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Effects of a chronic exposure to gemfibrozil in *Carassius auratus*

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ABSTRACT

Lipid regulators, such as fibrates, are pharmaceuticals manufactured to treat dyslipidaemias in humans. Their constant use and discard combined to their environmental persistence and poor removal rates from wastewater makes of these emergent contaminants ubiquitous in aquatic systems, with gemfibrozil (GEM) being the most commonly detected fibrate in water. The present study aimed to describe the effects of a 28day waterborne exposure to both an environmentally relevant concentration (1.5 μg/L) and a spiked concentration (15 mg/L) of GEM, in adult individuals of the model organism *Carassius auratus* (goldfish). To this end, bioaccumulation of this compound in liver and muscle, as well as variations on haematological parameters, plasma biochemistry endpoints, and the expression of relevant target genes in the liver were investigated. The results indicated that, following exposure to the highest concentration of GEM, this compound accumulated in both liver and muscle. Similarly, significant increases were observed in haemoglobin levels, and mean corpuscular haemoglobin concentrations in individuals exposed to 15 mg/L of GEM. The biochemical profiling of plasma revealed significant decreases of cortisol and glucose levels, as well as a significant increase in the total antioxidant capacity (TAC) together with significant upregulations of gene transcripts related to antioxidant defences (*gpx, gst*) and lipid metabolism (*apoa1*). However, no changes were observed between treatments in the relative expression of key pro-inflammatory markers (*il1β, il6* and *il8*) nor genetic markers of lipid metabolism (*pparα pparβ* and *pparγ*) or plasma levels of cholesterol and triglycerides. Overall, the results suggest that bioaccumulation of GEM in *C. auratus* triggers mild antioxidant responses at high doses but not at environmentally relevant ones.

Introduction

Lipid regulators, such as fibrates or statins, are pharmaceuticals commonly prescribed to treat dyslipidaemias (Pahan, 2006), and their production, use and discard rates are in accelerating growth (Fang et al., 2012). The constant input of these compounds in wastewater influents (Xia et al., 2005), combined with the common inefficacy of treatment plants to remove these type of compounds from effluents (Khalil et al., 2022; Martínez et al., 2012), and their environmental persistence (Fang et al., 2012), makes of lipid regulators ubiquitous in natural environments (Pahan, 2006; Zhang et al., 2020). Fibrates decrease blood cholesterol levels in humans by activating β-oxidation pathways (Pahan, 2006), acting primarily as agonists to the peroxisome proliferator system alpha (PPARα; Zhang et al., 2020), one of a class of transcription factors. Together with the β and γ PPAR isotypes, PPARα has a major role in mammalian lipid and carbohydrate metabolism, cell differentiation and growth, inflammatory onset and antioxidant responses during whole-body homoeostasis and neurogenesis (Di Giacomo et al., 2017). It has been reported that these lipid regulators act in a similar manner in aquatic vertebrates (Al-Habsi et al., 2016), and that prolonged exposure to these compounds might influence lipid metabolism, reproduction, development, and immunity in fishes (Blonç et al., 2023b; Skolness et al., 2012; Teles et al., 2016).

Concentrations of these pharmaceuticals in a series of aquatic systems have been quantified around Europe, with the most commonly detected being gemfibrozil (GEM), a fibrate derivative widely prescribed to regulate hypertriglyceridemia and cholesterol levels (Grenni et al., 2013; Loos et al., 2009). GEM is a persistent emergent contaminant with

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a half-life between 119 and 288 days in surface waters (Araujo et al., 2011). Measured values of GEM reach 1.7 ng/L in Italian tap water (Loos et al., 2007), 10.34 ng/L in Portuguese surface waters (Pereira et al., 2017), 758 ng/L in coastal waters (Gaw et al., 2014), up to 1 μg/L in Spanish rivers (Martínez et al., 2010), an average of 16.9 μg/L in commercial crop-derived irrigation waters (García-Valverde et al., 2023) and 5.315 μg/L in wastewaters derived from hospital practices in the Mediterranean region (Castaño-Trias et al., 2023). Insufficient or unspecific wastewater management and climate-derived water scarcities may alter the amount of pharmaceutically active compounds in water bodies, aggravating the effects on the normal physiology in fish, even in cultured ones, potentially having significant impacts on the fish production sector. A major part of the literature investigating the effects of fibrates in fish focuses precisely on GEM, its presence in the environment, and its short-term effects (Blonç et al., 2023b). However, more long-term studies are needed to ascertain the impact of persistent xenobiotics on marine organisms. Studies on the effects of prolonged exposures to GEM in fish have focused mainly on zebrafish (*Danio rerio*), a classical model species, with fewer studies dedicated to the effects of these lipid regulators in commercial species (Al-Habsi et al., 2016; Blonç et al., 2023b; Fraz et al., 2018; Galus et al., M. 2014; Hammill et al., 2018; Henriques et al., 2016).

The goldfish, *Carassius auratus*, also widely acknowledged as a model organism (Blanco et al., 2018; Filice et al., 2022), presents some advantages compared to zebrafish, such as larger size, and greater resistance to environmental extremes, facilitating handling and blood extraction for haematological studies (Blanco et al., 2018). In goldfish, chronic waterborne exposures to GEM may cause bioaccumulation of GEM in plasma (Mimeault et al., 2005, 2006) leading to decreased levels of plasmatic testosterone, and the activation of antioxidant defences, as well as alterations in the lipid metabolism. Exposure to GEM in several fish species seems to elicit alterations in swimming and social behaviour (Barreto et al., 2018; Galus et al., M. 2014). However, in goldfish, long-term co-exposure to GEM and serotonin uptake inhibitors not always correlate with neurotransmitter alterations and may not influence swimming activity (Simmons et al., 2017). In other fish species, GEM exposure tampers with the bioavailability of energetic resources (Al-Habsi et al., 2016; Fraz et al., 2018), and have implications on reproduction, transgenerational performance, development, and homoeostasis, although often with evident differences between male and female individuals (Fraz et al., 2019; Lee et al., 2019; Prindiville et al., 2011; Skolness et al., 2012).

The present study aims to expand the knowledge available on the effects of chronic exposures to GEM in fish, by challenging adult *C. auratus* to a waterborne exposure to GEM over a period of 28 days. To this end, the potential for bioaccumulation of GEM in muscle and liver, the possible haematological alterations as well as differences in plasma biochemical biomarkers linked to antioxidant defences and lipid metabolism were investigated. Additionally, relative expression of transcripts related to oxidative stress (catalase, *cat*; glutathione Peroxidase 1, *gpx*, and glutathione S-transferase, *gst*), inflammatory responses (interleukins 1β, 6 and 8, *il1β, il6, il8*) and lipid metabolism (apolipoprotein A, *apoa1*; lipoprotein lipase, *lpl*; peroxisome proliferator-activated receptors α, β and γ, *pparα, pparβ, pparγ*), were also assessed.

Materials & methods

Housing and husbandry

60 adult goldfish (14.03 ± 1.47 cm total length, 26.8 ± 5.45 g total weight) were acquired from Acuario Plantado, Pamplona, Spain. The fish were randomly allocated into 12 aerated experimental tanks, each containing 20 L of water and 5 individuals, and left to acclimate for 1 week prior to the start of the challenge. Throughout the acclimation period, fish were fed to satiation (Goldfish colour pellet, Tropical). Water parameters (Temperature, pH, Ammonium, nitrites, and nitrates) were monitored daily, and 75% of the medium was changed every 3 days in order to avoid the accumulation of metabolites, and to maintain water quality at optimal levels.

Exposure to gemfibrozil and sampling

Following the acclimation period, 75% of the water was replaced in all tanks, guaranteeing that water quality parameters were at optimal levels for the start of the experiment. The twelve experimental tanks were randomly assigned to the different experimental treatments (5 fish per tank, 4 replicates per treatment), namely "Control" (Ctrl), "Low concentration" (LC), and "High concentration" (HC). Individuals belonging to the Ctrl group were exposed solely to dimethyl sulfoxide (DMSO, CAS: 67–68–5, MERCK) at a concentration of 0.5 mL DMSO/L of water. The LC group was exposed to 1.5 μg/L GEM (CAS: 25,812–30–0, MERCK) dissolved in DMSO, an environmentally relevant concentration usually found on surface waters (Fang et al., 2012), and the HC group was exposed to 15 mg/L GEM (Barreto et al., 2018). The exposure lasted 28 days, during which fish were fed daily to satiation at 10 am. Water quality parameters, mortality, and abrupt changes in behaviour were monitored throughout the experimental period, and the tanks were maintained clean. Furthermore, 75% water changes were performed every 3 days to avoid the accumulation of metabolites, and the test compounds were added again to maintain experimental concentrations in each tank throughout the test period.

Once the 28-day exposure period elapsed, fish were individually euthanised by immersion in lethal doses (300 mg/L) of MS-222 (CAS 886–86–2) buffered with sodium bicarbonate (NaHCO3, CAS 144–55–8) at a 1:2 ratio. Once stage 4 of anaesthesia (medullar collapse) was reached, the fish were collected from the anaesthesia tank and blood was extracted through caudal puncture employing 1 mL heparinised syringes equipped with 25 G needles. 1 mL of blood was immediately transferred into two Eppendorf (500 μL each) containing heparin. One aliquot was destined for haematology, and therefore stored at 4° C until analysis, whereas the remaining of the blood was centrifuged at 2000 G for 10 min in order to obtain plasma for biochemical analyses. Following blood extraction, death of the fish was confirmed through spinal rupture and necropsy was performed. Liver was collected from each fish and immediately snap frozen in liquid nitrogen and stored at −80° C until further analysis.

