Ecotoxicology and Environmental Safety 208 (2021) 111394

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

# Exposure to hydrocarbons and chlorpyrifos alters the expression of nuclear receptors and antioxidant, detoxifying, and immune response proteins in the liver of the rainbow trout, *Oncorhynchus mykiss*

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ARTICLE INFO

Edited by - Dr. Caterina Faggio

*Keywords:*
Steroid receptors
Liver X receptor
Liver injury
Cytokines
Caspases
Fish

ABSTRACT

The development of oil and gas production together with the fruit production in nearby areas of North Patagonia, Argentina, suggests aquatic pollution scenarios which include permanent oil pollution combined with short events of pesticides application. It has been reported that oil hydrocarbons activate the aryl hydrocarbon receptor (AhR) pathway in the rainbow trout, *Oncorhynchus mykiss,* and that the insecticide Chlorpyrifos (CPF) interacts with these effects. Thus, it is interesting to investigate whether hydrocarbons and insecticides, applied by separate or combined, can affect fish health and reproductive signaling by acting on different nuclear receptors' regulatory pathways. To study this kind of interactions, we exposed juvenile rainbow trout to water accommodated fraction (WAF) of crude oil (62 μg $L^{-1}$ TPH) for 48 h and subsequently exposed the livers *ex vivo* to the insecticide Chlorpyrifos (CPF) (20 μg $L^{-1}$) for 1 h. We analyzed the mRNA expression of nuclear receptors and proteins involved in detoxifying, antioxidant, immune and apoptosis responses by qRT−PCR. We also performed histopathological analysis. WAF induced the expression of the androgen (AR) and the Liver X receptor (LXR) by 8- and 3-fold, respectively. AR induction was reversed by subsequent exposure to CPF. The progesterone receptor (PR) and glucocorticoid receptor (GR) were increased 2-fold and 3-fold by WAF respectively, while estrogen and mineralocorticoid receptors were not affected. GR was also induced by CPF with an additive effect in the WAF−CPF treatment. The antioxidant genes, gamma glutamyl transferase (GGT), superoxide dismutase (SOD1) were induced by WAF (2–3-fold). WAF upregulated the ATP Binding Cassette Subfamily C Member 2 (ABCC2, MRP2) (4-fold) and downregulated alkaline phosphatase. WAF also induced the inflammatory interleukins (IL) IL-8, and IL-6 and the anti-inflammatory IL-10, while CPF induced the inflammatory tumor necrosis factor (-α) and IL-6, and activated the intrinsic apoptotic pathway through the induction of caspases 3 and 9. Both, WAF and CPF downregulated the expression of the extrinsic apoptosis initiator caspase 8 and the inflammatory caspase 1. In conclusion, WAF hydrocarbons alter *O. mykiss* endocrine regulation by inducing AR, PR and GR. The subsequent exposure to CPF reverses AR, suggesting a complex interaction of different pollutants in contaminated environments, WAF hydrocarbons alter liver metabolism by inducing the expression of LXR, GR, antioxidant and detoxifying enzymes, and both inflammatory and anti-inflammatory cytokines, and causing mild hepatic steatosis. CPF activates inflammatory and stress responses associated with the induction of inflammatory cytokines together with apoptosis initiator and executioner caspases.

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https://doi.org/10.1016/j.ecoenv.2020.111394
Received 17 June 2020; Received in revised form 18 September 2020; Accepted 20 September 2020
Available online 5 October 2020

## 1. Introduction

Petroleum-derived hydrocarbons are major contributors to the pollution of aquatic environments (Kennedy and Farrell, 2005). Oil production through conventional and non-conventional methods is, in part, responsible for the introduction of pollutants into the natural water bodies (Zeliger, 2011). In North Patagonia, Argentina, beside the original conventional oil and gas production activity, a large non-conventional oil and gas reserve, stretching over the Rio Negro and part of its main tributary basins (Neuquén and Limay rivers), has started to be exploited in 2010. This area is upstream of and partially overlapping with an important fruit production area, in which pesticides are intensively applied in a seasonal basis (Loewy et al., 2011; Monza et al., 2013). Fig. 1 shows the Rio Negro−Neuquén−Limay basin, the oil and gas area, and the fruit production area. Both, pesticides and oil may reach aquatic ecosystems from punctual or diffuse sources, depending on landscape characteristics and meteorological conditions. For example, Ondarza et al. (2012) have found DDT and endosulfan in rainbow trout (*Oncorhynchus mykiss*) tissues in the Río Negro after a flood, despite those pesticides had ceased to be used before the study. Monza et al. (2013) have reported aliphatic hydrocarbons ranging from 0.41 μg $g^{-1}$ to 125 μg $g^{-1}$ dry mass in sediments of the rivers Neuquén and Limay. In this context, aquatic organisms could be exposed simultaneously or subsequently to hydrocarbons and pesticides. Sequential or simultaneous exposure to different kinds of pollutants can produce combined effects, *e.g.* a pollutant can modify the expression/activity of antioxidant and detoxification enzymes, exacerbating or ameliorating the effects of other pollutants (Clark and Di Giulio, 2012).

The rainbow trout (*Oncorhynchus mykiss*) was introduced in Argentina at the beginning of the 20th century (Marini and Mastarrigo, 1963) and is currently widely distributed in Patagonia with the presence of self-sufficient populations (Pascual et al., 2002). *O. mykiss* has great economic relevance in this region due its quality for sport fishing, and its suitability for aquaculture.

In a previous investigation, we have exposed juvenile *O. mykiss*, which is also a widely used ecotoxicological model (e.g Klaverkamp et al., 1977; Smith et al., 2007) to the water accommodated fraction of crude oil (WAF) and to the organophosphorus insecticide, chlorpyrifos (CPF), in a sequential fashion. We analyzed the *m*RNA expression of key genes in the aryl hydrocarbon receptor (AhR) pathway and the activity of antioxidant and detoxifying enzymes. *In vivo* exposure to WAF induced AhR and cytochrome P450 monooxygenase 1A (CYP1A) *m*RNA expression. Subsequent *ex vivo* exposure of liver slices from those fish exposed to CPF for 1 h reversed the induction of AhR (De Anna et al., 2018). Therefore, it became interesting to investigate whether this interaction between the activation of the AhR pathway by hydrocarbons and the short-term effect of CPF modulates the expression of other genes, which could be direct or indirect targets of the AhR signaling pathway. The study of these molecular interactions could help to understand the mechanisms involved in the response of the organism in a mixed pollution ecotoxicological context. Iwano et al. (2006) proposed that polycyclic aromatic hydrocarbons (PAHs) activated by AhR target enzymes such as CYP1A can produce DNA damage, which induces the transcription factor p53. This protein interferes with the signal transduction of nuclear receptors such as the androgen receptor beta (ARβ), the estrogen receptor 1 (ER1) (Sengupta and Wasylyk, 2004), the glucocorticoid receptor (GR) (Crochemore et al., 2002; Sengupta and Wasylyk, 2001) and the liver X receptor (LXR) (Iwano et al., 2006), affecting the regulation of a great number of genes, e.g., those related to metabolism, reproduction, detoxification, antioxidant defense, immune/inflammatory response and cellular stress.

