F., Luquet, C.M., Venturino, A., 2016. Comparative study of toxicity and biochemical responses induced by sublethal levels of the pesticide azinphosmethyl in two fish species from North-Patagonia, Argentina. Aquat. Toxicol. 177, 365–372. https://doi.org/10.1016/j.aquatox.2016.06.015.

Gupta, R.C., Dettbarn, W.D., 1993. Role of carboxylesterases in the prevention and potentiation of N-methylcarbamate toxicity. Chem. Biol. Interact. 87, 295–303. https://doi.org/10.1016/0009-2797(93)90057-6.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hanno, K., Oda, S., Mitani, H., 2010. Effects of dioxin isomers on induction of AhRs and CYP1A1 in early developmental stage embryos of medaka (*Oryzias latipes*). Chemosphere 78 (7), 830–839. https://doi.org/10.1016/j.chemosphere.2009.11.043.

Hasselberg, L., Westerberg, S., Wassmur, B., Celander, M.C., 2008. Ketoconazole, an antifungal imidazole, increases the sensitivity of rainbow trout to 17 alpha-ethynylestradiol exposure. Aquat. Toxicol. 86, 256–264. https://doi.org/10.1016/j.aquatox.2003.12.007.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2005. Glutathione transferases. Annu. Rev. Pharmacol. Toxicol. 45, 51–88. https://doi.org/10.1146/annurev.pharmtox.45.120403. 095857.

Jokanovic, M., 2001. Biotransformation of organophosphorus compounds. Toxicology 166, 139–160. https://doi.org/10.1016/S0300-483X(01)00463-2.

Jönsson, E.M., Gao, K., Olsson, J.A., Goldstone, J.V., Brandt, I., 2010. Induction patterns of new CYP1 genes in environmentally exposed rainbow trout. Aquat. Toxicol. 98, 311–321. https://doi.org/10.1016/j.aquatox.2010.03.003.

Kavitha, P., Rao, J.V., 2008. Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, *Gambusia affinis*. Environ.Toxicol. Pharmacol. 26, 192–198. https://doi.org/10.1016/j.etap.2008.03.010.

Kennedy, S.W., Jones, S.P., 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. Anal. Biochem. 222, 217–223. https://doi.org/10.1006/abio.1994.1476.

Kim, E.Y., Iwata, H., Suda, T., Tanabe, S., Amano, M., Miyazaki, N., Petrov, E.A., 2005. Aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) expression in Baikal seal (*Pusa sibirica*) and association with 2, 3, 7, 8-TCDD toxic equivalents and cyp1 expression levels. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 141, 281–291. https://doi.org/10.1016/j.cca.2005.07.007.

Kwong, T.C., 2002. Organophosphate pesticides: biochemistry and clinical toxicology. Ther. Drug Monit. 24, 144–149. https://doi.org/10.1097/00007691-200202000-00022.

Leggieri, L.R., De Anna, J.S., Cerón, G.A., Arias Darraz, L., Fuentes Monasterio, D., Cárcamo, J.G., 2017. CYPs molecular biomarkers in rainbow trout *Oncorhynchus mykiss* to assess oil contamination in a Patagonian stream. Rev. Int. Contam. Ambie. 33 (4), 681–690. https://doi.org/10.20937/RICA.2017.33.04.11.

Liu, Q., Rise, M.L., Spitsbergend, J.M., Hori, T.S., Mieritz, M., Geise, S., McGrawf, J.E., Goetzg, G., Larson, J., Reinhold, J.H., Carvan, J.M., 2013. Gene expression and pathologic alterations in juvenile rainbow trout due to chronic dietary TCDD exposure. Aquat. Toxicol. 0, 356–368. https://doi.org/10.1016/j.aquatox.2013.06.018.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using realtime quantitative pcr and the 2–δδct method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Loewy, R.M., Monza, L.B., Kirs, V.E., Savini, M.C., 2011. Pesticide distribution in an agricultural environment in Argentina. J. Environ. Sci. Health B 46, 662–670. https://doi.org/10.1080/03601234.2012.592051.

Mandl, M., Depping, R., 2014. Hypoxia-inducible Aryl hydrocarbon receptor nuclear translocator (ARNT) (HIF-1beta): is it a rare exception? Mol. Med. 20, 215–220.

Mandl, M., Depping, R., 2017. ARNT is a potential direct HIF-1 target gene in human Hep3B hepatocellular carcinoma cells. Cancer Cell Int. 17 (77). https://doi.org/10.1186/s12935-017-0446-2.