Gemfibrozil extraction

GEM was extracted from fish tissue samples according to Chen et al. (2015) with little modifications. The whole liver or muscle (between 0.08 and 0.5 g) was homogenised and introduced in an Eppendorf tube. Then, the matrix was spiked with 5 μl of Gemfibrozil D-16 (GEM-d16, MERCK), here used as an internal standard, at 10 mg/L and left to equilibrium for 20 min. Then, 1 ml of acetone (Chromasolv, USA) was added, homogenized in a vortex for 1 min and for 5 min in an ultrasonic bath. Then, the mixture was centrifuged at 12,000 rpm for 5 min, and 0.5 ml of the supernatant was collected and introduced in a LC-vial. This procedure was repeated twice, and the acetone collected (1.5 ml) was evaporated till dryness under nitrogen stream. Finally, the extracts were reconstituted in 0.5 ml of methanol:water (1:9; Chromasolv, USA) and preserved at −20 °C until instrumental analysis. In parallel to samples extraction, a blank consisting on acetone was used to monitor any possible cross contamination during the extraction process.

Gemfibrozil analysis

The analysis was carried out by means of liquid chromatography coupled to high resolution mass spectrometry (LC—HRMS, QExactive). The Acquity LC system was equipped with Purospher® STAR RP-18 end capped analytical column (5 μm, 2 × 125 mm, MERCK). The injection volume was 20 μL and the chromatographic separation was carried out

using gradient conditions with (A) methanol and (B) water. The starting gradient was 10% (A) for 30 s and then increased, reaching 90% (A) at 18.5 min. The gradient was maintained for 5 min in isocratic conditions, re-equilibrated until the initial gradient during 1 min and maintained for 30 s, resulting in a total run time of 25 min. The chromatographic system was coupled to the QExactive, equipped with an electrospray ionization source (ESI), operating in negative ionization mode for GEM and GEM-d16. Data acquisition was performed in Full MS (m/z 100 – 500) at a resolution of 70,000 FWHM and data dependant scan MS2 (FS-ddMS2) with a mass resolution of 17,500 FWHM for the most intense ion transition in both cases ($m/z = 121$).

The whole system was controlled by Xcalibur 3.0. The data was processed by means of Xcalibur 3.0 QuanBrowser and the quantification by means of calibration curve normalized by internal standard built in methanol:water (1:9) from 0.1 to 500 ng/ml.

Control and quality parameters

Quality and control parameters including recoveries for each matrix, reproducibility, expressed in relative standard deviation (%RSD), and method limit of detection were experimentally calculated by spiking real samples with labelled gemfibrozil pure standard (GEM-d6). Real samples were spiked at 50 ng/mL in water and with 5 µl of GEM-d6 at 1 mg/L in muscle and liver. The recoveries for matrices were 77 % for liver and 104 % for muscle, both extracted with acetone, and 90 % for water experiments extracted by SPE. The reproducibility expressed as%RSD was adequate, being 23 % for liver ($n = 10$), 6 % for muscle ($n = 10$) and 32 % for water ($n = 5$). In addition, the method's limits of quantification were settled at 0.22 ng/g for liver and muscle as well as 0.2 µg/L for water samples.

Haematology

Haematological analyses were performed less than 12 h after sampling employing the automated flow cytometer blood cell analyser Sysmex XN-1000 V, manufactured for veterinary purposes (Sysmex Corp., Kobe, Japan). The system undergoes a daily internal quality control using commercially available material specifically manufactured for this purpose: Sysmex XN Check level 1 (low range), level 2 (normal range), level 3 (abnormally high range).

Biochemistry

Total oxidative status (TOS) was determined as per Erel (2005), whereas total antioxidant capacity (TAC) was measured through analysis of the ferric reducing ability of the plasma (Benzie and Strain, 1996). Adenosine Deaminase (ADA) activity was determined with commercially available kits (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA). Cortisol levels were determined with a commercially available enzyme immunoassay involving solid-phase chemiluminescence (COR Cortisol, LKC01, Siemens Health Diagnostics, Deerfield, IL) in an automated analyser (Immulite 1000, Immulite System, Siemens Health Diagnostics; Franco-Martinez et al., 2019). Plasma glucose, cholesterol and triglyceride levels were measured using the automated analyser Olympus Diagnostica (GmbH, Freiburg, Germany) following the manufacturer's indications (Blonç et al., 2023a; Brandts et al., 2021).

Gene expression

Total RNA extraction was performed using TRI REAGENT (Sigma) as per manufacturer's recommended protocols. RNA concentration and purity was determined employing a NanoDropND-1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was generated through reverse transcription with a commercially available kit (iScript cDNA Synthesis Kit, Bio-Rad), following the manufacturer's indications. The selected primers were tested for efficiencies through RT-qPCR using serial dilutions of pooled cDNA (Pfaffl, 2001), and primers with efficiencies between 90% and 110% were selected (Table 1). RT-qPCRs were run in 384-well plates each containing 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 200 nM primers and 2 µL of cDNA, for a total volume of 20 µL, through the BIO-RADCFX384 Real-Time PCR Detection System (Bio-Rad). The selected housekeeping genes, namely *βactin, rps5* and *rpl7*, were assessed for suitability using the GENorm algorithm integrated into the Bio-Rad CFX Maestro software (Bio-Rad). The results indicated that the most stable genes were *rps5* and *rpl7*, which were therefore employed as reference genes. The gene expression was calculated relative to 0 as per the Bio-Rad CFX Maestro Used Guide's recommendations.

Statistical analyses

All collected data was analysed using the open-source software RStudio. Data was tested for homoscedasticity and normality employing the *Bartlett's test* and the *Shapiro-Wilk* test, respectively. As the data met the assumptions for parametric tests, one-way Analyses of Variance (ANOVA) were run to determine significant differences amongst groups (P-values < 0.05). For the post-hoc test, the authors employed a pairwise comparison with Bonferroni adjustment.

Results

Bioaccumulation of gemfibrozil in liver and muscle

The results from the quantification of GEM in both liver and muscle indicate that bioaccumulation occurred at significantly higher rates in the HC group, compared to both the Ctrl and the LC groups (Fig. 1). GEM was also detected in both organs of Ctrl and LC, but levels remained below the threshold for quantification. Therefore, no statistically significant differences were found between these two groups. Furthermore, no significant differences were detected in the bioaccumulation rates between liver and muscle.

Haematology

The results obtained from the haematological analyses indicate a significant increase of haemoglobin (HGB) in HC compared to Ctrl (Fig. 2). Mean corpuscular haemoglobin concentration (MCHC) resulted significantly lower in LC compared to HC. However, no significant differences were found between Ctrl and LC nor between Ctrl and HC. None of the other investigated parameters, including white and red blood cell counts (WBC; RBC), haematocrit (HCT), and platelet count (PT), displayed significant variations between experimental groups.

Biochemical markers in plasma

The results from the biochemical analyses of blood plasma (Fig. 3) indicate significant reductions in cortisol and glucose levels between the Ctrl and both exposed groups. No significant differences in these parameters were detected between LC and HC. Whereas no differences between treatments were found concerning TOS, TAC showed a significant increase in the HC group when compared to Ctrl and LC. No significant differences were found between treatment groups in ADA activity, triglycerides and cholesterol plasma levels between treatments.

Gene expression in liver

The results obtained through real time qPCR analysis (Fig. 4) revealed that *apoa1* transcripts were significantly upregulated in the HC group compared to the Ctrl and LC groups but the expression of the rest of transcripts associated to lipid metabolism (*lpl*, and *pparα, pparβ* and *pparγ*) remained unaltered at the end of the 28-day exposure to GEM.

Table 1
Primer sequences used in the real time qPCR analysis.

Gene	GenBank Accession number	Sequence (5′->3′)		Product size
rps5	XM_026226870.1	Fw	5′ CACGCCTTTGAGATCATCCAC	128
		Rv	5′ TGTCTCCTCACGGTTCCAG	
rlp7	XM_026286641.1	Fw	5′ ATGGTGTCAGCCCTAAAGTCC	190
		Rv	5′ ATCTTGCCAAATCCACGCTTG	
pparα	XM_026238527.1	Fw	5′ CCCCAGAGTCTCCAAATGACC	269
		Rv	5′ TGACTGGCTTTGTGAGGAGG	
pparβ	XM_026270284.1	Fw	5′ TGGCTTTGTGGATCTCTTCC	178
		Rv	5′ GATCTCGCTGAAAGGTTTGC	
pparυ	XM_026240742.1	Fw	5‘ TTCCACAGCTGTCAGTCTCG	201
		Rv	5′ CATGAAGATCTGTCCGTAGG	
lpl	XM_026198452.1	Fw	5′ GGCCAAGTTTGTCAACTGG	166
		Rv	5′ CTCAAAACTAGGGCCAGCA	
apoa 1	XM_026226007.1	Fw	5′ TCAACTGGGAGGAGACCAAG	201
		Rv	5′ TGCCCAACTCTTCCATCTTC	
il1β	XM_026220385.1	Fw	5′ GATGCGCTGCTCAGCTTCT	66
		Rv	5′ AGTGGGTGCTACATTAACCATACG	
il6	XM_026289280.1	Fw	5′ CTGGATGCAGTGCCTGTCTA	214
		Rv	5′ TCACGTCTTCAAACGCAGTC	
il8	XM_026204952	Fw	5′ CTGAGAGTCGACGCATTGGAA	75
		Rv	5′ TGGTGTCTTTACAGTGTGAGTTTGG	
cat	XM_026203265.1	Fw	5′ TACACCGATGAGGGCAACT	170
		Rv	5′ AACGATTCAGGACGCAAAC	
gpx	XR_003293223.1	Fw	5′ GAAGTGAACGGTGTGAACGC	94
		Rv	5′ GATCCCCCATCAAGGACACG	
gst	XM_026224781.1	Fw	5′ GTGGATTACTTCAATGGCAGAG	211
		Rv	5′ CAGCGATGTAGTTCAGGATGG	

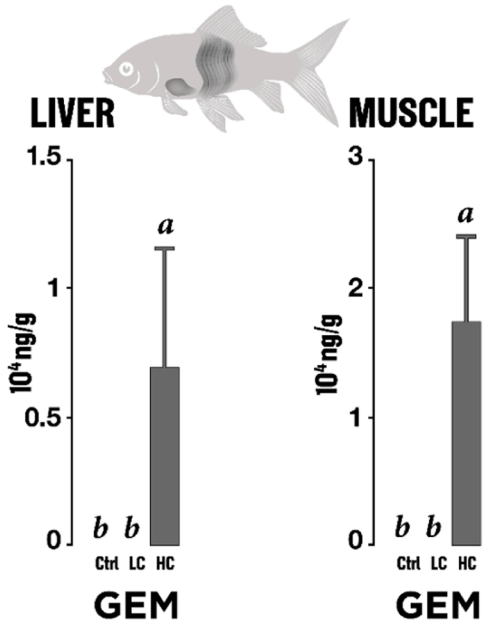

Fig. 1. Bioaccumulation of gemfibrozil (GEM; mean ± SD) in liver and muscle of adult *Carassius auratus* following a 28-day waterborne exposure ($n = 10$; Ctrl: 0 mg GEM/L; LC: 1.5 μg GEM/L; HC: 15 mg GEM/L). Significant differences ($P<0.05$) are depicted by different letters.