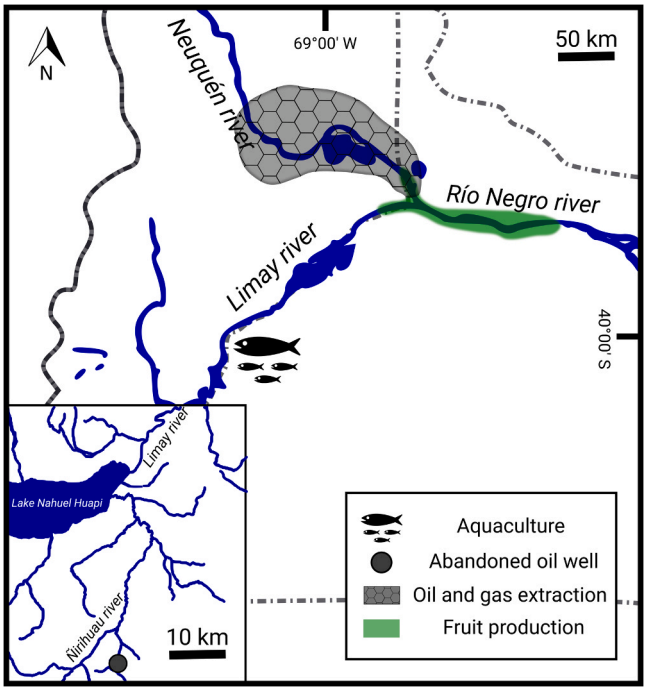


**Fig. 1.** Rio Negro−Neuquén−Limay basin. The oil and gas extraction area, and its overlapping with the nearby fruit production area are indicated in gray and green shaded areas, respectively. The rectangle in the lower left corner details the zone of the oil spill from an abandoned extraction well.

Steroid receptors of the nuclear receptors (NRs) superfamily, such as the mineralocorticoid (MR), ER, AR, GR and progesterone (PR) receptors are targets of environmental pollutants called endocrine disrupting chemicals (EDCs) (Scholz and Mayer, 2008). Reproductive hormones regulate all the aspects of reproductive development from sex differentiation to puberty; and are thus, critical for population viability. Among the EDCs, the most studied so far are those that bind to ERs producing estrogenic effects (xenoestrogens). In fish, mixtures of oil hydrocarbons and AhR agonists, such as 2,3,7,8-tetrachlorodibenzo-p--dioxin (TCDD) and β-naphthoflavone have been reported to affect estrogen-mediated regulatory functions (Navas and Segner, 2000; Bemanian et al., 2004; Gräns et al., 2010; Tollefsen et al., 2011). In this sense, the mRNA levels of zebrafish (*Danio rerio*) ERs decrease with increasing concentrations of WAF of crude oil (Arukwe et al., 2008; Salaberria et al., 2014), suggesting an interaction between AhR and ER.

Environmental pollutants can produce both androgenic and antiandrogenic effects on the aquatic biota. For example, Parks et al. (2001) reported the presence of fish with androgenic effects downstream of a Kraft mill. In contrast, the pesticide derivative dichlorodiphenyl dichloro ethylene (DDE) causes antiandrogenic effects by impairing AR transcriptional activity (Boelsterli et al., 2007). Martyniuk et al. (2020) have reviewed the endocrine disruption effects of organochlorine pesticides on fish and proposed a pathway by which combined agonistic and antagonistic interaction of pesticides with ER and AR, respectively, lead to tissue and organ disfunctions and, finally, to negative outcomes at the population level. Studies about PR in fish are yet scarce. Chen et al. (2010) characterized a zebrafish PR, which may regulate germ cell differentiation and steroidogenesis. This receptor is present in both, testes and ovary, and in other tissues including liver. Mineralocorticoids and glucocorticoids regulate diverse physiological functions in teleost fish by binding their specific receptors (MR and GR). Particularly, GR modulates metabolism and growth, osmoregulation, immune and inflammatory responses. “MR is important in behavioral responses related to osmotic stress and has a modest participation in osmoregulatory functions regulated by cortisol or 11-deoxycorticosterone (DOC). In addition, GR and MR signaling regulate cortisol levels during the stress response (Faught and Vijayan, 2018; Greenwood et al., 2003; Takahashi and Sakamoto, 2013, for a review).

LXR is a NR which regulates cholesterol homeostasis, lipid and carbohydrate metabolism, and modulates inflammatory and immune

responses (Schultz et al., 2000; Jakobsson et al., 2012; Fessler, 2018). In zebrafish, LXR has been reported to regulate the expression of genes related to cholesterol transport, lipid synthesis and visual perception (Archer et al., 2012; Sukardi et al., 2012). Besides, treatment with a pharmacological LXR agonist induces lipid accumulation (steatosis) in zebrafish liver, coinciding with results obtained with mammalian models (Schultz et al., 2000; Sukardi et al., 2012). Cruz-Garcia et al. (2011) have reported that LXR agonists increase LXR expression in trout myocytes, thereby indicating that this receptor is self-induced.

Paetzold et al. (2009) have reported that killifish exposed to PAHs and metals show increased expression of liver CYP1A1, glutathione-S-transferase-μ (GST-μ), the ATP Binding Cassette Subfamily C Member 2 (ABCC2) and breast cancer resistance protein (ABCG2), which would be involved in phase I and II biotransformation, and in biliary excretion of PAHs. The immune systems of vertebrates have been reported to respond to oil exposure, *e.g.* through AhR binding and CYP-mediated biotransformation mechanisms (Reynaud and Deschaux, 2006). The immunotoxicity of crude oil and derived products on fish has been addressed in the last decades. Functional immunological endpoints and gene expression respond to PAHs, in a species- and product-specific manner (reviewed by Tierney et al., 2013). In this context, cytokines appear as biomarkers of inflammation, immune reactivity, tissue injury and repair, and organic dysfunction (Lacour et al., 2005). Fish immune system is also susceptible to pesticides; particularly, the exposure to CPF for 40 days induces interleukins IL-1β and IL-1R, and interferon-γ (IFN-γ) mRNA expression in common carp (*Cyprinus carpio*) spleen and head kidney (Wang et al., 2011).

Caspases are cysteine proteases that play a key role as apoptotic factors and participate in the inflammatory response. Fish caspases share many characteristics with those of mammals and their mRNA expression has been proposed as an early marker of apoptosis (Jin et al., 2013; Luzio et al., 2013). According to their functional and structural similarities, caspases have been classified as inflammatory (Caspases 1, 4, 5 and 12), and apoptosis initiators (Caspases 2, 8, 9 and 10) or executioners (Caspases 3, 6 and 7) (Krumschnabel and Podrabsky, 2009; Spead et al., 2018, for reviews).

The aim of this work is to analyze the mRNA expression of a suit of genes related to i) key NRs involved in endocrine and metabolic functions; ii) oxidative balance and detoxification, and iii) molecules related to stress and immune response; in the liver of juvenile *O. mykiss* exposed *in vivo* to WAF and subsequently exposed *ex vivo* to CPF. The study also includes liver condition indicators, such as hepato-somatic index and histopathological aspects.

## 2. Materials and methods

### 2.1. Fish processing and experimental design

The utilized fish were juvenile *Oncorhynchus mykiss,* 16.24 ± 0.47 g; 11.49 ± 0.15 cm (mean ± SD), from a line bred in the CEAN aquaculture facility for at least 30 years; thus, having high homozygosity and similar genetic information among individuals. Fish were kept in 15 L tanks with continuously circulating filtrated Chimehuin River water (considered free of oil and pesticides pollution, De Anna et al., 2018) and fed daily with commercial trout pellets (1% body mass ratio). The physicochemical conditions of the acclimation water were: alkalinity 34 mg $L^{-1}$, conductivity 36 μS $cm^{-1}$, pH 7.4–7.6, dissolved oxygen 8.37 mg $L^{-1}$, temperature 10–18 °C. The photoperiod was set at 12 h light: 12 h dark.

