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Depressed, hypertense and sore: Long-term effects of fluoxetine, propranolol and diclofenac exposure in a top predator fish

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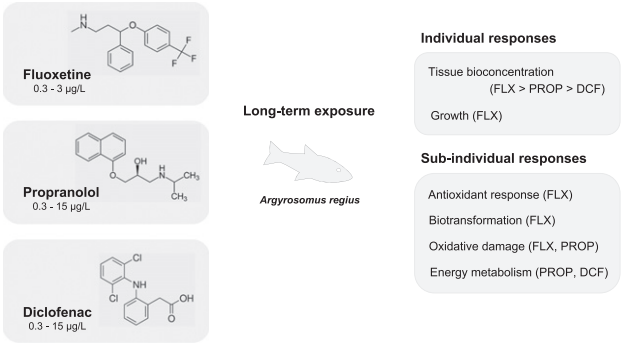
HIGHLIGHTS

- Long-term exposure of meagre to pharmaceuticals with different modes of action.
- Fluoxetine and propranolol bioconcentrated in fish muscle, but not diclofenac.
- Fluoxetine reduced growth, inhibited biotransformation and caused oxidative stress.
- Propranolol and diclofenac affected energy metabolism in meagre.
- Different MOA resulted in differently affected mechanisms in *Argyrosomus regius*.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical compounds are continuously released into the aquatic environment, resulting in their ubiquitous presence in many estuarine and coastal systems. As pharmaceuticals are designed to produce effects at very low concentrations and target specific evolutionary conserved pathways, there are growing concerns over their potential deleterious effects to the environment and specifically to aquatic organisms, namely in early life-stages. In this context, the long-term effects of exposure of juvenile meagre *Argyrosomus regius* to three different pharmaceuticals were investigated. Fish were exposed to environmental concentrations of one of three major used pharmaceuticals: the antidepressant fluoxetine (0.3 and 3 µg/L for 15 days), the anti-hypertensive propranolol and the non-steroidal anti-inflammatory agent diclofenac (0.3 and 15 µg/L for 30 days). Pharmaceuticals bioconcentration in fish muscle was examined, along with biomarkers in different tissues related with antioxidant and biotransformation responses (catalase, superoxide dismutase, ethoxyresorufin-*O*-deethylase and glutathione *S*-transferase), energetic metabolism (lactate dehydrogenase, isocitrate dehydrogenase and electron transport system activities), neurotransmission (acetylcholinesterase activity) and oxidative damage (DNA damage and lipid peroxidation levels). Overall, each pharmaceutical had different potential for bioconcentration in the muscle (FLX > PROP > DCF) and induced different biological responses: fluoxetine was the most toxic

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compound to juvenile meagre, affecting fish growth, triggering antioxidant defense responses, inhibiting detoxification mechanisms and increasing lipid peroxidation and DNA damage in the liver; propranolol exposure increased DNA damage and decreased aerobic metabolism in fish muscle; and diclofenac showed no potential to bioconcentrate, yet it affected fish metabolism by increasing cellular energy consumption in the muscle and consequently reducing fish net energy budget. The diverse response patterns evidence the need for future research focused on pharmaceuticals with different modes of action and their exposure effects on organismal physiological mechanisms and homeostatic status. Ultimately, the combination of sub-individual and individual responses is key for ecologically relevant assessments of pharmaceutical toxicity.

1. Introduction

Pharmaceutical compounds of human and veterinary use are often released into the aquatic environment, either directly or after incomplete removal by wastewater treatment plants, contributing to their continuous and persistent presence in many aquatic systems (Caldwell, 2016; Kümmerer, 2009). Hence, pharmaceuticals are commonly detected in surface, ground and drinking waters at concentrations in the ng/L and low μg/L range, yet maximum reported concentrations can reach hundreds of μg/L and up to mg/L (aus der Beek et al., 2016). Even if detected at low concentrations, these compounds may pose a risk to many species, as they are biologically active at very low concentrations and target specific pathways, most of them conserved throughout the tree of life, and in particular among vertebrates (Gunnarsson et al., 2008). Overall, pharmaceuticals have been found to affect various biological endpoints such as molecular and biochemical processes, including growth, metabolism, reproduction and behaviour (Duarte et al., 2019; Fabbri and Franzellitti, 2016; Sehonova et al., 2018). However, efforts have historically focused mainly on freshwater systems and acute exposure tests, with studies on chronical exposures and on estuarine and marine organisms still limited (Fent et al., 2006; Gaw et al., 2014; Reis-Santos et al., 2018).

With over 600 pharmaceuticals detected in the environment worldwide, therapeutic groups such as analgesics, antidepressants and antihypertensive drugs are prevalent (aus der Beek et al., 2016). Within these classes, diclofenac (DCF), fluoxetine (FLX) and propranolol (PROP) are among the most used and prescribed drugs, and therefore some of the most frequently detected compounds in the aquatic environment, at concentrations ranging from ng/L to μg/L (aus der Beek et al., 2016; Bonnefille et al., 2018; Mezzelani et al., 2018). Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) commonly prescribed to treat pain, fever and inflammation, whereas fluoxetine belongs to the antidepressant class of selective serotonin reuptake inhibitors (SSRIs) used to treat depression and other psychiatric disorders, and propranolol is a beta-adrenergic receptor antagonist (β-blocker), used to treat hypertension and heart-related diseases. Although with varying environmental degradation rates and retention efficiencies in water treatment plants (Luo et al., 2014), their continuous release ultimately results in the permanent exposure of non-target species (Arnold et al., 2014; Monteiro and Boxall, 2010). In this context, chronic exposure assessments at environmental concentrations are paramount to address the potential risks posed by these compounds to aquatic species. In particular, examining the effects of pharmaceuticals with different modes of action (MOA), not yet fully described in fish, and at different levels of organization (i.e. sub-individual/biochemical and individual responses) will ultimately contribute to a more comprehensive and ecologically relevant assessment of pharmaceutical toxicity.