Mandl, M., Lieberum, M.K., Depping, R., 2016. A HIF-1α-driven feed-forward loop augments HIF signalling in Hep3B cells by upregulation of ARNT. Cell Death Dis. 7, e2284. https://doi.org/10.1038/cddis.2016.187.

Maxwell, D.M., 1992. The specificity of carboxylesterase protection against the toxicity of organophosphate compounds. Toxicol. Appl. Pharmacol. 114, 306–312. https://doi.org/10.1016/0041-008X(92)90082-4.

Melbye, A.G., Brakstad, O.G., Hokstad, J.N., Gregersen, I.K., Hansen, B.H., Booth, A.M., Rowland, S.J., Tollefsen, K.E., 2009. Chemical and toxicological characterization of an unresolved complex mixture-rich biodegraded crude oil. Environ. Toxicol. Chem. 28, 1815–1824. https://doi.org/10.1897/08-545.1.

Monza, L.B., Loewy, R.M., Savini, M.C., Pechen de d'Angelo, A.M., 2013. Sources and distribution of aliphatic and polyaromatic hydrocarbons in sediments from the Neuquén River, Argentine Patagonia. J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng. 48 (4), 370–379. https://doi.org/10.1080/10934529.2013.728097.

Neal, R.A., 1980. Microsomal metabolism of thiono-sulfur compounds: mechanisms and toxicological significance. Rev. Biochem. Toxicol. 2, 131–171. https://doi.org/10.1016/B978-0-12-87702-6.50035-9.