In the laboratory, fish were placed in individual cylindrical 10 L tanks with aerated Chimehuin River water as described above, at 16–18 °C and 12 h light: 12 h dark photoperiod for 48 h. Then, water was changed to expose the fish in the same individual containers to four experimental treatments, with six fish per treatment (n = 6, in 24 containers). According to De Anna et al. (2018), two groups of six fish were exposed *in vivo* to WAF (62 μg $L^{-1}$ total petroleum hydrocarbons, TPH) for 48 h and another two groups were kept as exposure controls (C). Fish were not fed and water was not changed during the exposure period. Subsequently, fish were sacrificed by a blow to the head followed by decapitation, and weighed. Livers were immediately removed, weighed and rinsed in ice-cold Cortland saline (5 mmol $L^{-1}$ $NaHCO_3$, 5.55 mmol $L^{-1}$ glucose, pH 7.4). Part of each liver (~200 mg) was cut into thin slices and put in a glass vessel with 6 mL of aerated Cortland solution. Liver slices from six WAF and six control individuals were exposed *ex vivo* to 20 μg $L^{-1}$ CPF for 1 h (groups WAF−CPF and C−CPF). This concentration, which is c.a. two-fold higher than the CPF lethal concentration 50 (LC50 96 h) for rainbow trout (USEPA, 1996), was chosen in order to ensure biochemical effects upon a short-time exposure (1 h). The liver slices from the remaining WAF and control fish were exposed to 0.1% acetone in Chimehuin River water, as solvent control groups (WAF−SC and C−SC). This resulted in four treatments (experimental groups), C−SC, C−CPF, WAF−SC and WAF−CPF. After 1 h, liver slices from six individuals per treatment were preserved in RNA later (Life Technologies, Carlsbad, CA) at −30 °C for further studies of mRNA expression and histopathology. This experimental set up allowed the study of possible interactions between WAF and CPF in a sequential exposure of liver tissue from control and WAF exposed fish to a controlled CPF concentration, avoiding possible metabolization or accumulation in other tissues. The hepato-somatic index was calculated as HSI (%) = (liver mass x fish $mass^{-1}$) x 100. The experimental protocols were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

### 2.2. Water-accommodated fraction of crude oil

As described by De Anna et al. (2018), the WAF was made by mixing 4.75 g of crude oil with one L of Chimehuin River water. The crude oil utilized was obtained from the permanent spill from an abandoned oil extraction exploitation next to the La Mina stream, a tributary of Ñirihuau river, (41°17′34′’ S- 71°11′14′’ W, 1011 masl, Fig. 1), in the Limay River Basin, where sport fishing of introduced salmonid species and rainbow trout aquaculture constitute important economic resources. Oil samples were transported in a cooler to the laboratory and immediately used for WAF preparation. This oil was characterized by Cazau et al. (2005) as immature heavy oil (Ro = 0.44–0.53%, American Petroleum Institute (API) = 18°, sulfur = 0.45%;), with 33.7% saturated and 17.8% aromatic hydrocarbons, 5.9% asphaltenes and 42.6% NSOs (YPF, Argentina). The WAF was analyzed according to the method 3510C-8015D GC-FID (USEPA). Total petroleum hydrocarbons (TPH, C6 to C36, including 16 priority PAHs USEPA, without discrimination) were analyzed (detection and quantitation limits, 0.002 and 0.010 mg $L^{-1}$). The TPH (C6−C36) concentration in the WAF was 1.24 mg $L^{-1}$ (CV < 7%). For the experiment, the WAF was diluted at 5% in Chimehuin River water to obtain a nominal TPH concentration of 62 μg $L^{-1}$. This concentration was selected according to the environmental TPH concentration measured by Leggieri et al. (2017) at 0–1600 m downstream from the oil spill described above, where juvenile rainbow trout are abundant. The gas chromatography analysis of water accommodated fraction of petroleum is shown in De Anna et al. (2018), Supplementary file 1.

### 2.3. Chlorpyrifos

One mg of CPF (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl phosphorothioate], 99% purity, Chem Service, West Chester, Pennsylvania, USA) was dissolved in chromatographic grade acetone (Cicarrelli, Buenos Aires, Argentina) to obtain a 20 mg $L^{-1}$ solution. The analysis was performed in an Agilent Technologies 6890 gas chromatograph (Wilmington, USA), equipped with a flame photometric detector (GC-FPD). The GC column was a fused silica capillary column HP-5, 5% phenylmethylsiloxane, with the dimensions of 30 m × 0.32 mm i.d. and a 0.25 μm film thickness (Agilent Technologies). The temperature was

programmed to increase at 10 °C $min^{-1}$ from 100 °C to 200 °C and then at 4 °C $min^{-1}$ to a final temperature of 220 °C. A purified helium carrier gas was used 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.

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

Total RNA was obtained from the livers of three randomly chosen individuals from each experimental group with Trizol reagent (Invitrogen, CA, USA). RNA quality was analyzed by 1% agarose gel electrophoresis, and its concentration was measured by spectrophotometry at 260 and 280 nm. 2 μg of RNA were used as template for cDNA synthesis using RevertAid reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Each qPCR reaction mixture was prepared with 1 μL of cDNA template, 0.5 μmol $L^{-1}$ of each primer, 8 μL of water, and 10 μL 2 × SYBR green q-PCR Master Mix (Thermo Fisher, MA, USA). Table 1 shows the sequences of the primer pairs used for qPCR. Melting curve analysis was performed for each gene by reading fluorescence between 60 °C and 95 °C, to ensure that a single product had been amplified. We used a StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, USA) with a thermal cycling program of one denaturing step (95 °C, 3 min), and 40 cycles of denaturation (95 °C for 10 s), annealing (60 °C for 30 s) and extension (72 °C for 20 s). Each sample was analyzed in triplicate. B-actin was selected as housekeeping gene. The analysis of relative expression was performed by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Results (fold change) were calculated with the $2^{-\Delta\Delta CT}$ method:

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

where $Ct_{target}$ and $Ct_{\beta\text{-actin}}$ are the cycle thresholds for target and β-actin genes, t is the treatment group and c is the control group. $Avg$ = average.

### 2.5. Histopathological analysis

Six samples per experimental group (n = 6) were preserved in RNA later (Life Technologies, Carlsbad, CA, USA) and then fixed in 10% phosphate buffered formalin for 48 h. Tissues were paraffin embedded, sectioned at 3–5 μm, and stained with hematoxylin and eosin. Histopathological changes were assessed under a light microscope (Olympus CXe1). Ten microscopic fields were randomly chosen after general examination at 40x, 200x and 400x magnification. The scores were semi-quantitatively derived according to the severity and extent of the pathologies, and were reported as follows: none: - (no lesion), mild: + (1–3 microscopic fields with lesions), moderate: ++ (4–7 fields with lesions), severe: +++ (8–10 fields with lesions) (Topal et al., 2015).