This study provides an integrative view on the risks and toxicity of three pharmaceutical compounds with different MOA, the antihypertensive PROP, the non-steroidal anti-inflammatory DCF, and the antidepressant FLX, in the meagre *Argyrosomus regius* (Asso, 1801), a top predator fish species of high economic value. The specific aim of this study was to assess the effects of long-term exposure to two distinct environmentally relevant concentrations, integrating different levels of biological organization. Thus, following exposure, alterations at the individual level were investigated, including fish growth, condition, and pharmaceutical bioconcentration. At the sub-individual level, various responses were assessed, namely: activity levels of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD), involved in the detoxification of reactive oxygen species (ROS) thus reducing oxidative stress; the responses of biotransformation enzymes ethoxyresorufin O-deethylase (EROD) and glutathione-S-transferase (GST), responsible for the metabolism of xenobiotic compounds such as pharmaceuticals; the levels of oxidative stress effects such as lipid peroxidation (LPO), DNA damage (DNAd), and of neurotoxicity, acetylcholinesterase inhibition (AChE). Furthermore, energy-related parameters were assessed, including: the levels of each energy reserve (carbohydrates, proteins and lipids), and total energy available (EA); lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) enzyme activities, involved in anaerobic and aerobic metabolism pathways, respectively, as well as the LDH/IDH ratio; the electron transport system (ETS) activity, a proxy for cellular energy consumption; and finally the cellular energy allocation (CEA), for the quantification of organismal energetic tradeoffs.

2. Materials and methods

2.1. Experimental design

Argyrosomus regius juveniles (7.31 ± 0.58 cm, 3.84 ± 0.83 g), obtained from a fish farm, were randomly distributed among 21 experimental 40 L tanks, with 8 individuals per tank, and acclimated to exposure conditions for 15 days. The long-term semi-static toxicity test was performed according to OECD guidelines (test no. 210) with a 16:8 h light:dark photoperiod and UV-treated natural seawater (average 24.8 PSU and 17.3 °C). A control and two concentrations (low and high) were used for DCF, PROP and FLX exposures, with three replicate tanks per concentration. Fish were exposed for 30 days to nominal concentrations of 0.3 and 15 μg/L, for low and high concentrations, respectively. The exception was the high FLX treatment, which consisted of a separate 15 days' exposure to a 3 μg/L concentration with fish from the initial batch and with an independent control group (controls high FLX). This was due to early distress signs (swimming and feeding) evident within 48 h exposure in a preliminary test run with a 15 μg/L FLX concentration. Nonetheless, all concentrations used in this study cover the range of reported environmental concentrations for the different pharmaceutical classes (aus der Beek et al., 2016; Mezzelani et al., 2018).

Pharmaceutical stock solutions were prepared with milli Q-grade water and stored at −20 °C. Daily water renewals were performed (25%), and pharmaceutical concentrations appropriately restored to maintain nominal pharmaceutical concentrations in tanks. Water parameters, namely dissolved oxygen, temperature, salinity, pH, ammonia and nitrites, as well as any fish mortalities were recorded daily. Fish were fed daily with pellets developed for hatchery feeds (WinFast by Sparos), with portion adjustments throughout the experiment to maintain a 2% ratio with mean fish weight.

All experimental procedures were performed in accordance with animal testing guidelines (EU Directive 2010/63, Portuguese DL 113/2013), licensed by the animal welfare committee at the Faculty of Sciences of the Lisbon University, and by national authorities.

2.2. Growth and condition indices

Total fish length (Lt, in cm) and weight (Wt, in g) were recorded at the beginning and end of the experiment. Fulton' condition factor K was determined according to Ricker (1975): $K = Wt/Lt^3$; where Wt is total weight and Lt is total length. Specific growth rates in weight were determined per tank, in % per day, using the formula: $G = 100 * (\ln Wt_f - \ln Wt_i) / (t_f - t_i)$; where Wt_f and Wt_i are fish total weights at final (t_f) and initial (t_i) days of exposure, respectively (Kroon et al., 2017).

2.3. Concentration of pharmaceuticals in water and fish tissues

Water samples were collected from each tank every week for pharmaceutical quantification. Sample extraction, purification, and concentration were adapted from Pereira et al. (2015) and Sousa et al. (2011). Samples (500 mL) were sequentially filtered through 3 membranes (110 mm, 0.45 μm and 0.2 μm), purified with OASIS HLB cartridges and subsequently washed with 5 mL of methanol:water (10:90) and eluted with 6 mL of methanol. The extract was dried under a gentle stream of N_2 at 40 °C. Prior to analysis, extracts were dissolved in 500 μL of methanol:water (3:97), filtered through a PVDF Mini-uniprep™ filter (0.45 μm), injected and quantified through ultra-high performance liquid chromatography and time-of-flight mass spectrometry (UHPLC-TOF-MS). Results are presented as μg of pharmaceutical compound per liter of water.

Portions of fish dorsal muscle tissue (approximately 2 g) were sampled for pharmaceutical quantification, i.e. bioconcentration (expressed as μg/kg in fish tissue). Sample extraction, purification, and concentration were performed as an extension of the method from Freitas et al. (2014). Briefly, tissues were homogenized, and extraction was performed with 5 mL of acetonitrile and 1 mL of 0.1 M EDTA. Samples were centrifuged and the supernatant evaporated to near dryness (until 0.5 mL) under a gentle stream of N_2 at 40 °C. After adding 500 μL of 0.1% formic acid to the residue, a filtration step through a PVDF mini-uniprep™ filter (0.45 μm) was performed, followed by the injection into the UPHLC-TOF-MS for detection and quantification. Results are presented as μg of pharmaceutical per kg of wet weight. For a full description of methodology and instrumentation used for pharmaceutical quantification, including limits of quantification (LOQ) and recovery (%) in water and fish muscle samples, see Appendix A, Table A1.