Similarly, the expression of transcripts related to inflammatory responses (*il1β, il6* and *il8*) did not change the expression levels between treatments. Concerning the anti-oxidative mediators, *gpx* and *gst*, but not *cat,* transcripts appeared significantly upregulated in LC compared to Ctrl and in HC compared to LC.

Discussion

Once absorbed by the gastrointestinal tract, GEM exerts its multi-tissue hypolipidemic effects in mammals by activating the PPAR receptors, inducing an up-regulation of *apoa1* and *lpl* mainly in muscle and adipose tissues. In turn, this decreases the levels of plasma triglycerides, inhibits peripheral lipolysis, and reduces the hepatic removal of fatty acids by mechanisms still not entirely understood (Roy and Pahan, 2009). The present results showed that GEM bioaccumulates at quantifiable concentrations in the liver and muscle of fish at high (15 mg/L) exposure concentrations (Fig. 1). However, GEM was also detected in the control group and in fish challenged with LC (1.5 μg/L) concentrations of this compound, although below the threshold for quantification. The bioaccumulation of GEM observed in goldfish tissues is in accordance with the described bioaccumulation of GEM in muscular and fatty tissues of fish(X. Zhang et al., 2010), plasma (Mimeault et al., 2005), and liver (Ramirez et al., 2009; Skolness et al., 2012), at environmentally relevant, and spiked waterborne concentrations. In addition, a previous study described bioretention of GEM in whole-fish samples collected from wild populations, with lower concentrations being quantified in fish tissues than in the surrounding water (Meador et al., 2016). The detection of GEM in the tissues of the control group could be explained by the possible current presence of this compound in Spanish tap water, as described in other European countries (Loos et al., 2007). Furthermore, it is likely that, as in humans, GEM undergoes hepatic metabolism, being transformed into inactive metabolites in the liver (Quintanilla Rodriguez and Correa, 2019). Therefore, the evident differences in accumulation rates between the analysed organs might be directly linked to the pharmacokinetic properties of this compound, and the enzymatic differences between liver and muscle. In fact, the bioaccumulation properties of pharmaceuticals and other xenobiotics in fish seem to differ between organs and tissues, due to species-specific metabolic needs (Chen et al., 2017), and usually the liver seem to be more efficient (due to a more complex enzymatic pool of xenobiotic degradative components) than muscle in the short-term clearance of xenobiotics and unwanted pharmaceuticals, lessening the outcome of oxidative stress (Beghin et al., 2021). This may explain the observed accumulation of GEM in the muscle vs. liver in goldfish (the former being a bona fide indicator of chronic contamination, as suggested by Orias et al. (2015), and may also relate to the appearance on myopathologies and muscle disorders related to an excess of Gemfibrozil in mammals, mainly due to inhibition of the glucuronidation pathway and cytochrome P4502C8 (CYP2C8) activity (Dubińska-Magiera et al.,

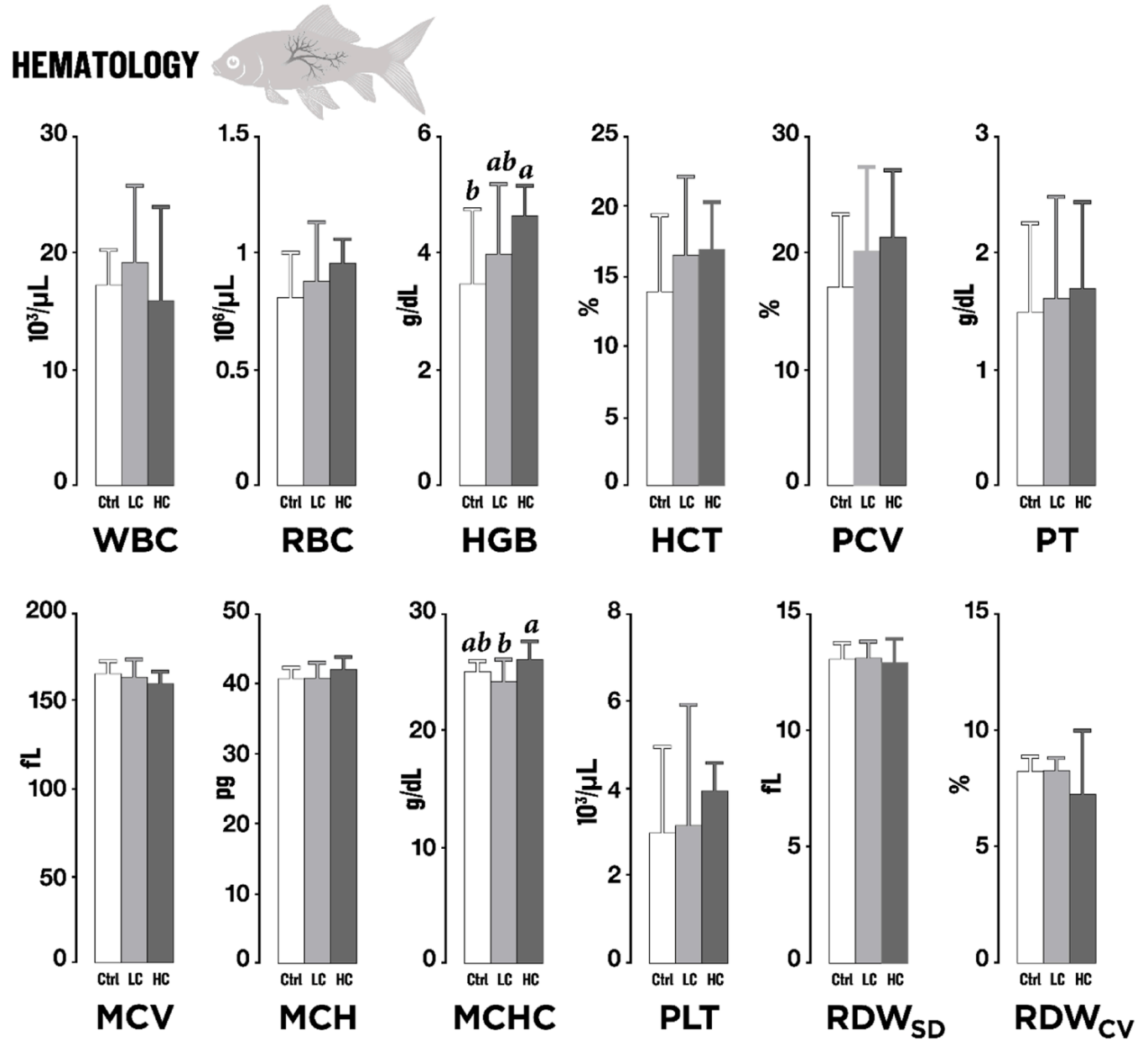

Fig. 2. Haematological parameters of adult *Carassius auratus* following a 28-day exposure to gemfibrozil (GEM; $n = 10$; Ctrl: 0 mg GEM /L; LC: 1.5 µg GEM/L; HC: 15 mg GEM/L; mean ± SD). Significant differences ($P<0.05$) are depicted by different letters. The investigated parameters were: White Blood Cell count (WBC), Red Blood Cell count (RBC), Haemoglobin (HGB), Haematocrit (HCT), Packed Cell Volume (PCV), Prothrombin Time test (PT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Platelet count (PLT), Red Cell Distribution Width (RDW).

2021) Present results, however, contrast with a previous report of bioaccumulation in *C. auratus* plasma following a 14-day waterborne exposure to 1.5 µg/L and 1.5 mg/L of GEM (Mimeault et al., 2005). Altogether this suggests a variable pattern of bioaccumulation during chronic exposures to the drug that, once taken up through the gills (Mimeault et al., 2005), is then transported by blood and accumulated within the plasma at first, accumulating in organs (e.g. muscle) only after longer exposures, or at higher doses.