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

| Gene | | Primer pair secuence 5′ → 3′ | Amplicon length (pb) | NCBI reference secuence |
|---|---|---|---|---|
| AR-β | FW | TAATGCGGGACATGACCATTGC | 108 | NM_001124185.1 |
| | RV | CCTTTGGCCCACTTGACCACTTT | | |
| ER | FW | ACTCTGGTGCCTTCTCCTTCTGTT | 127 | XM_021598359.1 |
| | RV | ACAGAGGCTCCTGAATGGCTGAT | | |
| PR | FW | CCTCCGTAGTGCGTAGTTTTGGAA | 144 | XM_021588568.1 |
| | RV | GGTCAGAGCGGTCATAAAACGCTT | | |
| MR | FW | AGA GGA CCA AAT CAC CCT GAT CCA | 99 | NM_001124483.1 |
| | RV | TAG AGC ATC TGT CCG TTGGTG T | | |
| GR | FW | TTGTGAGGCTGCAGGTGTCCTATG | 138 | XM_021617180.1 |
| | RV | TCCCCAGCTCCTTTATGTAGGTCA | | |
| LXR | FW | AGTGCCCAGTATTCGTGCAAGAAC | 122 | FJ470291.1 |
| | RV | AGAACGCATTGCTCCAGCAT | | |
| ABCC2/MRP2 | FW | ATGCTGGGAAGATCGTGGAGTTTG | 142 | XM_021604527.1 |
| | RV | GAGAGCTGTGCTATCCAATGCAGT | | |
| AP | FW | TATTTCATCCGCTCCCTCGACCTA | 195 | XM_021609750.1 |
| | RV | AGTGCCTCCGGGAAGTATGTACAA | | |
| GGT | FW | CCTGGTGAAAGCAACATCACTC | 128 | XM_021602658.1 |
| | RV | CTGCTCCTTGTTCTGCAATGGT | | |
| SOD1 | FW | AGGACCATGGTGATCCATGAGAAG | 115 | NM_001123587.1 |
| | RV | GGGCAATGCCAATAACTCCACA | | |
| GPX | FW | GAACCTGGCACTGAAGCTCAGAT | 157 | XM_021585624.1 |
| | RV | TGCCATTTCCCAGTAAGCCTTTCC | | |
| IL-1β | FW | CAAGCTGCCTCAGGGTCT | 101 | NM_001124347 |
| | RV | CGCCACCCTTTAACCTCTCC | | |
| IL-4 | FW | CAACCCAACCAAAGATGAAGACGG | 135 | NM_001246341.1 |
| | RV | GCAGGCAGAGTTCCAGAGTCAAAT | | |
| IL-6 | FW | GGAGGAGTTTCAGAAGCCCG | 101 | HF913655.1 |
| | RV | TGGTGGTGGAGCAAAGAGTCT | | |
| IL-8 | FW | TGAGACGGAAAGCAGACGAA | 135 | AY160985.1 |
| | RV | GCGCTGACATCCAGACAAAT | | |
| IL-10 | FW | CGCTATGGACAGCATCCTGAAGTT | 119 | NM_001245099.1 |
| | RV | CGTGGAAGATGTTTCCGATGGAGT | | |
| TNF-α | FW | GGCGAGCATACCACTCCTCT | 125 | NM_001124357.1 |
| | RV | TCGGACTCAGCATCACCGTA | | |
| CAS 1 | FW | AGATGCCGACCAAAGACAGA | 124 | XM_021582336.1 |
| | RV | ATATGCGGCTCCCTTATTCCTC | | |
| CAS 8 | FW | TTCACCGACGCAATCCTGTAAGAC | 188 | XM_021588594.1 |
| | RV | CCACTTTAAACCCCAACCTGGAGA | | |
| CAS 9 | FW | TCGGGCCTTCCCAGCTTTAATA | 147 | NM_001124647.1 |
| | RV | GAGTAAACTGGCAAAGGCCTAACG | | |
| CAS 3 | FW | TCGAACGTTTGGGGTACAACGTGA | 101 | NM_001246335.1 |
| | RV | TGACTGGCTGTGGTTGTCTTGA | | |
| β-actin | FW | TGAAGTGTGACGTGGACATCCGTA | 108 | NW_018528586.1 |
| | RV | AGGTGATCTCCTTCTGCATCCTGT | | |

### 2.6. Data analysis

Statistical analyses were done using GNU PSPP 1.2.0 software (GNU general public license). Results were expressed as mean ± standard deviation. Normality and homogeneity of variance were analyzed by Kolmogorov–Smirnov and Levene's tests, respectively. mRNA expression data were compared by two-way ANOVA. The treatments analyzed in the two-way ANOVA were WAF (*in vivo*) and its control medium (C), and CPF (*ex vivo*) and its solvent control (SC) followed by Tukey HSD posttest comparisons when appropriate. Results were considered significant with $p < 0.05$.

## 3. Results

### 3.1. Nuclear steroid hormone receptors

AR mRNA expression was 8-fold higher in liver of fish exposed only to WAF (WAF−SC) than in control fish (C−SC), (ANOVA: $F = 172.6$; $P < 0.0001$ for interaction, Tukey's multiple comparisons $P < 0.0001$). There was no significant effect of CPF alone (C−CPF) or WAF−CPF. PR expression was significantly (2-fold) induced by WAF (ANOVA: $F = 20.61$; $P < 0.01$) and there was no effect of CPF. In contrast, the glucocorticoid receptor (GR) mRNA expression was significantly induced by both WAF and CPF with additive effects (ANOVA: $F = 19.36$, $P < 0.01$; $F = 10.86$, $P < 0.05$, respectively). Neither MR nor ER expression were significantly affected by the experimental treatments (Fig. 2).

### 3.2. Liver condition

During the study, fish appeared in good health and no mortality was observed. Furthermore, there were no significant differences in HSI among treatments (Supplementary Figure 1). The liver tissue from C−SC fish showed 83% of mild atrophy and 17% of moderate atrophy. Moderate atrophy increased up to 60% in livers from the CPF treatment and up to 67% in those from the WAF treatment. In addition, the latter group showed 33% of mild hepatic steatosis and 17% of moderate hepatic congestion. Finally, the livers from the WAF−CPF group showed 80% of moderate atrophy, 20% of mild steatosis, 20% of moderate congestion, and 20% of mild focal hemorrhage (Fig. 3a, Fig. 4).

### 3.3. LXR and liver function-related genes

LXR mRNA expression was induced 3-fold by WAF and was not affected by CPF alone. However, there was a significant interaction between both treatments (ANOVA: $F = 11.16$; $P < 0.01$), which is evident in the partial reversion of the WAF-induced expression effect in the WAF−CPF group. LXR expression was significantly different from C−SC only in the WAF−SC group ($P < 0.01$; Fig. 3b). ABCC2 expression was 3–4-fold induced in livers from WAF-exposed fish, irrespectively of the subsequent *ex vivo* exposure to CPF (ANOVA: $F = 12.31$; $P < 0.05$ for WAF; Fig. 3c). Alkaline phosphatase (AP) expression was reduced by 50% by WAF treatment (ANOVA: $F = 9.502$; $P < 0.05$; Fig. 3d) but was not affected by *ex vivo* exposure to CPF.

### 3.4. Antioxidant enzymes

Gamma glutamyl transferase (GGT) and superoxide dismutase 1 (SOD1) mRNA expression showed significant induction in the liver of fish exposed to WAF (ANOVA: $F = 6.912$, $P < 0.05$; $F = 18.95$, $P < 0.01$, respectively) with no significant effect of CPF. Finally, glutathione peroxidase (GPX) mRNA expression did not change with the exposure to WAF or CPF, but there was significant interaction between treatments (ANOVA: $F = 13.43$, $P < 0.01$), in which GPX mRNA expression in livers of fish exposed to both treatment (WAF−CPF) was 4-fold higher than when the exposure occurred independently (WAF-SC or