2.4. Biomarkers quantification

For biomarkers quantification different fish tissues were dissected, namely liver, brain, muscle, and heart. Tissue samples were homogenized in cold 100 mM monobasic potassium phosphate/dibasic potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (pH 7.4) containing 0.15 M KCl (potassium chloride), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiothreitol) and 1 mM EDTA (ethylenediaminetetraacetic acid) to avoid protein degradation. Liver homogenates were aliquoted for DNA damage (DNAd), lipid peroxidation (LPO) quantification, superoxide dismutase (SOD), catalase (CAT), ethoxyresorufin-*O*-deethylase (EROD) and glutathione *S*-transferase (GST) determination.

Muscle homogenates were used for determination of LPO, DNAd, electron transport system activity (ETS), lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) activities, as well as for determination of total carbohydrates (CBH), proteins (PT) and lipids (LP) content. Heart homogenates were used for ETS, LDH and IDH activity measurements. Brain homogenates were used for the measurement of acetylcholinesterase (AChE) activity.

All biomarker responses were determined using a Sinergy HT Microplate Reader (BioTek Instruments, Vermont, USA), and each reading was done in triplicate using homogenization buffer as blank reaction. Superoxide dismutase (SOD) activity was measured according to (Mccord and Fridovich, 1969), and was expressed as U mg^{-1} of total protein concentration, where one unit is the amount of enzyme required to inhibit the reduction of cytochrome *c* by 50%. Catalase (CAT) activity was determined according to Aebi (1974), following substrate consumption, as a decrease in absorbance at 240 nm. CAT activity was then calculated as the difference in absorbance per unit of time ($\varepsilon = -0.04\ mM^{-1}\ cm^{-1}$) and expressed as μmol per minute per mg of total protein concentration. Ethoxyresorufin-O-deethylase (EROD) activity was determined following Burke and Mayer (1974) method, with few adaptations by Fernandes et al. (2002). Activity was calculated as the amount of resorufin (ρmol) generated per mg of total protein per minute of reaction time. Glutathione S-transferase (GST) activity was determined following Habig et al. (1974), and activity was expressed as nmol CDNB conjugate formed per mg of total protein per minute of reaction. Lipid peroxidation (LPO) was determined according to Ohkawa et al. (1979) and was expressed as nmol of TBARS formed per mg of wet weight. DNA damage (DNAd) was determined following the DNA alkaline precipitation assay by Olive (1988). DNA concentration in the supernatant was determined following the addition of Hoechst dye and fluorescence values were compared to a DNA standard curve. DNAd was expressed as μg DNA per mg of wet weight. Acetylcholinesterase (AChE) was determined according to Ellman et al. (1961), adapted to microplate (Guilhermino et al., 1996). The enzymatic activity was expressed in nmol of substrate hydrolyzed per minute per mg of total protein. LDH activity was assessed using the methods described by Vassault (1983) and Diamantino et al. (2001) and results were expressed as nmol min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$). IDH activity was determined following Ellis and Goldberg (1971) method, adapted by Lima et al. (2007), and results were expressed as nmol min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$). Aerobic and anaerobic pathways were also assessed through LDH/IDH ratio. Cellular energy allocation (CEA) was calculated as in Verslycke et al. (2004a, 2004b): CEA = Ea/Ec, where Ea (available energy) = carbohydrate + lipid + protein (mJ mg ww^{-1}), and Ec (energy consumption) = ETS activity (mJ h^{-1} mg ww^{-1}). Following De Coen and Janssen (2003, 1997), total content of carbohydrates, lipids and proteins were measured and transformed into energetic equivalents using enthalpy combustion (39.5 kJ g^{-1} lipid, 24 kJ g^{-1} protein, 17.5 kJ g^{-1} glycogen, respectively). Results were expressed as mJ mg wet $weight^{-1}$. ETS activity in the mitochondria was determined according the method of De Coen and Janssen (1997). Oxygen consumption was calculated using a stochiometrical relationship: 2 μmol of formazan formed = 1 μmol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 480 kJ mol O_2^{-1} for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983). Protein content was quantified following Bradford's method, adapted to microplate, and bovine serum albumin solution (1 mg mL^{-1}) was used as protein standard. For further protocol details see Appendix B.

2.5. Data analyses

Differences in fish responses in DCF and PROP experiments were tested through permutational analyses of variance (PERMANOVA) followed by pair-wise tests (results presented as Pseudo-F and t_{pw}, respectively), whereas in FLX experiment, differences were tested with Mann-Whitney-Wilcoxon test (results presented as W). A multivariate nested design was initially considered, with treatment and tank treated as the fixed and nested (random) factors, respectively. Tank effects were absent for the majority of fish responses, except for 1 and 3 out of 25 responses analyzed for PROP and DCF treatments, respectively. Since no statistical differences were found when considering nested or

one-factor design for these responses, we decided to use the less complex univariate design, with treatment as the fixed factor. Differences in specific growth rates (G) and pharmaceutical bioaccumulation were tested with Welch's *t*-test (results presented as t), considering its robustness when a reduced number of samples is being tested (minimum $N = 3$). Spearman rank correlation (r) analysis was performed to test for correlations between fish responses. Analyses were performed in PRIMER 6 and R software (R Core Team, 2018), and a significance level of 0.05 was considered for all statistical tests used.