A common side effect of GEM in humans is the development of anaemia (Strauss et al., 2012). Present results indicate that chronic exposure to GEM did not induce significant changes in the number of erythrocytes, leukocytes nor haematocrit in goldfish after the 28-day treatment. Nonetheless, total haemoglobin (HGB), as well as mean corpuscular haemoglobin concentration (MCHC) resulted higher in the HC group. This could indicate that, as opposed as what has been reported in humans (Scatena et al., 1995), haemoconcentration occurred due to a reduced affinity of red blood cells to haemoglobin. Therefore, the observed response is similar to that observed in fish subjected to hypoxic conditions (Tervonen et al., 2006), or to an acidic medium (Kulkarni and Barad, 2015). However, it cannot be ruled out that the present results reflected the remains of an over-compensatory erythropoietin (EPO)-mediated effect regulated by the activation of hypoxia-inducible factor (HIF) as described for PPARα-knockout mice exposed to GEM (Estrela et al., 2020). The authors reported a transient (14 days) PPARα-dependant anaemia, leukopenia and changes in haemoglobin and haematocrit levels that were reverted on day 21 after GEM treatment, concurrent with elevated EPO plasma levels and increased expression of *HIF-2α* and EPO in renal tissue of treated mice. The mechanisms of GEM-induced anaemia are still far from being resolved, and the EPO-dependant regulation of erythropoiesis in fish differs from

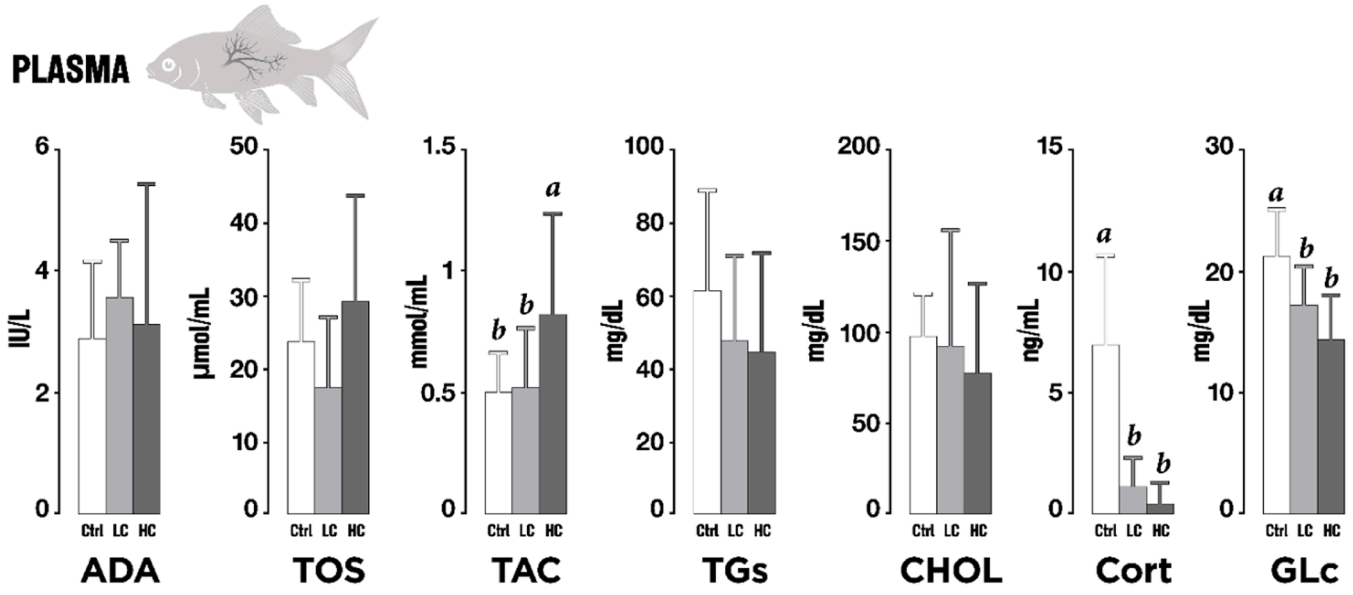

Fig. 3. Biochemical parameters in *Carassius auratus* blood plasma following a 28-day waterborne exposure to gemfibrozil (GEM; $n = 10$; Ctrl: 0 mg GEM/L; LC: 1.5 µg GEM/L; HC: 15 mg GEM/L; mean ± SD). Statistical differences ($P<0.05$) are represented by different letters above the corresponding columns. The investigated endpoints were Adenosine Deaminase (ADA), Total Antioxidant Capacity (TAC), Total Oxidative Status (TOS), Triglycerides (TGs), Cholesterol (CHOL), Cortisol (Cort), and Glucose (GLc).

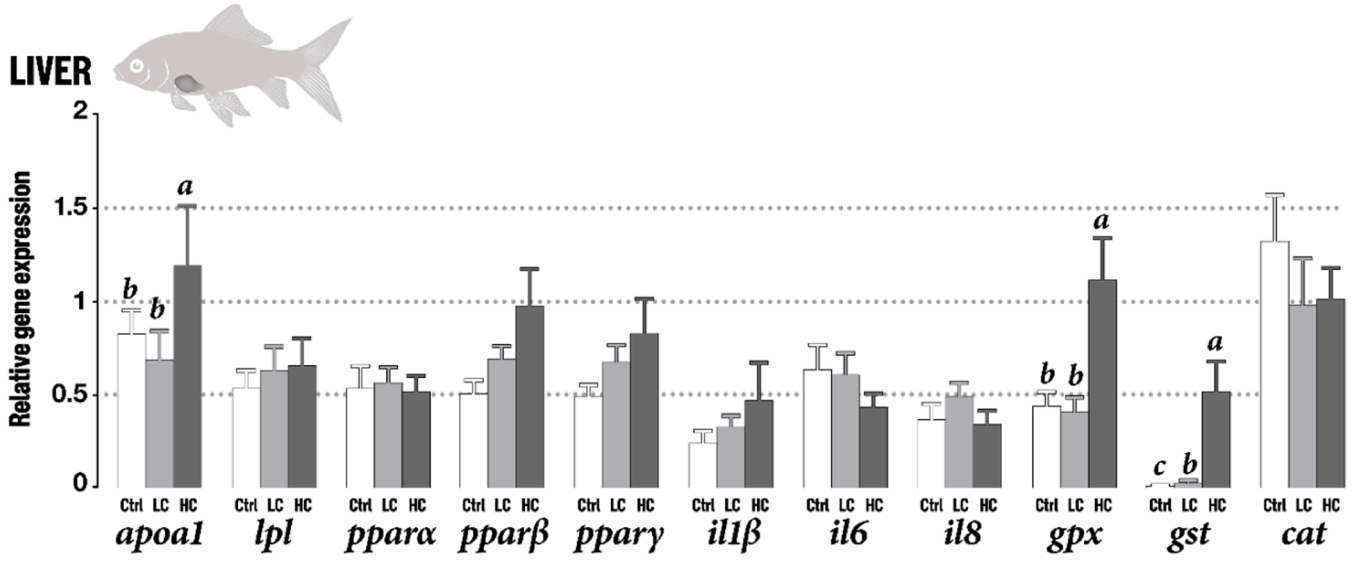

Fig. 4. Relative gene expression of *Carassius auratus* liver homogenates following a 28-day waterborne exposure to gemfibrozil (GEM; $n = 10$; Ctrl: 0 mg GEM/L; LC: 1.5 µg GEM/L; HC: 15 mg GEM/L; mean ± SD). Statistical differences ($P<0.05$) are represented by different letters above the corresponding columns. The investigated endpoints were Catalase, *cat*; Glutathione Peroxidase 1, *gpx,* glutathione *S*-transferase, *gst*, interleukins 6, 8 and 1β, *il6, il8, il1β;* apolipoprotein A, *apoa1*; lipoprotein lipase, *lpl*; peroxisome proliferator-*activated* receptors α, β, and ϒ, *pparα, pparβ, pparγ*.

that of mammals (Kulkeaw and Sugiyama, 2012), but present results seem to agree with the timing of haematological variations described for short-term/chronic GEM exposure in mice.

Absence of major changes in lipid metabolism and immune biomarkers in goldfish after chronic exposure to gemfibrozil

The 28-day exposure to GEM did not lead to significant changes in plasma levels of neither cholesterol nor triglycerides. These results agree with previously published literature, stating that no significant changes in plasma cholesterol and triglycerides occurred following a waterborne exposure of *Pimephales promelas* (fathead minnow) to GEM (Skolness et al., 2012). In contrast, it has been reported that *Oncorhynchus mykiss* (rainbow trout) exposed to this emergent contaminant through injection did display significant alterations in these parameters (Prindiville et al., 2011). This suggests a species-specific scenario of lipid physiology in fish that also involves the regulatory adipogenesis networks. The management of lipids in fish results from a balance between hypertrophy and hyperplasia of adipocytes in visceral adipose tissues (Salmerón, 2018). This is orchestrated by *pparγ* and assisted by adipogenic gene markers such as *lpl*, involved in the hydrolysis of circulating triglycerides to produce the free fatty acids (FFA) required for growth and metabolism (Rakhshandehroo et al., 2010), or *apoa1*, implicated in the transport of cholesterol (Xie et al., 2020). *Pparα* and *pparβ* complete the minimal

genetic repertoire to adequately process lipids, with varied tissue representation but similar ligands in fish species (Páscoa et al., 2022). However, the pleiotropic effects of the PPAR family extend beyond the management of lipids and affect general homoeostasis. For instance, the activation of PPARα upon feeding on high-fat content diets enhances the expression of antioxidant regulators, such as *cat* and *gpx*. Furthermore, it inhibits the expression of pro-inflammatory cytokines such as *il1β* or *il6* in an effort to control the upsurge of reactive oxygen species (ROS) derived from the oxidative metabolism of lipids and FFA absorption (Ahmadian et al., 2013). *Pparγ*, in turn, may be inhibited by an excess of ROS, adding layers of complexity to the regulation of lipid metabolism in presence of xenobiotics (Li et al., 2022). In the present experiment, a significant increase in the expression of *apoa1* mRNA in goldfish liver exposed to GEM was observed, being consistent with previously published literature. It has been reported that after a short-term exposure to 150 μg/L of GEM in water, *Sparus aurata* (gilthead seabream) displayed a significant upregulation of this gene in the liver (Teles et al., 2016). Concerning PPARs, the significant differences in the expression of *pparα* transcripts were somewhat expected, as it has been demonstrated that PPARs may be affected, to some extent, by GEM. In addition, they may regulate the expression of other genes involved in lipid metabolism (e.g. *apoa1, lpl*) in mammals (Singh et al., 2011; Staels et al., 1998),. However, previous studies on fishes have stated that GEM does not necessarily induce the activation of *ppar* pathways in the liver, although it may alter the abundance of other genes related to lipid metabolism, such as *lpl* and *apoa1* (Teles et al., 2016). This was reported, for instance, in rainbow trout exposed to GEM through injections over a period of 15 days, where an increase in *lpl*, was observed, although not accompanied by up- or down-regulations of *pparα* (Prindiville et al., 2011). This is consistent with the results obtained through the present experiment. Additionally, *C. auratus* exposed to waterborne GEM at concentrations reaching 1500 μg/L failed to display increased levels of *pparα* and *pparγ*. However, the expression of *pparβ* mRNA resulted significantly downregulated (Mimeault et al., 2006). On the other hand, an experiment challenging *D. rerio* with dietary exposure to GEM over a 30-day period resulted in significant upregulations of *pparα,* only in females, and of *pparγ*, regardless of sex (Al-Habsi et al., 2016). In addition to *pparα, pparβ* and *pparγ* transcripts remained unresponsive to GEM that, together with the lack of differences in plasma cholesterol and triglyceride levels between treatments, indicates a lack of altered overall lipid metabolism in response to long-term exposure to GEM in goldfish.