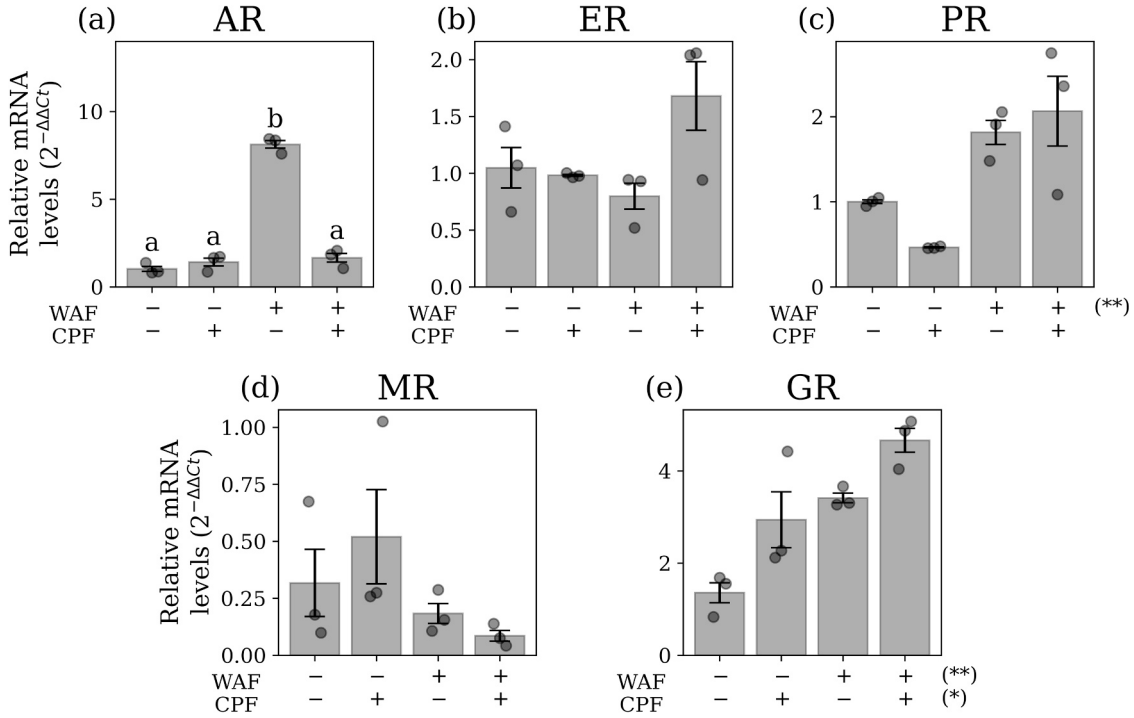


**Fig. 2.** Effects of *ex vivo* exposure to 20 μg $L^{-1}$ chlorpyrifos (CPF) on the relative mRNA expression of nuclear receptors in liver tissue from rainbow trout previously exposed to control medium or to 62 μg $L^{-1}$ total petroleum hydrocarbons of water accommodated fraction (WAF) or to control medium. (a) Androgen receptor (AR), (b) estrogen receptor (ER), (c) progesterone receptor (PR), (d) mineralocorticoid receptor (MR), (e) glucocorticoid receptor (GR). + or – indicate the presence or absence of each treatment. Two-way ANOVA, **$p < 0.01$ for WAF effects in (c) and (e); *$p < 0.05$ for CPF effect in (e). Different letters indicate significant differences between treatments (*post hoc* Tukey's multiple comparisons). Each point corresponds to one independent sample ($n = 3$).

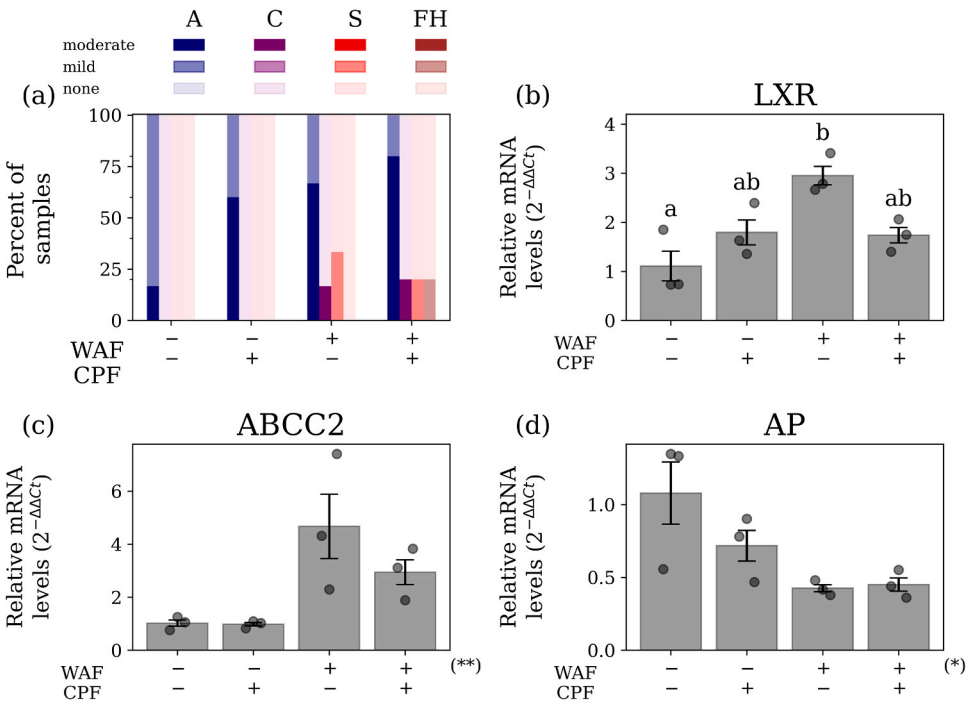


**Fig. 3.** Effects of *ex vivo* exposure to 20 μg $L^{-1}$ chlorpyrifos (CPF) on liver condition and relative mRNA expression of liver function-related genes in rainbow trout previously exposed to 62 μg $L^{-1}$ total petroleum hydrocarbons of water accommodated fraction (WAF) or to control medium (a) percentage values were calculated as number of microscopic fields (out of a total of 10) in which each pathology was observed, A = atrophic, C = congestion, S = steatosis and FH = focal hemorrhage. The color code indicates the grade of severity of each pathology (n = 6). (b) Relative mRNA levels of Liver X receptor (LXR), (c) ATP Binding Cassette Subfamily C Member 2 (ABCC2), (d) Alkaline phosphatase (AP). + or – indicate the presence or absence of each treatment. Two-way ANOVA, *$p < 0.05$, **$p < 0.01$ for WAF effects in (c) and (d). Different letters indicate significant differences between treatments (*post hoc* Tukey's multiple comparisons) in (b). Each point corresponds to one independent sample (n = 3).