3. Results

3.1. Water quality parameters and pharmaceutical exposure concentrations

Water parameters were measured daily, and temperature (17.3 ± 0.2 °C), salinity (24.8 ± 0.2), pH (8.1 ± 0.02) and dissolved oxygen (96.8 ± 0.1%) were constant throughout the experiment, and ammonia and nitrite levels were maintained below 0.2 mg/L. Measured pharmaceutical concentrations were slightly lower than nominal concentrations, and evidenced low variation among measurements (Table 1). Average concentrations (μg/L) in the water were 0.13 and 9.25 for DCF; 0.15 and 2.52 for FLX and 0.27 and 14.74 for PROP, for low and high concentrations respectively (Table 1).

3.2. Individual responses

3.2.1. Growth and condition indices

Mortality in all treatments and controls was lower than 10%, where two fish died in control and high FLX treatments, and one fish in low DCF treatment.

Fish length, weight and specific growth rates (G) were significantly reduced by FLX at the highest concentration ($W = 119.5$, $p < .05$; $W = 132$, $p < .01$; $t = 6.2$, $p < .01$, respectively; Fig. 1a-c), whereas Fulton' condition factor (K) showed the same pattern, yet without statistical significance ($W = 103$, $p > .05$; Fig. 1d). Contrarily, exposure to either low or high DCF and PROP concentrations caused no significant morphometric changes in fish (Pseudo-F > 0.09, p > .05; Fig. 1), nor on growth rates ($t > -0.4$, p > .05; Fig. 1c).

3.2.2. Pharmaceutical bioconcentration

Bioconcentration of pharmaceuticals in fish muscle tissues was observed for both low and high FLX concentrations ($t_{low\ FLX} = -77.2$, $p < .001$ and $t_{high\ FLX} = -18.1$, $p < .01$; Fig. 1e and Table 1) as well as for PROP ($t_{low\ PROP} = -15.3$, $p < .001$ and $t_{high\ PROP} = -23.5$, $p < .001$; Fig. 1e and Table 1), yet no bioconcentration was observed for DCF (Fig. 1e and Table 1).

3.3. Sub-individual responses

3.3.1. Fluoxetine

After long-term exposure to high FLX concentration, liver antioxidant enzyme SOD activity was significantly increased ($W = 13$, $p < .05$; Fig. 2a), whereas the same pattern was observed for CAT activity, but not statistically significant ($W = 14$, $p > .05$; Fig. 2b). Activity levels of biotransformation enzymes GST and EROD were significantly reduced after exposure to high FLX concentration ($W = 59$ and $W = 60$, $p < .05$, respectively; Fig. 2c and d). Low FLX concentration had no effects on antioxidant and biotransformation enzymes ($W > 206$, $p > .05$; Fig. 2).

Concerning damage, LPO levels were significantly reduced in the liver at low FLX concentration but increased at high concentration ($W = 357$, $p < .01$; Fig. 3a) and DNA damage was significantly increased at high FLX concentration ($W = 12$, $p < .05$; Fig. 3b). Contrarily, no changes in LPO and DNA damage were observed in muscle at low concentration ($W > 251$, respectively, $p > .05$; Fig. 3c and d) and no neurotoxic effects, namely changes in acetylcholinesterase activity, were observed in fish brain at both FLX concentrations ($W > 51$, $p > .05$; Fig. 3e).

FLX did not affect fish heart nor muscle energetic metabolism, i.e., aerobic and anaerobic pathways, assessed through LDH/IDH ratio ($W > 157$, $p > .05$; Appendix C, Fig. C1; Fig. 4a). Moreover, the amount of energy reserves available (EA, i.e. total sugar, protein and lipids) and the electron transport system (ETS) activity, a proxy for energy consumption, also remained unchanged in muscle at low FLX concentration ($W > 137$, $p > .05$; Fig. 4b and Appendix C, Fig. C2) which, consequently, revealed no significant changes in cellular energy allocation (CEA) ($W = 152$, $p > .05$; Fig. 4c). Few significant correlations could be observed among fish responses to FLX (Appendix C, Table C3). Muscle and heart ETS activity were negatively correlated with both fish length and weight (Muscle: $r = -0.40$, $p < .01$ and $r = -0.34$, $p < .05$, respectively. Heart: $r = -0.32$ and $r = -0.32$, $p < .05$, respectively).

3.3.2. Diclofenac

Long-term exposure to DCF caused no significant effects in liver antioxidant enzymes SOD and CAT and in biotransformation enzymes GST and EROD activities (Pseudo-F > 0.002, $p > .05$; Fig. 2a–d), as well as no damage to liver and muscle lipids and DNA, nor to brain AChE activity (Pseudo-F > 0.2, p > .05; Fig. 3a–e). However, DCF significantly increased ETS activity in fish muscle at both low and high concentrations (Pseudo-F = 3.9, $p < .05$; Fig. 4b), but not in the heart (Pseudo-F = 0.38, p > .05; Appendix C, Fig. C1). Since muscle energy reserves were unaffected (Pseudo-F > 0.19, p > .05; Appendix C, Fig. C2), a significant reduction in fish net energy budget (CEA) was observed following the increase in ETS (Pseudo-F = 5.1, $p < .01$ and Pseudo-F = 3.88, $p < .05$, respectively; Fig. 4a and b), yet with no significant changes to LDH/IDH ratio (Pseudo-F = 3.2, p > .05; Fig. 4c). Few significant correlations among biomarker responses were observed (Appendix C, Table C4).

3.3.3. Propranolol

Exposure to PROP caused no changes in liver antioxidant CAT and SOD enzymes and biotransformation GST and EROD enzymes responses (Pseudo-F > 0.004, p > .05: Fig. 2a–d). Also, no effects on lipids and DNA damage were observed in the liver (Pseudo-F > 1.82, p > .05; Fig. 3a and b), whereas in muscle, DNA damage was significantly increased at high PROP concentration ($t_{pw} = 2.2$, $p < .05$; Fig. 3a–d). No neurotoxicity was found at both concentrations (Pseudo-F = 0.7, p > .05; Fig. 3e).