The absence of changes in the expression of *pparα* transcripts may explain the absence of inflammation at day 28, as indicated by the lack of changes in the expression of key pro-inflammatory immune transcripts, namely *il1β, il6*, and *il8*. Previously published studies have reported a significant increase in the expression of *il1β* following a short-term waterborne exposure to GEM, both in the liver (Teles et al., 2016), and in the gills and head kidney of *S. aurata* (Oliveira et al., 2018). However, the authors described this effect as time- and dose-dependant. Similarly, the expression of *il6* did not change between groups in the present study. Oliveira et al. (2018) described a significant increase in the abundance of this gene in the head kidney of *S. aurata*. However, this was only observed at 1500 μg/L, and not at lower nor higher concentrations in this organ. In addition, the levels of this gene remained unchanged in the gills of gilthead seabream, regardless of the exposure concentration (Oliveira et al., 2018). This further corroborates that the response to this emergent contaminant is dependant on the dose and the organ investigated. Lastly, the expression of *il8* did not display any significant differences amongst groups in the present study. In humans, GEM has been demonstrated to significantly reduce the expression levels of this pro-inflammatory cytokine, either as a direct effect (Wägner et al., 2011), or through the inhibition of *il1β*-induced expression of *il8*, as observed with other fibrates (Stolarz et al., 2015). However, as the present results did not indicate any significant decrease in either *il1β* or *il8*, it can be hypothesized that the mechanism of actions of GEM, as other fibrates, may differ from mammals to fish. Therefore, further studies are required to fully understand the effects GEM may have on the immune response amongst these taxa.

Impact of long-term exposure to gemfibrozil on cortisol levels in goldfish

More intriguing is the downfall of plasma cortisol levels at day 28 in goldfish exposed to low and high concentrations of GEM (see Fig. 3). The expected physiological response to stressors is an increase of cortisol levels in plasma, resulting from an activation of the hypothalamic-pituitary-interrenal (HPI) axis (Aluru and Vijayan, 2009). Enduring a chronic exposure to GEM, which also acts as an endocrine disruptor, could alter the availability of energetic resources, such as steroids, and affect the HPI axis as observed with other endocrine disruptors (Teles et al., 2006), impairing the cortisol-mediated response. Alterations of stress neuroendocrine axis in fish exposed to pharmaceutical xenobiotics have been described previously in zebrafish (Al-Habsi et al., 2016). Indeed, low levels of cortisol were also demonstrated in females, but not in males, treated chronically (30 days) with GEM and other anti-dyslipidaemics. The authors argued that this could be due to altered signalling in reproductive-related triglyceride and cholesterol balance and abundance (Al-Habsi et al., 2016), which were unaltered in the present study. Instead, present results seem to correlate with the effects described in zebrafish exposed to antipsychotic drugs such as diazepam and fluoxetine, that diminish cortisol levels at short- and long-term challenges, effectively blocking the cortisol-mediated behavioural responses to acute stress and anxiety (Egan et al., 2009), whereas risperidone and aripiprazole increase the plasma cortisol levels in the same species (reviewed by Mauro et al., 2021). In the present research study, glucose follows the same pattern described for cortisol, but, considering the lack of expression of *pparα* transcripts, that once active are known to decrease glucose levels (Chou et al., 2002), the diminishing pattern probably reflects the decrease in the cortisol-dependant hepatic gluconeogenesis (Jentoft et al., 2005). Reduced plasma glucose levels may also reflect the depletion of an energetic resource due to a higher energetic demand of exposed individuals undergoing a series of highly energetically demanding processes. These may include antioxidant mechanisms to counter the effects of ROS associated with xenobiotic exposure (Liang et al., 2020). Clearly, the effects of pharmaceutically active compounds, such as GEM, on the stress physiology of fish are species-, sex-, drug-, dose-, and developmental stage-dependant and may be influenced by the combined interaction between several xenobiotics. The underlying physiological mechanisms that lower plasma cortisol levels in presence of pharmaceutical xenobiotics are still unresolved in fish but may correlate with the affectation of serotonergic signalling in the central nervous system (Porretti et al., 2022), that partially determines behavioural patterns. In this sense, male zebrafish early exposed to fluoxetine maintained hypocortisolism and exploratory behaviour in the offspring (Vera-Chang et al., 2018). This suggests that, if persistent, the potential anxiolytic effects induced by chronic exposure to both low and high levels of GEM in goldfish may be of special concern. Furthermore, these may be difficult to predict in terms of behavioural coping with stressful environments prone to biomagnification and unpredictable changes in physicochemical variables. It has been suggested that, in fish, plasmatic cortisol levels are tightly linked to the hierarchical position of individuals (Sloman et al., 2004), and that, consequently, cortisol-linked social interactions might be hampered by prolonged exposure to GEM. Similarly, a wide range of cortisol-related processes, such as anti-predatory behaviour, responses to environmental stressors, or growth, might be impaired, having strong deleterious effects on the fitness of individuals, and significantly affecting their survival. (Gagnon et al., 2006; Teles et al., 2006). Therefore, the presence of GEM in aquatic systems may have effects not only at an individual level, but also at a population or species scale.

Oxidative management after long-term exposure to gemfibrozil in goldfish

GEM has been considered an agonist of PPARα and thus been added to the formulation of fish diets with high content of carbohydrates, in an attempt to control for the undesirable oxidative and immune effects of such diets in such dysregulated metabolism scenarios (Luo et al., 2020). However, the present results indicate that the agonistic effects of GEM on goldfish were only partially fulfilled: the apparent immune quiescence contrasts with the upregulated expression of oxidative-stress-related transcripts at high doses, together with an enhanced TAC. TAC and TOS reflect the combined action of different antioxidants, and the presence of different oxidant species, respectively, and have been suggested as valuable endpoints to assess the possible oxidative damage in fish following an exposure to chemicals (Teles et al., 2016). In the present study, TAC, but not TOS nor ADA, a biomarker for both oxidative stress and immune response (Blonç et al., 2023a), increased in the HC group, compared to both the LC and Ctrl groups. The observed TAC values may reflect, in part, the upregulation of transcripts related to antioxidant defences. The relative expression of hepatic *gpx* mRNAs was significantly higher in the HC group when compared to the Ctrl and LC groups, agreeing with the previous findings in the same species (Mimeault et al., 2006). However, in contradiction with the results of the present experiment, the authors described a significant increase of *gpx* related enzymes at the lowest tested concentrations, equivalent to the one hereby imposed on the LC group, compared to the control, but not between exposed groups. In *S. aurata*, previous findings have reported an upregulation of this antioxidant defence-related transcript, as well as superoxide dismutase (*sod2*), in gills and head kidney after a 96-h exposure to 15 mg/L GEM (Oliveira et al., 2018). However, the authors stated that no other antioxidant indicator resulted significantly altered by this exposure. In contrast, Teles et al. (2016), following a similar experimental design, described no significant changes in the activity of any antioxidant genes in the liver of the same species, under the same exposure conditions. On the other hand, a study replicating the experiment found significant increases in the hepatic expression of *gpx* at concentrations ranging from 15 μg/L to 15 mg/L GEM (Barreto et al., 2018). On the contrary, intraperitoneal (IP) injection of GEM in *Solea senegalensis* resulted in significantly lower *gpx* activity compared to the control group (Solé et al., 2014). The upregulation of hepatic *gst* transcripts observed in the present study with increasing GEM concentration agrees partly with previously published results stating significant increases in this gene's activity only at 1.5 μg/L, but not at higher concentrations (Mimeault et al., 2006). Nonetheless, no changes in the expression of this antioxidant indicator were reported in either liver, gills nor head kidney of *S. aurata* and *Anguilla anguilla* (European eel; Barreto et al., 2018; Lyssimachou et al., 2014; Oliveira et al., 2018; Teles et al., 2016). As with *gpx, gst* activity in the liver of *S. senegalensis* was significantly lowered by IP injection of GEM (Solé et al., 2014). Lastly, no significant differences were found in abundance of *cat* mRNA in the present study. These results agree with previous findings on *S. aurata* challenged with an acute waterborne exposure to GEM, where no significant alterations in catalase activity were described (Oliveira et al., 2018; Teles et al., 2016). Nonetheless, a study on *C. auratus* with similar experimental design as the present, resulted in a significant increase in the expression of hepatic *cat* with increasing GEM concentration. Similarly, Barreto et al. (2018) observed an increase in *cat* at 15 mg/L GEM in *S. aurata.* In addition, Lyssimachou et al. (2014) reported an upregulation of *cat*, although only in *A. anguilla* injected with 0.1 μg/L, and not at higher concentrations. In contrast, in *S. senegalensis* injected with GEM, a significant decrease in catalase activity was reported (Solé et al., 2014).