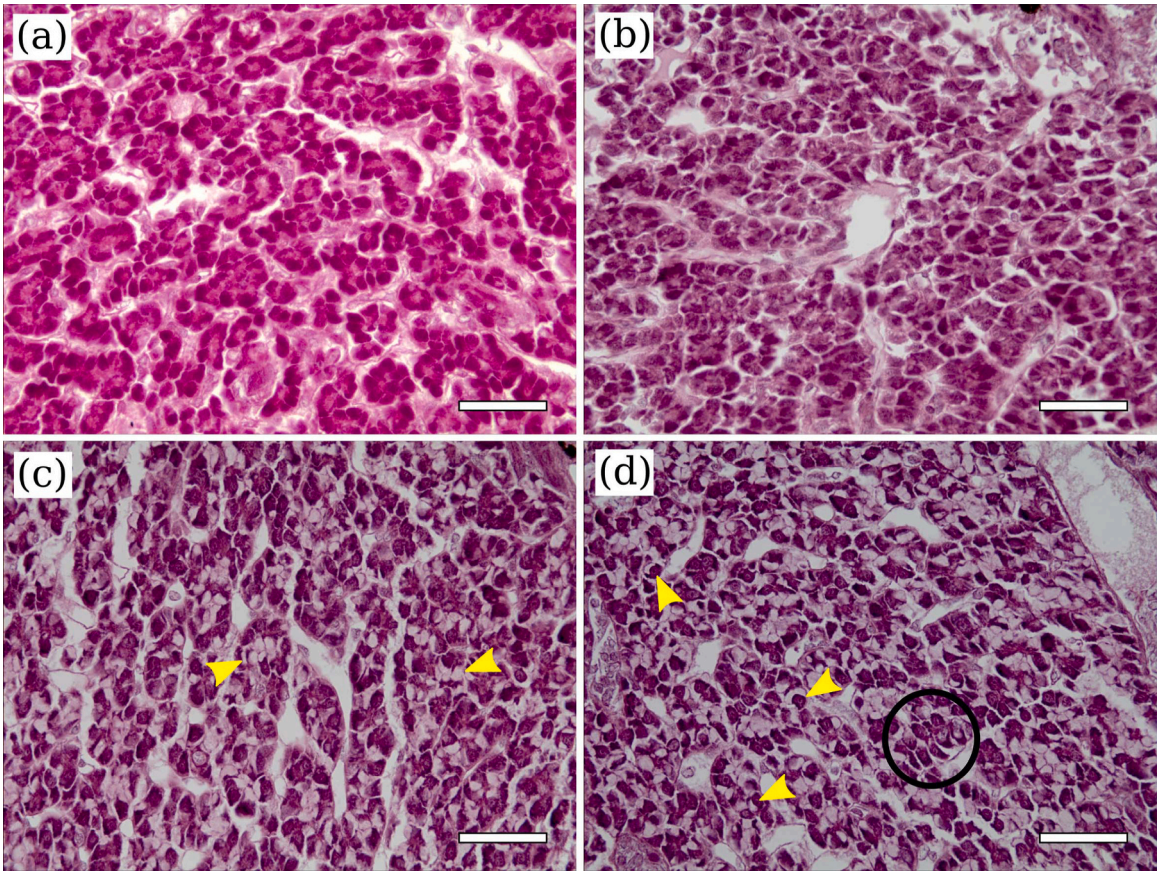


**Fig. 4.** Histopathological aspects of livers from juvenile rainbow trout exposed to each experimental treatment. (a) exposed *in vivo* for 48 h to control medium and subsequently exposed *ex vivo* for 1 h to the solvent control, 0.1% acetone. (b) exposed *in vivo* for 48 h to control medium and subsequently exposed *ex vivo* for 1 h to 20 μg $L^{-1}$ chlorpyrifos (CPF). (c) exposed *in vivo* for 48 h to 62 μg $L^{-1}$ total petroleum hydrocarbons of water accommodated fraction of oil (WAF) and subsequently exposed *ex vivo* for 1 h to the solvent control. (d) exposed *in vivo* for 48 h to 62 μg $L^{-1}$ total petroleum hydrocarbons of WAF and subsequently exposed *ex vivo* for 1 h to 20 μg $L^{-1}$ CPF. Yellow arrowheads show hepatocytes with lipid vesicles (steatosis). The circle shows atrophic hepatocytes. Hematoxylin and Eosin, bar = 50 μm.

C-CPF) ($P < 0.05$), however did not significantly differ from C−SC (Fig. 5).

### 3.5. Cytokines

Among the inflammatory cytokines (Fig. 6a−e), IL-8 mRNA expression was induced 2–3-fold in the livers of fish exposed to WAF (ANOVA: $F = 12.35$; $P < 0.01$) but was not affected by CPF, while tumor necrosis factor-alpha (TNF-α) expression was induced only by CPF (ANOVA: $F = 7.066$; $P < 0.05$). IL-6 expression was significantly induced by both treatments (ANOVA, effect for WAF: $F = 47.83$; $P < 0.0001$, and effect for CPF: $F = 12.16$; $P < 0.01$), and these effects were additive. Neither IL-1β nor IL-4 expression were significantly affected by any treatment. The anti-inflammatory cytokine IL-10 (Fig. 6f) was significantly induced by WAF exposure (ANOVA: $F = 6.303$; $P < 0.05$).

### 3.6. Caspases

Caspase 1 expression did not show significant effects by any treatment. However, there was a significant interaction effect (ANOVA: $F = 18.45$; $P < 0.01$), in which CPF alone reduced the expression of caspase 1 to half of the control value ($P < 0.01$), but this effect was absent in livers from individuals previously exposed to WAF (Fig. 7a). There were also interaction effects for caspase 8 expression (ANOVA: $F = 108.6$; $P < 0.0001$), the three groups with livers of fish exposed to WAF and/or CPF showed reductions in this caspase expression of 6 to 10 fold with respect to C-SC ($P < 0.001$; Fig. 7b). Finally, the expressions of caspases 9 and 3 were significantly induced by *ex vivo* exposure to CPF (ANOVA: $F = 4.199$; $P < 0.05$; and $F = 7.869$; $P < 0.05$; Fig. 7c, d, respectively). There was no significant WAF exposure effect on these caspases.

## 4. Discussion

Our results show clear signs of endocrine disruption by *in vivo* exposure to WAF, which include the induction of mRNA expression of androgen and progesterone receptors. The eight-fold induction of AR expression by WAF, which is completely reversed by subsequent *ex vivo* exposure to CPF for one hour, closely resembles the results obtained in our previous paper (De Anna et al., 2018) for AhR expression in juvenile rainbow trout obtained from the same aquaculture facility and exposed to WAF and CPF with same protocol as in the present study. This suggests that the transcription of AR is induced by AhR signaling. Alternatively, WAF and CPF could have modulated both receptors in a similar way. In this regard, Vinggaard et al. (2000) have found that dibenzo[*a*,*h*]anthracene (DB[*a*,*h*]A) was a potent agonist of both receptors in humans, although other PAHs were AhR agonists and AR antagonists. The reversion of the WAF effect by CPF coincides with the reported antiandrogenic activity of related compounds such as the organophosphorus insecticide fenitrothion (Tamura et al., 2001). This effect of CPF could involve reduced mRNA stability or inhibited transcription activity, e.g. by binding of CPF or a CPF metabolite to AR, impairing its interaction with the androgen response element (ARE), as it has been described by Boelsterli (2007) for DDE. In our study, PR expression is also induced by *in vivo* exposure to WAF but is not affected by CPF. The interaction between the effects of WAF and CPF on AR, together with the simultaneous induction of AR and PR by WAF, suggests a complex interplay of masculinizing and femininizing signals in polluted environments.

Exposure to WAF produces a three-fold induction of LXR mRNA, which is partially reversed by further *ex vivo* exposure to CPF, showing a similar pattern to those of AR (this study) and AhR (De Anna et al., 2018). This similarity suggests that the binding of hydrocarbons to AhR induces both, AR and LXR pathways, although no direct crosstalk has been reported yet. The alternative explanation based on similar effects of WAF components and CPF on the three pathways seems less likely but cannot be discarded, e.g., in mammals, AhR and LXR share some potential agonists, such as phthalates and indole derivatives (Minzaghi et al., 2019). On the other hand, both nuclear receptors have anti-inflammatory activity and share targets in the cholesterol metabolism and in the promotion of lipogenesis through the induction of the protein CD36, which mediates lipogenic effects of LXR and is critical for the induction of steatosis by pharmacological LXR agonists and by AhR ligands (Degrace et al., 2006; He et al., 2011). In zebrafish, a transcriptional and lipid profile study by Mu et al. (2018) reports that diesel contamination can affect lipid homeostasis and endocrine functions, and Sukardi et al. (2012) report mild hepatic steatosis induced by agonists of mammalian LXR. Accordingly, our histopathological results show mild steatosis in the livers of the WAF treated fish with the same pattern of LXR expression, since steatosis is not evident in livers treated only with CPF *ex vivo* and is lower in the WAF−CPF than in the WAF treatment. Congestion seems also to be produced only by WAF exposure. In contrast, moderate atrophy occurs in all the treatments in similar extent, while focal hemorrhage is evident only when *in vivo* exposure to WAF and *ex vivo* exposure to CPF are combined (WAF−CPF).