Muscle metabolic ratio LDH/IDH was significantly increased after exposure to high PROP concentration ($t_{pw} = 4.3$, $p < .001$; Fig. 4c) due to a decrease in aerobic metabolism, i.e. IDH activity (Pseudo-F = 14.68, $p < .01$; Appendix C, Fig. C2). However, no significant changes in energy reserves or ETS activity followed PROP exposure (Pseudo-F > 1.04, p > .05; Appendix C, Fig. C2; and Fig. 4b), hence CEA also remained unchanged (Pseudo-F = 2.1, p > .05; Fig. 4a). Correlations among *A. regius* responses were observed (Appendix C, Table C5). In the heart, LDH was negatively correlated with fish length and weight ($r = -0.30$, $p < .05$ and $r = -0.37$, $p < .01$, respectively).

Table 1
Average (± standard deviation) concentrations of pharmaceuticals in water (μg/L) and in fish muscle (μg/kg) samples, for low and high treatments of fluoxetine (FLX), diclofenac (DCF) and propranolol (PROP).

	Water (μg/L)		Muscle (μg/kg)	
	Low	High	Low	High
FLX	0.15 ± 0.02	2.52 ± 0.27	66.3 ± 10.6	425.5 ± 215.8
DCF	0.13 ± 0.03	9.25 ± 1.18	< LOQ	< LOQ
PROP	0.27 ± 0.01	14.74 ± 2.65	1.39 ± 0.3	58.39 ± 22.8

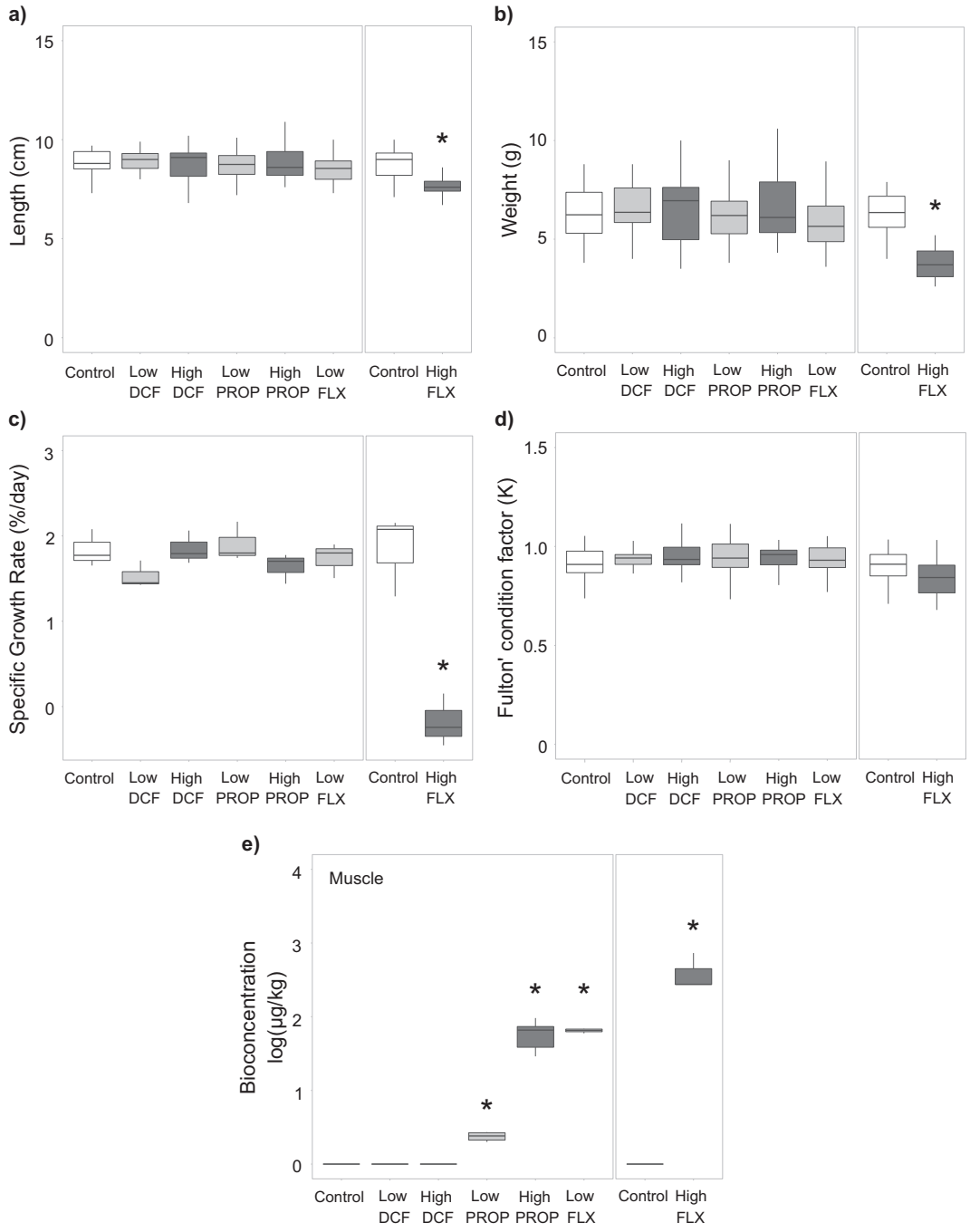

Fig. 1. Individual responses of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of responses measured: a) length (Lt), b) weight (Wt), c) specific growth rate (G), d) Fulton' condition factor (K) and e) bioconcentration in fish muscle. Asterisks indicate significant differences between treatments and respective controls.