Overall, the anti-oxidative response in goldfish enduring 28 days of GEM seems restricted to high doses and limited in intensity and recruitment of antioxidant markers. This may indicate a decaying defence against ROS during the initial exposure to GEM that trends towards normal homoeostasis at day 28. Alternatively, this could indicate a mildly oxidative stress elicited by continuous exposure to GEM. However, as discussed above, *pparγ* and pro-inflammatory transcripts remained unaltered, which suggests an absence of a strong immune-related ROS production that may eventually inhibit *pparγ* (Li et al., 2022). On the other hand, this could result from the activation of macrophages and other cellular components on the defence response against a xenobiotic. Therefore, the anti-oxidative response to GEM may obey the complex effects of the breakdown of GEM at high but sublethal doses following the bioaccumulation in liver and muscle of goldfish. In mammals, drug and xenobiotic biotransformation involves the cytochrome P450 (CYP)-dependant phase I and the conjugation-dependant phase II enzymatic reactions. The former includes hydrolysis and oxidation of molecules and the later the presence of endogenous polar components, such as glutathione, that links to the xenobiotic due to transferases such as glutathione S-transferases (Xu et al., 2005). Both enzymatic complexes oxidize GEM to highly reactive phenolic and glucuronide metabolites and induce oxidative stress that, in turn, requires the activation of antioxidant biomarkers (Stading et al., 2020). Activity of CYP enzymes was not hereby evaluated. However, considering the upregulation of *gst* transcripts observed in goldfish exposed to high doses of GEM, and the quiescence of the pro-inflammatory mRNAs analysed, the overrepresentation of reactive metabolites derived from the biotransformation of chronic exposure to high doses of GEM may account for the mild anti-oxidative responses observed. However, it is worth mentioning that the biotransformation reactions are complex, still poorly studied in fish and strongly organ-, species-, sex-, and xenobiotic type-dependant.

Concluding remarks

To summarize, the present results show that a chronic (28-day) exposure to environmentally relevant and high sublethal doses of GEM induced bioaccumulation of this pharmaceutical in the goldfish liver and muscle. The latter is of particular importance to the aquaculture industry, as it is the most relevant in terms of fish consumption. Therefore, it represents an additional exposure and biomagnification pathway for humans through the consumption of contaminated seafood products. Both prolonged exposure and bioconcentration may have played a role in the observed differences in biochemical plasma endpoints between treatments, suggesting (1) a mild anti-oxidative response to high but not low doses of GEM that maybe related to the biotransformation enzymatic reactions to GEM, (2) the possibility of transient anaemia during the treatment, (3) an unaltered expression of lipid metabolism biomarkers, including key regulators such as *pparα* and *pparγ*, and pro-inflammatory cytokine transcripts, notably *il1β*, a canonical telltale of inflammatory outbursts, and (4) an intriguing reduction of plasma cortisol levels. This indicates that GEM may act, to some extent, as a stress-inducing agent, and, as described in other fish species, may tamper with reproduction, development, stress responses, and overall fitness of both wild and cultured populations, affecting the bioavailability of energetic resource in a strongly species-specific manner. The effects of GEM depend upon the developmental stage, sex, exposure concentration and duration. Therefore, further studies are needed to describe the in-deep mechanisms of physiological disturbance that high doses of GEM. These disturbances may originate from inappropriate wastewater management or unregulated bioaccumulation in natural and cultured systems. The present findings should serve as a foundation for future research, which is needed to understand their potential effects at different life stages of different species, both wild and cultured, and may help shape future legislations concerning pharmaceutical waste management and sewage treatment practices. Furthermore, the relevance of long-term exposure in terms of adaptive phenotypes in the population and persistent transgenerational physiological changes must be looked into. Moreover, investigating the synergistic effect of GEM in combination with other contaminants (Andrzejczyk et al., 2020), or environmental factors, may give a more comprehensive insight on the

mechanism of action of this compound, and their implications, not only from an environmental point of view, but for the aquaculture industry, and ultimately global food security.

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Author contribution

Conceptualization: MB, NR, MT, LT; Funding acquisition: LT, MT, MF; Investigation: MB, NR, JCB, LT, MF, ML, AT, MT; Writing – original draft: MB, NR, JCB, ML, AT; Writing – review & editing: All authors, Supervision: LT, MT.

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.

Data availability

Data will be made available on request.

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References

Ahmadian, M., Suh, J.M., Hah, N., Liddle, C., Atkins, A.R., Downes, M., Evans, R.M., 2013. PPARγ signaling and metabolism: the good, the bad and the future. Nat. Med. 19 (5), 557–566. https://doi.org/10.1038/nm.3159.

Al-Habsi, A.A., Massarsky, A., Moon, T.W., 2016. Exposure to gemfibrozil and atorvastatin affects cholesterol metabolism and steroid production in zebrafish (Danio rerio). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 199, 87–96. https://doi.org/10.1016/j.cbpb.2015.11.009.

Aluru, N., Vijayan, M.M., 2009. Stress transcriptomics in fish: a role for genomic cortisol signaling. Gen. Comp. Endocrinol. 164 (2–3), 142–150. https://doi.org/10.1016/j.ygcen.2009.03.020.

Andrzejczyk, N.E., Greer, J.B., Nelson, E., Zhang, J., Rimoldi, J.M., Gadepalli, R.S.V., Edwards, I., Schlenk, D., 2020. Novel disinfection byproducts formed from the pharmaceutical gemfibrozil are bioaccumulative and elicit increased toxicity relative to the parent compound in marine polychaetes (*Neanthes arenaceodentata*. Environ. Sci. Technol. 54 (18), 11127–11136. https://doi.org/10.1021/acs.est.0c01080.

Araujo, L., Villa, N., Camargo, N., Bustos, M., García, T., Prieto, A., de, J., 2011. Persistence of gemfibrozil, naproxen and mefenamic acid in natural waters. Environ. Chem. Lett. 9 (1), 13–18. https://doi.org/10.1007/s10311-009-0239-5.

Barreto, A., Luis, L.G., Paíga, P., Santos, L.H.M.L.M., Delerue-Matos, C., Soares, A.M.V. M., Hylland, K., Loureiro, S., Oliveira, M., 2018. A multibiomarker approach highlights effects induced by the human pharmaceutical gemfibrozil to gilthead seabream Sparus aurata. Aquat. Toxicol. 200, 266–274. https://doi.org/10.1016/j.aquatox.2018.05.012.

Beghin, M., Schmitz, M., Betoulle, S., Palluel, O., Baekelandt, S., Mandiki, S.N.M., Gillet, E., Nott, K., Porcher, J.-M., Robert, C., Ronkart, S., Kestemont, P., 2021. Integrated multi-biomarker responses of juvenile rainbow trout (Oncorhynchus mykiss) to an environmentally relevant pharmaceutical mixture. Ecotoxicol. Environ. Saf. 221, 112454 https://doi.org/10.1016/j.ecoenv.2021.112454.

Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power. The FRAP Assay. Anal. Biochem. 239 (1), 70–76. https://doi.org/10.1006/abio.1996.0292.

Blanco, A.M., Sundarrajan, L., Bertucci, J.I., Unniappan, S., 2018. Why goldfish? Merits and challenges in employing goldfish as a model organism in comparative endocrinology research. Gen. Comp. Endocrinol. 257, 13–28. https://doi.org/10.1016/j.ygcen.2017.02.001.

Blonç, M., Brandts, I., Cánovas, M., Franco-Martínez, L., Rubio, C.P., Tort, L., Tvarijonaviciute, A., Gravato, C., Teles, M., 2023a. Evaluation of a chronic exposure to nanoplastics in goldfish (Carassius auratus): analytical validation of automated assays for the measurement of biochemical markers. Ecol. Indic. 147, 109966 https://doi.org/10.1016/j.ecolind.2023.109966.

Blonç, M., Lima, J., Balasch, J.C., Tort, L., Gravato, C., Teles, M., 2023b. Elucidating the effects of the lipids regulators fibrates and statins on the health status of finfish species: a review. Animals 13 (5), 792. https://doi.org/10.3390/ani13050792.

Brandts, I., Barría, C., Martins, M.A., Franco-Martínez, L., Barreto, A., Tvarijonaviciute, A., Tort, L., Oliveira, M., Teles, M., 2021. Waterborne exposure of gilthead seabream (Sparus aurata) to polymethylmethacrylate nanoplastics causes effects at cellular and molecular levels. J. Hazard. Mater. 403, 123590 https://doi.org/10.1016/j.jhazmat.2020.123590.

Castaño-Trias, M., Rodríguez-Mozaz, S., Buttiglieri, G., 2023. A decade of water monitoring in a Mediterranean region: pharmaceutical prioritisation for an upgraded analytical methodology. Environ. Nanotechnol. Monitor. Manag. 20, 100850 https://doi.org/10.1016/j.enmm.2023.100850.

Chen, F., Gong, Z., Kelly, B.C., 2015. Rapid analysis of pharmaceuticals and personal care products in fish plasma micro-aliquots using liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1383, 104–111. https://doi.org/10.1016/j.chroma.2015.01.033.

Chen, F., Gong, Z., Kelly, B.C., 2017. Bioaccumulation behavior of pharmaceuticals and personal care products in adult zebrafish (*Danio rerio*): influence of physical-chemical properties and biotransformation. Environ. Sci. Technol. 51 (19), 11085–11095. https://doi.org/10.1021/acs.est.7b02918.

Chou, C.J., Haluzik, M., Gregory, C., Dietz, K.R., Vinson, C., Gavrilova, O., Reitman, M.L., 2002. WY14,643, a peroxisome proliferator-activated receptor α (PPARα) agonist, improves hepatic and muscle steatosis and reverses insulin resistance in lipoatrophic A-ZIP/F-1 Mice. J. Biol. Chem. 277 (27), 24484–24489. https://doi.org/10.1074/jbc.M202449200.

Di Giacomo, E., Benedetti, E., Cristiano, L., Antonosante, A., d'Angelo, M., Fidoamore, A., Barone, D., Moreno, S., Ippoliti, R., Cerù, M.P., Giordano, A., Cimini, A., 2017. Roles of PPAR transcription factors in the energetic metabolic switch occurring during adult neurogenesis. Cell Cycle 16 (1), 59–72. https://doi.org/10.1080/15384101.2016.1252881.