Pelkonen, O., Nebert, D.W., 1982. Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. Pharmacol. Rev. 34, 189–222.
Peluso, M., Srivatanakul, P., Munnia, A., Jedpiyawongse, A., Meunier, A., Sangrajrang, S., Piro, S., Ceppi, M., Boffetta, P., 2008. DNA adduct formation among workers in a Thai industrial estate and nearby residents. Sci. Total Environ. 389, 283–288. https://doi.org/10.1016/j.scitotenv.2007.09.012.
Powell, W.H., Karchner, S.I., Bright, R., Hahn, M.E., 1999. Functional diversity of vertebrate ARNT proteins: identification of ARNT2 as the predominant form of ARNT in the marine teleost, *Fundulus heteroclitus*. Arch. Biochem. Biophys. 361, 156–163. https://doi.org/10.1006/abbi.1998.0992.
Rivadeneira, P.R., Agrelo, M., Otero, S., Kristoff, G., 2013. Different effects of subchronic exposure to low concentrations of the organophosphate insecticide chlorpyrifos in a freshwater gastropod. Ecotoxicol. Environ. Saf. 90, 82–88.
Salgado Costa, C., Ronco, A.E., Trudeau, V.L., Marino, D., Natale, G.S., 2018. Tadpoles of the horned frog Ceratophrys ornata exhibit high sensitivity to chlorpyrifos for conventional ecotoxicological and novel bioacoustics variables. Environ. Pollut. 235, 938–947. https://doi.org/10.1016/j.envpol.2017.12.096.
Sams, C., Cocker, J., Lennard, M.S., 2004. Biotransformation of chlorpyrifos and diazinon by human liver microsomes and recombinant human cytochrome P450s (CYP). Xenobiotica 34, 861–873. https://doi.org/10.1080/00498250400017273.
Sanchez-Hernandez, J.C., 2007. Ecotoxicological perspectives of B-esterases in the assessment of pesticide contamination. In: Plattenberg, R.H. (Ed.), Environmental Pollution: New Research. Nova Science Publishers, New York, USA, pp. 1–45.
Singer, M.M., Aurand, D., Bragin, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L., Tjeerdema, R.S., 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. Mar. Pollut. Bull. 40, 1007–1016. https://doi.org/10.1016/S0025-326X(00)00045-X.
Sogorb, M.A., Vilanova, E., 2002. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol. Lett. 128, 215–228. https://doi.org/10.1016/S0378-4274(01)00543-4.
Somnuek, C., Boonphakdee, C., Cheevaporn, V., Tanaka, K., 2009. Gene expression of acetylcholinesterase in hybrid catfish (*Clarias gariepinus X Clarias macrocephalus*) exposed to chlorpyrifos and carbaryl. J. Environ. Biol. 30, 83–88. https://doi.org/10.14456/ea.2012.14.
Tang, J., Chambers, J.E., 1999. Detoxication of paraoxon by rat liver homogenate and serum carboxylesterases and A-esterases. J. Biochem. Mol. Toxicol. 13, 261–268. https://doi.org/10.1002/(SICI)1099-046.
Tang, J., Cao, Y., Rose, R.L., Brimfield, A.A., Dai, D., Goldstein, J.A., Hodgson, E., 2001. Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse and rat liver microsomes. Drug Metab. Dispos. 29, 1201–1204.
Tang, J., Cao, Y., Rose, R.L., Hodgson, E., 2002. In vitro metabolism of carbaryl by human cytochrome P450 and its inhibition by chlorpyrifos. Chem. Biol. Interact. 141, 229–241. https://doi.org/10.1016/S0009-2797(02)00074-1.
Tanguay, R.L., Abnet, C.C., Heideman, W., Peterson, R.E., 1999. Cloning and characterization of the zebrafish (*Danio rerio*) aryl hydrocarbon receptor. Biochim. Biophys. Acta 1444 (1), 35–48.
Testai, E., Buratti, F.M., Di Consiglio, E., 2010. Chlorpyrifos. In: Krieger, R. (Ed.), Hayes' Handbook of Pesticide Toxicology, Third ed. Elsevier Inc, London, pp. 1505–1526.
U. S. Environmental Protection Agency, 1996. Ecological Effects Test Guidelines: OPPTS 850.1075 Fish Acute Toxicity Test, Freshwater and Marine. U. S. Environmental Protection Agency, Washington, D.C. US (EPA 712-C-96-118).
Uno, S., Dalton, T.P., Derkenne, S., Curran, C.P., Miller, M.L., Shertzer, H.G., Nebert, D.W., 2004. Oral exposure to benzo[a]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. Mol. Pharmacol. 65, 1225–1237. https://doi.org/10.1124/mol.65.5.1225.
Van Veld, P.A., Westbrook, D.J., 1995. Evidence for depression of cytochrome P4501A in a population of chemically resistant mummichog (*Fundulus heteroclitus*). Environ. Sci. 3, 221–234.
Wassmur, B., Gräns, J., Norström, E., Wallin, M., Celander, M.C., 2012. Interactions of pharmaceuticals and other xenobiotics on key detoxification mechanisms and cytoskeleton in *Poeciliopsis lucida* hepatocellular carcinoma, PLHC-1 cell line. Toxicol. in Vitro 27, 111–120. https://doi.org/10.1016/j.tiv.2012.10.002.
Wheelock, C.E., Eder, K.J., Werner, I., Huang, H., Jones, P.D., Brammell, B.F., Elskus, A.A., Hammock, B.D., 2005. Individual variability in esterase activity and CYP1A levels in Chinook salmon (*Oncorhynchus tshawytscha*) exposed to esfenvalerate and chlorpyrifos. Aquat. Toxicol. 74, 172–192. https://doi.org/10.1016/j.aquatox.2005.05.009.
Whitehead, A., Dubansky, B., Bodinier, C., Garcia, T.I., Miles, S., Pilley, C., Raghunathan, V., Roach, J.L., Walker, N., Walter, R.B., Rice, C.D., 2011. Genomic and physiological footprint of the Deepwater horizon oil spill on resident marsh fishes. Proc. Natl. Acad. Sci. U. S. A. 109, 20298–20302. https://doi.org/10.1073/pnas.1109545108.
Whyte, J.J., Jung, R.E., Schmitt, C.J., Tillitt, D.E., 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit. Rev. Toxicol. 30 (4), 347–570. https://doi.org/10.1080/10408440091159239.
Wiseman, S.B., Vijayan, M.M., 2007. Aryl hydrocarbon receptor signaling in rainbow trout hepatocytes: role of hsp90 and the proteasome. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 146 (4), 484–491. https://doi.org/10.1016/j.cbpc.2007.05.006.
Wogram, J., Sturm, A., Segner, H., Liess, M., 2001. Effects of parathion on acetylcholinesterase, butyrylcholinesterase, and carboxylesterase in three-spined stickleback (*Gasterosteus aculeatus*) following short-term exposure. Environ. Toxicol. Chem. 20, 1528–1531. https://doi.org/10.1002/etc.5620200716.
Woźny, M., Brzuzan, P., Wolińska, L., Góra, M., Łuczyński, M.K., 2010. Preliminary evaluation of ER- and AhR-mediated gene expression patterns in rainbow trout (*Oncorhynchus mykiss*) liver after short-term exposure to zearalenone in binary mixtures. Environ. Biotechnol. 6, 16–23.
Xing, H., Wang, X., Sun, G., Gao, X., Xu, S., Wang, X., 2012. Effects of atrazine and chlorpyrifos on activity and transcription of glutathione S-transferase in common carp (*Cyprinus carpio L.*). Environ. Toxicol. Pharmacol. 33, 233–244. https://doi.org/10.1016/j.etap.2011.12.014.