4. Discussion

Exposure to pharmaceuticals from different therapeutic groups at environmentally relevant concentrations had distinct effects in juvenile meagre *Argyrosomus regius.* Fluoxetine (FLX) was the most toxic pharmaceutical of the three, affecting fish growth, increasing antioxidant response, inhibiting liver biotransformation enzymes and triggering lipid peroxidation and DNA damage in the liver. On the other hand, Diclofenac (DCF) affected fish metabolism, by increasing cellular energy consumption in the muscle and reducing fish net energy budget. Effects of Propranolol (PROP) exposure were observed only at high concentration in muscle, where DNA damage increased, and a higher energy demand caused a shift to anaerobic metabolism.

4.1. Individual responses

4.1.1. Growth and condition

Exposure to FLX (3 µg/L) resulted in decreased length, weight and growth rate in juvenile *A. regius*. Few earlier studies have also showed decreased fish growth after long-term waterborne exposure to FLX in the µg/L range (0.03 to 200 µg/L) (Mennigen et al., 2010; Pelli and Connaughton, 2015), yet no effects on growth, condition or weight

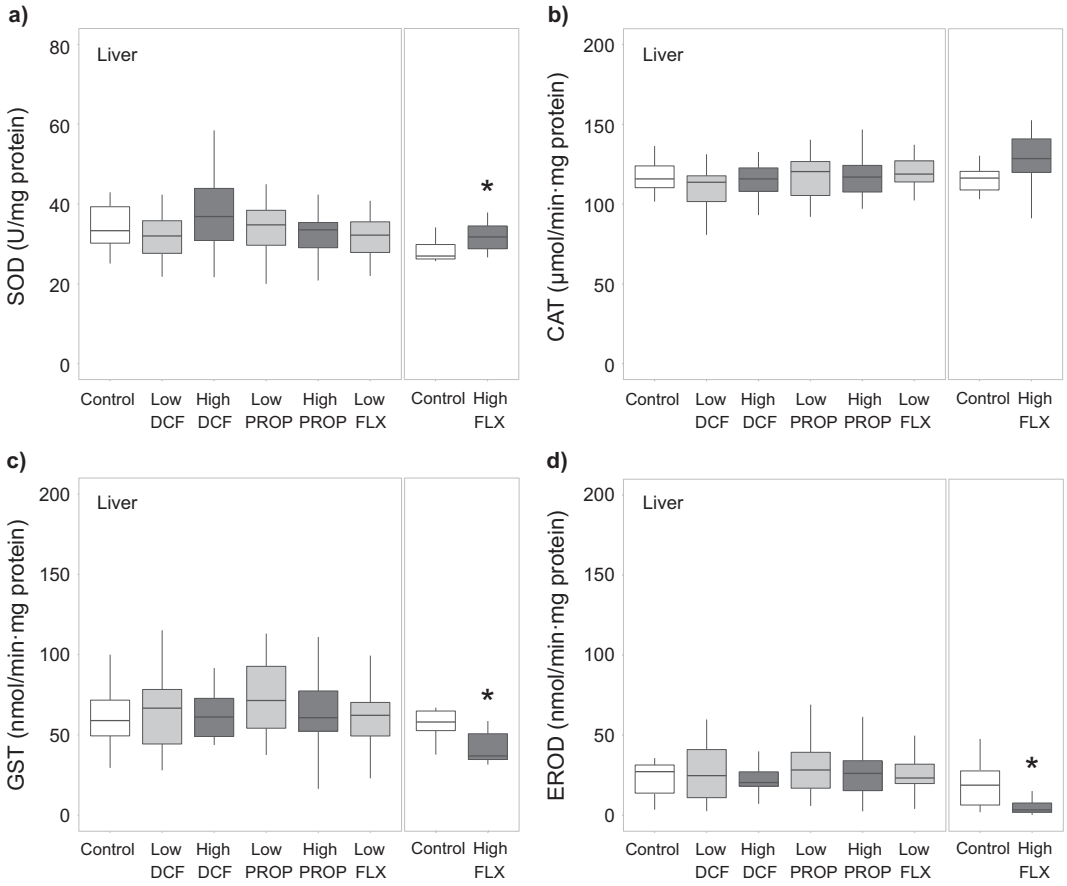

Fig. 2. Biomarker responses of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of enzymes' activities measured in the liver: a) superoxide dismutase (SOD), b) catalase (CAT), c) glutathione S-transferase (GST) and d) ethoxyresorufin-O-deethylase (EROD). Asterisks indicate significant differences between treatments and respective controls.

have also been reported (Chen et al., 2018; Foran et al., 2004). These different responses are likely associated with inter-species differences in metabolic efficiency (Smith et al., 2010), albeit this study is the first to consider a brackish-marine species. Nonetheless, FLX effect on fish growth and condition may be linked to serotonin-mediated appetite suppression as well as to altered behaviours (McDonald, 2017).

Detrimental effects of PROP on fish growth have been reported at much higher concentrations (above 500 µg/L) (Giltrow et al., 2009; Huggett et al., 2002; Owen et al., 2009). Accordingly, no significant effects of PROP in fish length, weight, condition or growth rates, were found in the present work, even though a slight decrease in growth rate could be perceived at high concentration, it was not statistically significant. Accordingly, these results suggest that PROP may not likely affect fish growth or condition at environmentally relevant concentrations.

Similarly, long-term exposure to high DCF concentrations was shown to decrease fish weight and growth rates (Memmert et al., 2013; Praskova et al., 2014), whereas lower concentrations caused no significant effects on fish length, weight, growth rates or condition (Lee et al., 2011). As discussed for PROP, our results suggest that DCF exposure do not affect meagre morphometrics at present environmentally relevant concentrations.