Dubińska-Magiera, M., Migocka-Patrzałek, M., Lewandowski, D., Daczewska, M., Jagla, K., 2021. Zebrafish as a model for the study of lipid-lowering drug-induced myopathies. Int. J. Mol. Sci. 22 (11), 5654. https://doi.org/10.3390/ijms22115654.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205 (1), 38–44. https://doi.org/10.1016/j.bbr.2009.06.022.

Erel, O., 2005. A new automated colorimetric method for measuring total oxidant status. Clin. Biochem. 38 (12), 1103–1111. https://doi.org/10.1016/j.clinbiochem.2005.08.008.

Estrela, G.R., Arruda, A.C., Torquato, H.F.V., Freitas-Lima, L.C., Perilhão, M.S., Wasinski, F., Budu, A., Fock, R.A., Paredes-Gamero, E.J., Araujo, R.C., 2020. Gemfibrozil induces anemia, leukopenia and reduces hematopoietic stem cells via PPAR-α in Mice. Int. J. Mol. Sci. 21 (14), 5050. https://doi.org/10.3390/ijms21145050.

Fang, Y., Karnjanapiboonwong, A., Chase, D.A., Wang, J., Morse, A.N., Anderson, T.A., 2012. Occurrence, fate, and persistence of gemfibrozil in water and soil. Environ. Toxicol. Chem. 31 (3), 550–555. https://doi.org/10.1002/etc.1725.

Filice, M., Cerra, M.C., Imbrogno, S., 2022. The goldfish Carassius auratus: an emerging animal model for comparative cardiac research. J. Comparat. Physiol. B 192 (1), 27–48. https://doi.org/10.1007/s00360-021-01402-9.

Franco-Martinez, L., Tvarijonaviciute, A., Martinez-Subiela, S., Teles, M., Tort, L., 2019. Chemiluminescent assay as an alternative to radioimmunoassay for the measurement of cortisol in plasma and skin mucus of Oncorhynchus mykiss. Ecol. Indic. 98, 634–640. https://doi.org/10.1016/j.ecolind.2018.11.046.

Fraz, S., Lee, A.H., Pollard, S., Srinivasan, K., Vermani, A., Wilson, J.Y., 2019. Parental gemfibrozil exposure impacts zebrafish F1 offspring, but not subsequent generations. Aquat. Toxicol. 212, 194–204. https://doi.org/10.1016/j.aquatox.2019.04.020.

Fraz, S., Lee, A.H., Wilson, J.Y., 2018. Gemfibrozil and carbamazepine decrease steroid production in zebrafish testes (Danio rerio). Aquat. Toxicol. 198, 1–9. https://doi.org/10.1016/j.aquatox.2018.02.006.

Gagnon, A., Jumarie, C., Hontela, A., 2006. Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical cells of rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 78 (1), 59–65. https://doi.org/10.1016/j.aquatox.2006.02.004.

Galus, M., Rangarajan, S., Lai, A., Shaya, L., Balshine, S., Wilson, J.Y., 2014a. Effects of chronic, parental pharmaceutical exposure on zebrafish (Danio rerio) offspring. Aquatic Toxicol. 151, 124–134. https://doi.org/10.1016/j.aquatox.2014.01.016.

Galus, M., Rangarajan, S., Lai, A., Shaya, L., Balshine, S., Wilson, J.Y., 2014b. Effects of chronic, parental pharmaceutical exposure on zebrafish (Danio rerio) offspring. Aquatic Toxicol. 151, 124–134. https://doi.org/10.1016/j.aquatox.2014.01.016.

García-Valverde, M., Aragonés, A.M., Andújar, J.A.S., García, M.D.G., Martínez-Bueno, M.J., Fernández-Alba, A.R., 2023. Long-term effects on the agroecosystem of using reclaimed water on commercial crops. Sci. Total Environ. 859, 160462 https://doi.org/10.1016/j.scitotenv.2022.160462.

Gaw, S., Thomas, K.V., Hutchinson, T.H., 2014. Sources, impacts and trends of pharmaceuticals in the marine and coastal environment. Philos. Trans. R. Soc. B: Biol. Sci. 369 (1656), 20130572 https://doi.org/10.1098/rstb.2013.0572.

Grenni, P., Patrolecco, L., Ademollo, N., Tolomei, A., Barra Caracciolo, A., 2013. Degradation of Gemfibrozil and Naproxen in a river water ecosystem. Microchem. J. 107, 158–164. https://doi.org/10.1016/j.microc.2012.06.008.

Hammill, K.M., Fraz, S., Lee, A.H., Wilson, J.Y., 2018. The effects of parental carbamazepine and gemfibrozil exposure on sexual differentiation in zebrafish (Danio rerio). Environ. Toxicol. Chem. 37 (6), 1696–1706. https://doi.org/10.1002/etc.4120.

Henriques, J.F., Almeida, A.R., Andrade, T., Koba, O., Golovko, O., Soares, A.M.V.M., Oliveira, M., Domingues, I., 2016. Effects of the lipid regulator drug gemfibrozil: a toxicological and behavioral perspective. Aquat. Toxicol. 170, 355–364. https://doi.org/10.1016/j.aquatox.2015.09.017.

Jentoft, S., Aastveit, A.H., Torjesen, P.A., Andersen, Ø., 2005. Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (Perca fluviatilis) and domesticated rainbow trout (Oncorhynchus mykiss). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 141 (3), 353–358. https://doi.org/10.1016/j.cbpb.2005.06.006.

Khalil, A.M.E., Han, L., Maamoun, I., Tabish, T.A., Chen, Y., Eljamal, O., Zhang, S., Butler, D., Memon, F.A., 2022. Novel Graphene-based foam composite as a highly reactive filter medium for the efficient removal of gemfibrozil from (Waste) water. Adv. Sustain. Syst. 6 (8), 2200016 https://doi.org/10.1002/adsu.202200016.

Kulkarni, R., & Barad, V. (2015). Haematological and blood biochemical changes in the fresh water fish, <i>Notopterus</i> <i>notopterus</i> (Pallas) exposed to acidic medium. *Int. Lett. Natural Sci.*, 45, 27–33. doi:10.18052/www.scipress.com/ILNS.45.27.

Kulkeaw, K., Sugiyama, D., 2012. Zebrafish erythropoiesis and the utility of fish as models of anemia. Stem Cell Res. Ther. 3 (6), 55. https://doi.org/10.1186/scrt146.

Lee, G., Lee, S., Ha, N., Kho, Y., Park, K., Kim, P., Ahn, B., Kim, S., Choi, K., 2019. Effects of gemfibrozil on sex hormones and reproduction related performances of Oryzias latipes following long-term (155 d) and short-term (21 d) exposure. Ecotoxicol. Environ. Saf. 173, 174–181. https://doi.org/10.1016/j.ecoenv.2019.02.015.

Li, X., Bai, R., Bai, Y., Shi, X., Yang, Y., Xu, S., 2022. ROS-mediated PPAR/RXR inhibition contributes to acetochlor-induced apoptosis and autophagy in Ctenopharyngodon idella hepatic cells. Fish Shellfish Immunol. 128, 684–694. https://doi.org/10.1016/j.fsi.2022.08.053.

Liang, Y., Liang, N., Yin, L., Xiao, F., 2020. Cellular and molecular mechanisms of xenobiotics-induced premature senescence. Toxicol. Res. (Camb.) 9 (5), 669–675. https://doi.org/10.1093/toxres/tfaa073.

Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G., 2009. EU-wide survey of polar organic persistent pollutants in European river waters. Environ. Pollut. 157 (2), 561–568. https://doi.org/10.1016/j.envpol.2008.09.020.

Loos, R., Wollgast, J., Huber, T., Hanke, G., 2007. Polar herbicides, pharmaceutical products, perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and nonylphenol and its carboxylates and ethoxylates in surface and tap waters around Lake Maggiore in Northern Italy. Anal. Bioanal. Chem. 387 (4), 1469–1478. https://doi.org/10.1007/s00216-006-1036-7.

Luo, Y., Hu, C.-T., Qiao, F., Wang, X.-D., Qin, J.G., Du, Z.-Y., Chen, L.-Q., 2020. Gemfibrozil improves lipid metabolism in Nile tilapia Oreochromis niloticus fed a high-carbohydrate diet through peroxisome proliferator activated receptor-α activation. Gen. Comp. Endocrinol. 296, 113537 https://doi.org/10.1016/j.ygcen.2020.113537.

Lyssimachou, A., Thibaut, R., Gisbert, E., Porte, C., 2014. Gemfibrozil modulates cytochrome P450 and peroxisome proliferation-inducible enzymes in the liver of the yellow European eel (Anguilla anguilla). Environ. Sci. Pollut. Res. 21 (2), 862–871. https://doi.org/10.1007/s11356-013-1944-y.

Martínez, Bueno, M.J., Hernando, M.D., Herrera, S., Gómez, M.J., Fernández-Alba, A.R., Bustamante, I., García-Calvo, E, 2010. Pilot survey of chemical contaminants from industrial and human activities in river waters of Spain. Int. J. Environ. Anal. Chem. 90 (3–6), 321–343. https://doi.org/10.1080/03067310903045463.

Mauro, M., Lazzara, V., Arizza, V., Luparello, C., Ferrantelli, V., Cammilleri, G., Inguglia, L., Vazzana, M., 2021. Human drug pollution in the aquatic system: the biochemical responses of danio rerio adults. Biology (Basel) 10 (10), 1064. https://doi.org/10.3390/biology10101064.

Meador, J.P., Yeh, A., Young, G., Gallagher, E.P., 2016. Contaminants of emerging concern in a large temperate estuary. Environ. Pollut. 213, 254–267. https://doi.org/10.1016/j.envpol.2016.01.088.

Mimeault, C., Trudeau, V.L., Moon, T.W., 2006. Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARβ) mRNA levels in male goldfish (Carassius auratus). Toxicology 228 (2–3), 140–150. https://doi.org/10.1016/j.tox.2006.08.025.