The modulation of regulatory, detoxifying and antioxidant proteins is also pertinent to liver damage. For example, in mammalian hepatocytes and bile duct cells, AP participates in the regulation of bile alkalinization through purinergic signaling (Poupon, 2015). Thus, the downregulation of AP by *in vivo* exposure to WAF in rainbow trout could affect liver and intestine functions. On the other hand, WAF-exposed fish show up to four-fold higher expression of ABCC2, which is an important transporter for bile secretion and xenobiotics excretion. In accordance, Paetzold et al. (2009) report increased hepatic mRNA expression of ABCC2 and other detoxifying proteins in killifish environmentally exposed to PAHs, PCBs and metals. In mammals, ABCC2 expression is regulated by several nuclear receptors, such as the pregnane X receptor (PXR), the farnesoid X receptor (FXR) and LXR, and also by the transcription factor erythroid 2- related factor 2 (Nrf2) (Arana et al., 2016; Unoki et al., 2018 for reviews). Although the regulation of ABCC2 by

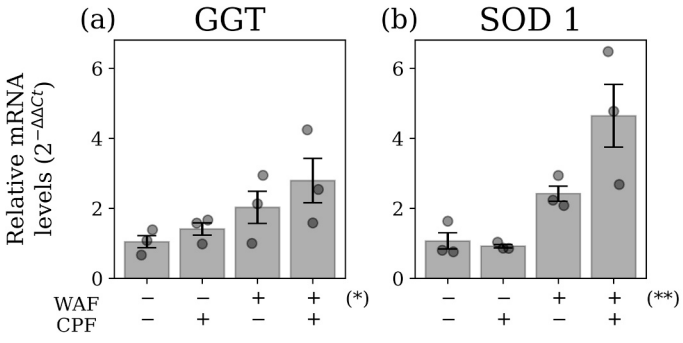


**Fig. 5.** Effects of *ex vivo* exposure to 20 μg $L^{-1}$ chlorpyrifos (CPF) on the relative mRNA expression of antioxidant enzymes in livers from rainbow trout previously exposed *in vivo* to 62 μg $L^{-1}$ total petroleum hydrocarbons of water accommodated fraction (WAF) or to control. (a) Gamma glutamyl transferase (GGT), (b) superoxide dismutase 1 (SOD1), (c) glutathione peroxidase (GPX). + or – indicate the presence or absence of each treatment. Two-way ANOVA, *$p < 0.05$, **$p < 0.01$ for WAF effects. Different letters indicate significant differences between treatments (*post hoc* Tukey's multiple comparisons). Each point corresponds to one independent sample ($n = 3$).

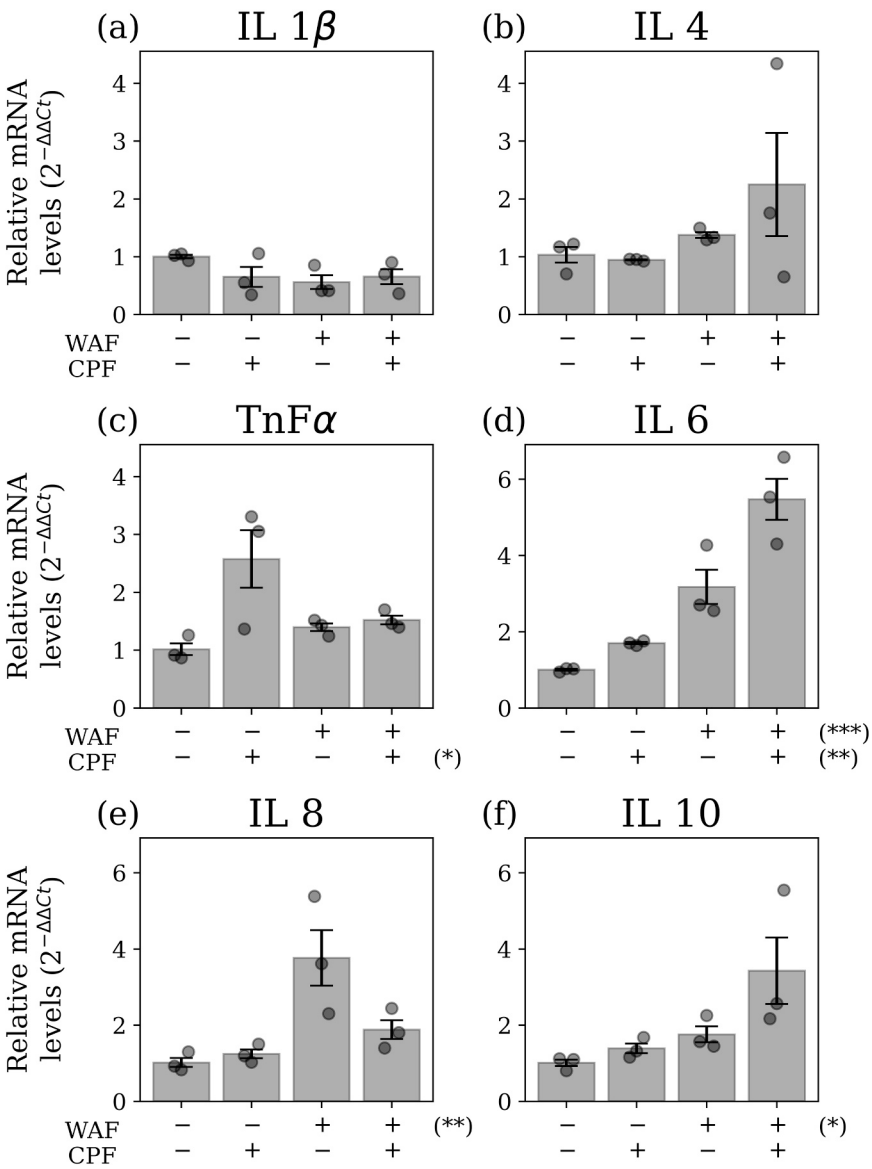


**Fig. 6.** Effects of *ex vivo* exposure to 20 μg $L^{-1}$ chlorpyrifos (CPF) on the relative mRNA expression of cytokines in livers from rainbow trout previously exposed *in vivo* to 62 μg $L^{-1}$ total petroleum hydrocarbons of water accommodated fraction (WAF) or to control medium. Inflammatory cytokines: interleukin (IL) 1β (a), IL-4 (b), IL-8 (c), tumor necrosis factor-alpha (TnFα) (d), IL-6 (e), (f) Anti-inflammatory interleukin, IL-10. + or – indicate the presence or absence of each treatment. Two-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001 for WAF or CPF effects. Each point corresponds to one independent sample (n = 3).

LXR in fish has not been described so far, the fact that both genes expression is similarly induced by *in vivo* exposure to WAF suggests that such regulatory pathway should be investigated. On the other hand, AhR has not been implied in ABCC2 regulation but there are reports on the binding of AhR to XRE elements in the Nrf2 promoter in mammals (Miao et al., 2005; Timme-Laragy et al., 2009; Rousseau et al., 2015). In this sense, Painefilú et al. (2020) report that arsenite induces the transcription of liver and intestine ABCC2 in rainbow trout. Moreover, Fuse et al. (2016) report that, in zebrafish, the induction of antioxidant enzymes and ABCC2 expression by arsenite occurs through Nrf2 signaling, and Afifi et al. (2017) have reported a significant correlation between the levels of total petroleum hydrocarbons and gene expression of SOD, catalase (CAT), GPx and GST in the liver of *Siganus canaliculatus* and *Epinephelus morio*. In the present work, ABCC2, GGT and SOD1 are induced but GPx is downregulated by *in vivo* exposure to WAF. In accordance, bisphenol a, which has been related to an Nrf2-mediated response in mouse liver (Shimpi et al., 2017), increases SOD and CAT and decreases GPx activities in the tilapia, *Oreochromis niloticus* (Hamed and Abdel-Tawwab, 2017). Although we have not evaluated Nrf2 expression, we can speculate that, in rainbow trout liver, these detoxification and antioxidant genes could be induced through oxidative stress- and/or AhR-activated Nrf2 signaling.

We have observed an additive induction of GR expression by both treatments, *in vivo* exposure to WAF and *ex vivo* exposure to CPF. Accordingly, Aluru and Vijayan (2004, 2008) have reported that the AhR agonist β-naphthoflavone (BNF) increases plasma cortisol levels and induces brain GR mRNA expression in *O. mykiss*. Wang et al. (2009) have reported that AhR activation in HO23 cells results in enhanced GR transactivation, while transactivation of AhR is inhibited by GR. Therefore, considering that the exposure to WAF *in vivo* induces AhR and CYP1A (De Anna et al., 2018), we can speculate that the activation of AhR signaling by WAF results in the induction of GR. On the other hand, to our knowledge, there are no reports on the effects of CPF on GR expression. Zhang et al. (2016) have reported antagonistic effects by several pyrethroid and organochlorine pesticides on GR expression and activity in mammalian cells, but the organophosphate pesticides tested by them, including CPF, have shown no effect.