4.2. Pharmaceutical bioconcentration

Bioconcentration in fish muscle differed between the three pharmaceuticals tested, likely due to differences in biotransformation capacity. The metabolism of these three pharmaceuticals has been tested in fish, both *in vitro* (e.g. Baron et al., 2017; Connors et al., 2013; Smith et al., 2012) and *in vivo* (e.g. Ding et al., 2015; Lahti et al., 2011; Margiotta-Casaluci et al., 2014). Yet, differences in metabolic rates were demonstrated *in vitro* by Connors et al. (2013), where extensive metabolism of PROP and DCF by fish hepatocytes was observed, whilst FLX was not metabolized, therefore supporting greater potential for bioaccumulation. Likewise, *in vivo*, accumulation of FLX but not DCF was observed in rainbow trout (Zhang et al., 2010), and higher PROP bioconcentration, when compared to DCF, was described in zebrafish embryos (Bittner et al., 2019). Similarly, in this study, FLX was noticeably bioconcentrated in fish muscle, in comparison to PROP and DCF. FLX uptake in fish occurs within few hours of exposure (Paterson and Metcalfe, 2008), and it bioconcentrates in different tissues (Nakamura et al., 2008; Pan et al., 2018; Schultz et al., 2011). Moreover, its concentration in fish tissues increases with exposure time (Ding et al., 2016), probably due to the low biotransformation rates reported for FLX.

Likely as a result of efficient metabolism and depuration, DCF has low potential for bioconcentration in juvenile fish (Memmert et al., 2013; Schwarz et al., 2017), as observed in low or untraceable muscle DCF concentrations of fish exposed under controlled conditions to similar ranges of concentrations (µg/L) (e.g. Daniele et al., 2016; Memmert et al., 2013). On the other hand, there is limited information regarding PROP bioconcentration in fish. Uptake of PROP into fish blood plasma has been reported (e.g. Bartram et al., 2011; Giltrow et al., 2009; Owen et al., 2009), however, with low ensuing PROP bioconcentration in muscle tissues (Ding et al., 2015), probably due to rapid and efficient PROP metabolism, as observed *in vitro* by several authors (Baron et al., 2017; Connors et al., 2013; Gomez et al., 2010). In fact, decreasing PROP concentrations in fish tissues with exposure time (Ding et al., 2016, 2015) further corroborates efficient PROP metabolism in fish.

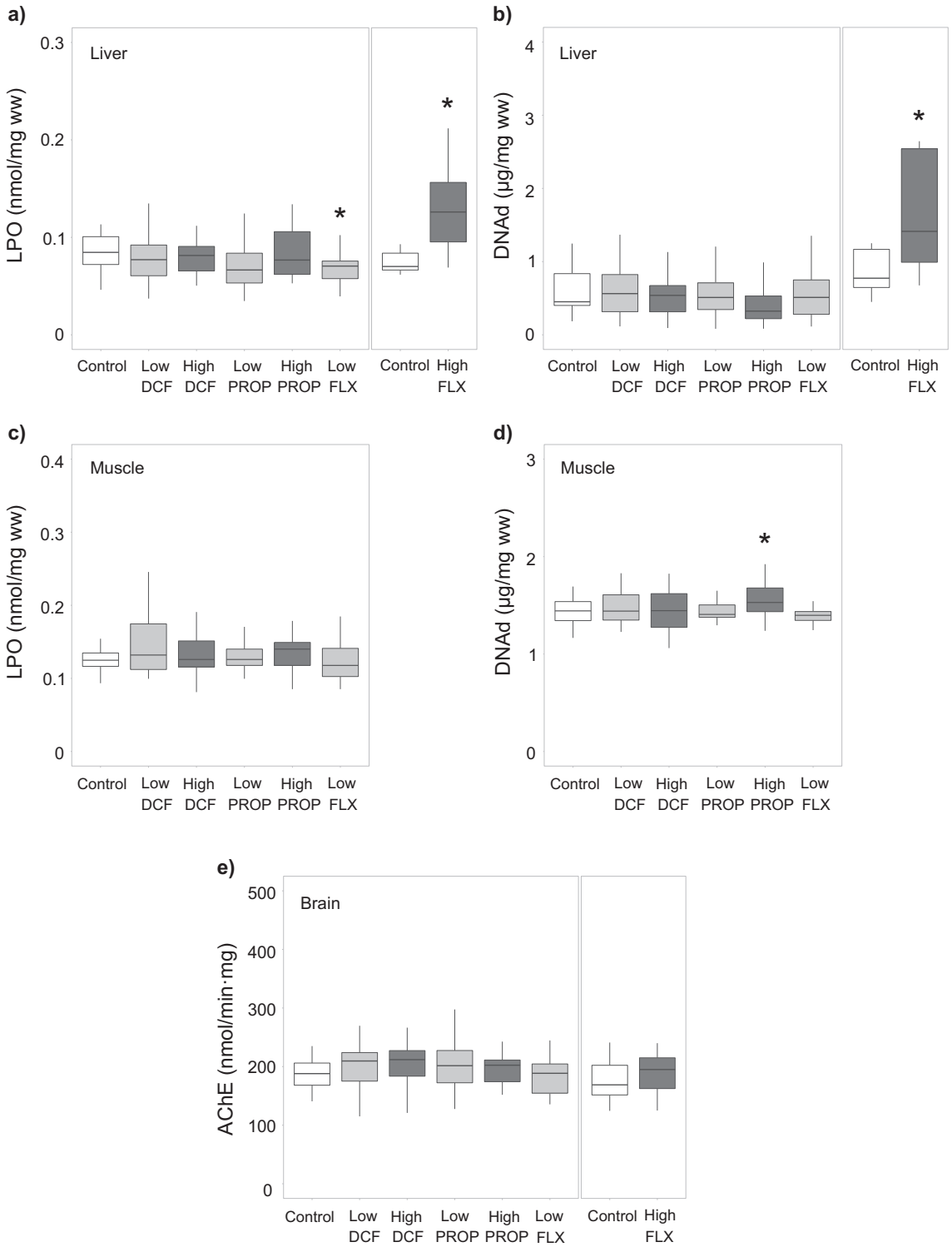

Fig. 3. Oxidative and neurotoxic effects of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of effects measured in the liver, muscle and brain: a) liver lipid peroxidation (LPO), b) liver DNA damage (DNAd), c) muscle lipid peroxidation (LPO), d) muscle DNA damage (DNAd) and e) brain acetylcholinesterase activity (AChE). Asterisks indicate significant differences between treatments and respective controls.