Mimeault, C., Woodhouse, A.J., Miao, X.-S., Metcalfe, C.D., Moon, T.W., Trudeau, V.L., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus. Aquat. Toxicol. 73 (1), 44–54. https://doi.org/10.1016/j.aquatox.2005.01.009.

Oliveira, M., Franco, L., Balasch, J.C., Fierro-Castro, C., Tvarijonaviciute, A., Soares, A. M.V.M., Tort, L., Teles, M., 2018. Tools to assess effects of human pharmaceuticals in fish: a case study with gemfibrozil. Ecol. Indic. 95, 1100–1107. https://doi.org/10.1016/j.ecolind.2017.12.051.

Orias, F., Simon, L., Mialdea, G., Clair, A., Brosselin, V., Perrodin, Y., 2015. Bioconcentration of 15N-tamoxifen at environmental concentration in liver, gonad and muscle of Danio rerio. Ecotoxicol. Environ. Saf. 120, 457–462. https://doi.org/10.1016/j.ecoenv.2015.06.033.

Pahan, K., 2006. Lipid-lowering drugs. Cell. Mol. Life Sci. 63 (10), 1165–1178. https://doi.org/10.1007/s00018-005-5406-7.

Páscoa, I., Fonseca, E., Ferraz, R., Machado, A.M., Conrado, F., Ruivo, R., Cunha, I., Castro, L.F.C., 2022. The Preservation of PPARγ genome duplicates in some teleost lineages: insights into lipid metabolism and xenobiotic exploitation. Genes (Basel) 13 (1), 107. https://doi.org/10.3390/genes13010107.

Pereira, A.M.P.T., Silva, L.J.G., Laranjeiro, C.S.M., Meisel, L.M., Lino, C.M., Pena, A., 2017. Human pharmaceuticals in Portuguese rivers: the impact of water scarcity in the environmental risk. Sci. Total Environ. 609, 1182–1191. https://doi.org/10.1016/j.scitotenv.2017.07.200.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), 45e–445. https://doi.org/10.1093/nar/29.9.e45.

Porretti, M., Arrigo, F., Di Bella, G., Faggio, C., 2022. Impact of pharmaceutical products on zebrafish: an effective tool to assess aquatic pollution. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 261, 109439 https://doi.org/10.1016/j.cbpc.2022.109439.

Prindiville, J.S., Mennigen, J.A., Zamora, J.M., Moon, T.W., Weber, J.-M., 2011. The fibrate drug gemfibrozil disrupts lipoprotein metabolism in rainbow trout. Toxicol. Appl. Pharmacol. 251 (3), 201–208. https://doi.org/10.1016/j.taap.2010.12.013.

Quintanilla Rodriguez, B.S., & Correa, R. (2019). Gemfibrozil. StatPearls Publishing.

Rakhshandehroo, M., Knoch, B., Müller, M., Kersten, S., 2010. Peroxisome proliferator-activated receptor alpha target genes. PPAR Res 2010, 1–20. https://doi.org/10.1155/2010/612089.

Ramirez, A.J., Brain, R.A., Usenko, S., Mottaleb, M.A., O'Donnell, J.G., Stahl, L.L., Wathen, J.B., Snyder, B.D., Pitt, J.L., Perez-Hurtado, P., Dobbins, L.L., Brooks, B.W., Chambliss, C.K, 2009. Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the united states. Environ. Toxicol. Chem. 28 (12), 2587. https://doi.org/10.1897/08-561.1.

Roy, A., Pahan, K., 2009. Gemfibrozil, stretching arms beyond lipid lowering. Immunopharmacol. Immunotoxicol. 31 (3), 339–351. https://doi.org/10.1080/08923970902785253.

Salmerón, C., 2018. Adipogenesis in fish. J. Exp. Biol. (Suppl_1), 221. https://doi.org/10.1242/jeb.161588.

Scatena, R., Nocca, G., Messana, I., Sole, P., Baroni, S., Zuppi, C., Castagnola, M., Giardina, B., 1995. Effects of gemfibrozil on the oxygen transport properties of erythrocytes. Br. J. Clin. Pharmacol. 39 (1), 25–30. https://doi.org/10.1111/j.1365-2125.1995.tb04405.x.

Simmons, D.B.D., McCallum, E.S., Balshine, S., Chandramouli, B., Cosgrove, J., Sherry, J. P., 2017. Reduced anxiety is associated with the accumulation of six serotonin reuptake inhibitors in wastewater treatment effluent exposed goldfish Carassius auratus. Sci. Rep. 7 (1), 17001. https://doi.org/10.1038/s41598-017-15989-z.

Singh, M.P., Pathak, D., Sharma, G.K., Sharma, C.S., 2011. Peroxisome proliferator-activated receptors (PPAR S): a target with a broad therapeutic potential for human diseases: an overview. Pharmacology Y 2, 58–89.

Skolness, S.Y., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2012. Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 31 (11), 2615–2624. https://doi.org/10.1002/etc.1989.

Sloman, K.A., Scott, G.R., McDonald, D.G., Wood, C.M., 2004. Diminished social status affects ionoregulation at the gills and kidney in rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat.Sci. 61 (4), 618–626. https://doi.org/10.1139/f04-032.

Solé, M., Fortuny, A., Mañanós, E., 2014. Effects of selected xenobiotics on hepatic and plasmatic biomarkers in juveniles of Solea senegalensis. Environ. Res. 135, 227–235. https://doi.org/10.1016/j.envres.2014.09.024.

Stading, R., Chu, C., Couroucli, X., Lingappan, K., Moorthy, B., 2020. Molecular role of cytochrome P4501A enzymes in oxidative stress. Curr. Opin. Toxicol. 20–21, 77–84. https://doi.org/10.1016/j.cotox.2020.07.001.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., Fruchart, J.-C., 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 98 (19), 2088–2093. https://doi.org/10.1161/01.CIR.98.19.2088.

Stolarz, A.J., Farris, R.A., Wiley, C.A., O'Brien, C.E., Price, E.T., 2015. Fenofibrate attenuates neutrophilic inflammation in airway epithelia: potential drug repurposing for cystic fibrosis. Clin. Transl. Sci. 8 (6), 696–701. https://doi.org/10.1111/cts.12310.

Strauss, V., Mellert, W., Wiemer, J., Leibold, E., Kamp, H., Walk, T., van Ravenzwaay, B., 2012. Increased toxicity when fibrates and statins are administered in combination–a metabolomics approach with rats. Toxicol. Lett. 211 (2), 187–200.

Teles, M., Fierro-Castro, C., Na-Phatthalung, P., Tvarijonaviciute, A., Soares, A.M.V.M., Tort, L., Oliveira, M., 2016. Evaluation of gemfibrozil effects on a marine fish (Sparus aurata) combining gene expression with conventional endocrine and biochemical endpoints. J. Hazard. Mater. 318, 600–607. https://doi.org/10.1016/j.jhazmat.2016.07.044.

Teles, M., Pacheco, M., Santos, M.A., 2006. Biotransformation, stress and genotoxic effects of 17β-estradiol in juvenile sea bass (Dicentrarchus labrax L. Environ. Int. 32 (4), 470–477. https://doi.org/10.1016/j.envint.2005.11.006.

Tervonen, V., Vuolteenaho, O., Nikinmaa, M., 2006. Haemoconcentration via diuresis in short-term hypoxia: a possible role for cardiac natriuretic peptide in rainbow trout. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 144 (1), 86–92. https://doi.org/10.1016/j.cbpa.2006.02.014.

Vera-Chang, M.N., St-Jacques, A.D., Gagné, R., Martyniuk, C.J., Yauk, C.L., Moon, T.W., Trudeau, V.L., 2018. Transgenerational hypocortisolism and behavioral disruption are induced by the antidepressant fluoxetine in male zebrafish *Danio rerio*. Proc. Natl. Acad. Sci. (52), 115. https://doi.org/10.1073/pnas.1811695115.

Wägner, A.M., Sánchez-Quesada, J.L., Benítez, S., Bancells, C., Ordóñez-Llanos, J., Pérez, A., 2011. Effect of statin and fibrate treatment on inflammation in type 2 diabetes. A randomized, cross-over study. Diabetes Res. Clin. Pract. 93 (1), e25–e28. https://doi.org/10.1016/j.diabres.2011.03.009.

Xia, K., Bhandari, A., Das, K., Pillar, G., 2005. Occurrence and Fate of Pharmaceuticals and Personal Care Products (PPCPs) in Biosolids. J. Environ. Qual. 34 (1), 91–104. https://doi.org/10.2134/jeq2005.0091.

Xie, S., Yin, P., Tian, L., Liu, Y., Niu, J., 2020. Lipid metabolism and plasma metabolomics of juvenile largemouth bass Micropterus salmoides were affected by dietary oxidized fish oil. Aquaculture 522, 735158. https://doi.org/10.1016/j.aquaculture.2020.735158.

Xu, C., Li, C.Y.-T., Kong, A.-N.T., 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch. Pharm. Res. 28 (3), 249–268. https://doi.org/10.1007/BF02977789.

Zhang, K., Zhao, Y., Fent, K., 2020. Cardiovascular drugs and lipid regulating agents in surface waters at global scale: occurrence, ecotoxicity and risk assessment. Sci. Total Environ. 729, 138770 https://doi.org/10.1016/j.scitotenv.2020.138770.

Zhang, X., Oakes, K.D., Cui, S., Bragg, L., Servos, M.R., Pawliszyn, J., 2010. Tissue-specific in vivo bioconcentration of pharmaceuticals in rainbow trout (*Oncorhynchus mykiss*) using space-resolved solid-phase Microextraction. Environ. Sci. Technol. 44 (9), 3417–3422. https://doi.org/10.1021/es903064t.

Further reading

Bueno, M.M., Gomez, M.J., Herrera, S., Hernando, M.D., Agüera, A., Fernández-Alba, A. R., 2012. Occurrence and persistence of organic emerging contaminants and priority pollutants in five sewage treatment plants of Spain: two years pilot survey monitoring. Environ. Pollut. 267–273.