GR mediates the modulation of many physiological processes, including immunosuppressive and anti-inflammatory effects and apoptosis. For example, GR can inhibit the transcription of the inflammatory cytokines IL-1β, IL-8 and TNF-α and induce the anti-inflammatory IL-10 (Necela and Cidlowski, 2004, for a review). In fish, Quabius et al. (2005) exposed *O. mykiss* head kidney cells to the AhR agonist PCB126 for up to 24 h and observed a concentration

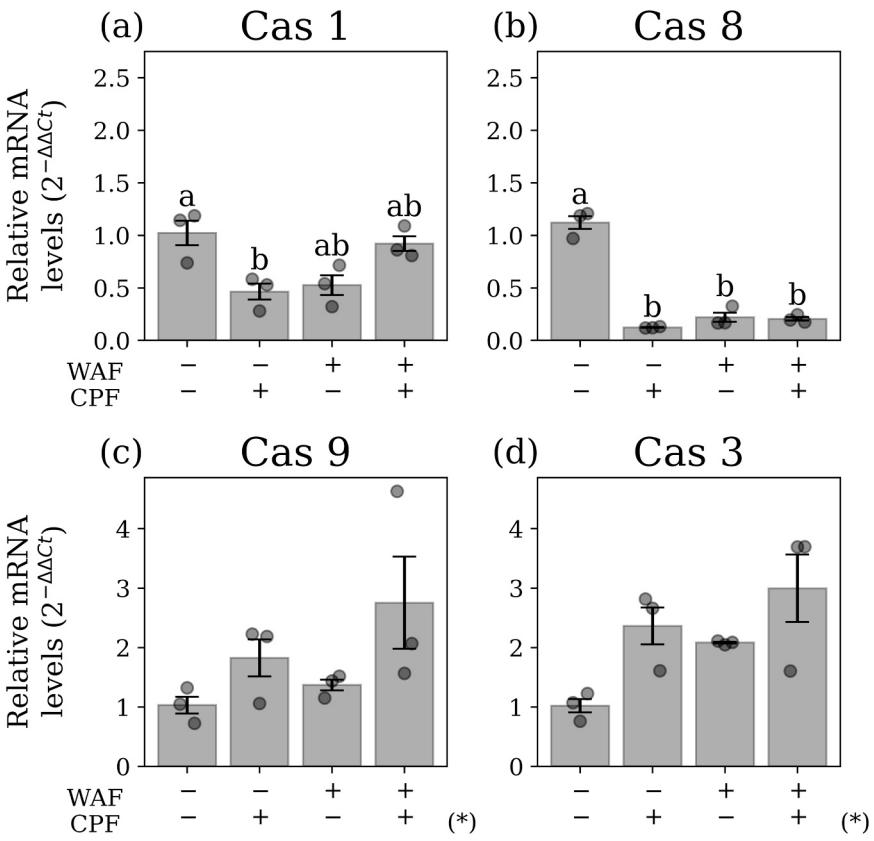


**Fig. 7.** Effects of *ex vivo* exposure to 20 μg $L^{-1}$ chlorpyrifos (CPF) on the relative mRNA expression of caspases in liver from rainbow trout previously exposed *in vivo* to 62 μg $L^{-1}$ total petroleum hydrocarbons of water accommodated fraction (WAF) or to control medium. (a) Caspase 1, (b) Caspase 8, (c) Caspase 9, (d) Caspase 3. + or – indicate the presence or absence of each treatment. Two-way ANOVA, *$p < 0.05$ for CPF effects. Different letters indicate significant differences between treatments (*post hoc* Tukey's multiple comparisons). Each point corresponds to one independent sample (n = 3).

dependent-induction of CYP1A and GR, and only a transient increase (up to 2 h) in IL-1 β mRNA expression. Hur et al. (2013) have reported that in *Paralichthys olivaceus,* the exposure to the AhR agonist benzo [a] pyrene upregulates the expression of the inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α. The upregulation of these cytokines has been directly associated with inflammation and liver injury in fish (Jia et al., 2014). In this sense, we observe an induction of the inflammatory IL-6 and IL-8 and the anti-inflammatory IL-10 by WAF exposure, together with moderate hepatic injury in *O. mykiss.* On the other hand, WAF does neither induce IL-1β nor TNFα, while exposure to CPF for 1 h induces IL-6 and TNF-α, and produces moderate tissue atrophy. The lack of IL-1β or TNF-α induction and the induction of IL-10 in WAF-exposed fish could be explained by the induction of GR (Necela and Cidlowski, 2004, for a review). Accordingly, the possible activation of the nuclear factor κB (NF-κB) pathway, leading to IL-1β, TNF-α and IL-8, by the increased expression of TNF-α in *O. mykiss* exposed to CPF alone, could have been blocked by interaction with GR, which is also induced by CPF.

The induction of TNF-α by CPF has been previously reported for fish (Wang et al., 2011; Zahran et al., 2018). Moreover, Yang et al. (2020) have shown that in *C. carpio* lymphocytes, CPF induces apoptosis through the upregulation of TNF-α, Bax, and caspases 3, 8 and 9. This pesticide has also been reported to induce the expression of caspases 3 and 9 in *C. carpio* kidney (Zhang et al., 2019) and in *O. mykiss* hepatocytes and Kupffer cells (Topal et al., 2014). In our study, the mRNA expression of caspase 9 and caspase 3 is induced by *ex vivo* exposure to CPF, suggesting a rapid activation and execution of the intrinsic apoptosis pathway. On the other hand, the extrinsic apoptosis initiator caspase 8 is strongly downregulated by all the treatments (WAF, CPF and WAF−CPF). In addition, the expression of the inflammatory caspase 1 is not increased by WAF and is downregulated by CPF.

## 5. Conclusions

The exposure to WAF hydrocarbons for 48 h produces endocrine disruption in juvenile rainbow trout, with both masculinizing and femininizing signals, through the induction of AR and PR. The addition of CPF reverses AR induction by WAF; thus, in an environment polluted by both kinds of compounds together, the overall effect would be feminizing. In addition, the exposure to WAF hydrocarbons affects lipid metabolism through the induction of the nuclear receptor LXR, with liver damage in juvenile rainbow trout, suggesting an important risk for fish population at longer times of exposure. There is also a detoxifying and antioxidant response to hydrocarbons and this effect is reinforced in part in the presence of CPF. Finally, both kind of pollutants downregulate the extrinsic apoptosis pathway, while CPF rapidly activates the intrinsic apoptosis pathway through the induction of caspases 9 and 3, together with the induction of inflammatory cytokines. The induction of GR in all the treatment groups seems to counterbalance the inflammatory effects of both hydrocarbons and CPF.

## CRediT authorship contribution statement

**Julieta S. De Anna:** Conceptualization, Methodology, Investigation, Writing - original draft. **Juan Manuel Castro:** Investigation. **Luis Arias Darraz:** Methodology, Investigation. **Federico Daniel Elías:** Visualization, Formal analysis. **Juan G. Cárcamo:** Resources, Writing - review & editing. **Carlos M. Luquet:** Conceptualization, Resources, Supervision, Writing - reviewing & editing,

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

This work was supported by CONICET, Argentina [PIP 11220130100529CO]; ANPCYT, Argentina [PICT 2013–1415 and PICT-2018–02653] to CML; FONDAP, Chile [15110027] and FONDECYT, Chile [1150934] to JGC. We gratefully acknowledge the technical assistance and material support from Institute of Biochemistry and Microbiology, Austral University of Chile (Valdivia, Chile) and from CEAN (Neuquén, Argentina). We appreciate the valuable help of Dr. Enrique Paredes Herbach in the histopathological study.

### *Declarations*

Conflicts of interest/Competing interests: the authors declare that they have no conflict of interest.

Ethics approval: the experimental protocols were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

## Appendix A. supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111394.

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