4.3. Sub-individual responses

4.3.1. Antioxidant and biotransformation enzymes

FLX toxicity in fish hepatocytes has been linked to increased reactive oxygen species (ROS) production but also to its inhibitory effect on biotransformation enzymes of the cytochrome P450 family, including EROD (Fernández et al., 2013; Laville et al., 2004). Likewise, *in vivo* inhibition of biotransformation enzymes GST and EROD by FLX has previously been reported (e.g. Chen et al., 2018; Ding et al., 2016), as well as increased CAT and SOD activities at low µg/L concentrations (Pan et al., 2018). Accordingly, in this study exposure to high FLX treatment also triggered an increased antioxidant response and inhibited both

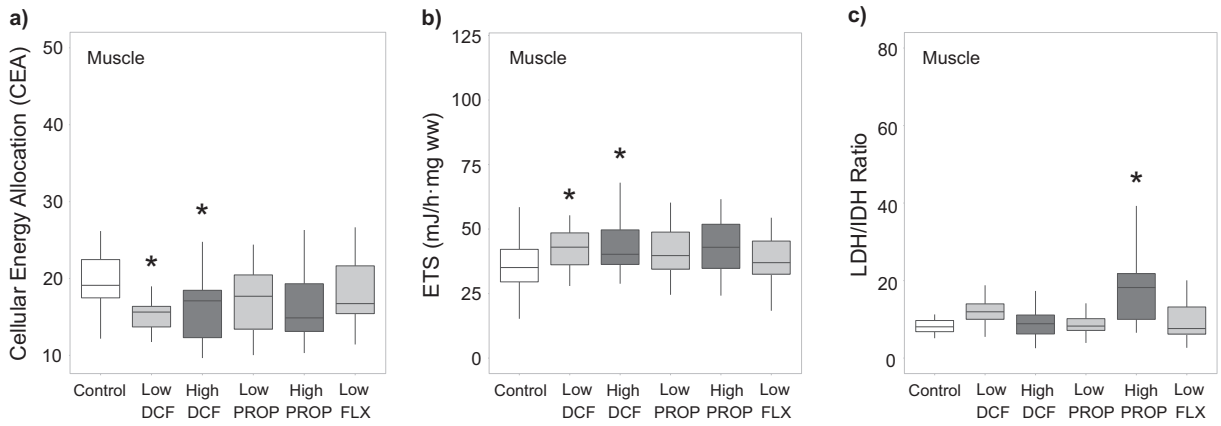

Fig. 4. – Energy related responses of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of responses measured in fish muscle: a) cellular energy allocation (CEA), b) electron transport system activity (ETS) and c) LDH/IDH ratio. Asterisks indicate significant differences between treatments and respective controls.

biotransformation enzymes activities, revealing enhanced oxidative stress and FLX toxicity at environmentally relevant concentrations. Likewise, SSRI venlafaxine, sharing the same mode of action of FLX, also increased liver CAT activity and inhibited GSTs in *A. regius* juveniles after 28 days of waterborne exposure at 20 μg/L (Maulvault et al., 2018b).

Contrarily to FLX, Laville et al. (2004) found that DCF did not increase ROS production in fish hepatocytes, whilst increased DCF exposure ensued fish antioxidant responses only at higher DCF concentrations (high μg/L to mg/L range) (e.g. Islas-Flores et al., 2013; McRae et al., 2019; Pandey et al., 2017). Moreover, either no changes or inhibition of EROD enzyme activity were reported in vitro (Laville et al., 2004; Thibaut et al., 2006) and in vivo (Guiloski et al., 2017; Prokkola et al., 2015), with GST induced after long-term exposure to similar concentrations but inhibited at higher μg/L (Guiloski et al., 2017; Stancova et al., 2017). In this study, no differences in liver antioxidant and biotransformation enzymes were found after exposure to DCF, suggesting low potential to cause oxidative stress in *A. regius* at environmental concentrations tested. Similar results were also observed by Maulvault et al. (2018a) in top predator *Dicentrarchus labrax* juveniles after dietary exposure to DCF.

PROP also failed to increase ROS production in fish hepatocytes (Laville et al., 2004). Yet, only a few studies have measured antioxidant and biotransformation enzymes responses *in vivo*, without significant alterations after waterborne exposure (Bartram et al., 2011; Pereira et al., 2018). Likewise, we observed no changes on antioxidant and biotransformation enzymes activities, suggesting low PROP toxicity at tested concentrations.

4.3.2. Oxidative damage and neurotoxic effects

In line with the enzymatic responses described above, FLX exposure (3 μg/L) resulted in both LPO and DNA damage in the liver of juvenile meagre. Similarly, exposure to FLX in the μg/L range have been shown to increase LPO levels in *Carassius auratus* and *Pseudorasbora parva* after 7 and 42 days, respectively (Chen et al., 2018; Ding et al., 2016), whilst no significant effects were observed in *Pomatoschistus microps* after only 4 days (Duarte et al., 2019). Increased lipid peroxidation was also observed in *A. regius* juveniles after 28 days of waterborne exposure to venlafaxine (20 μg/L), another SSRI compound (Maulvault et al., 2018b). To the best of our knowledge, genotoxic effects of FLX in fish were firstly assessed in our previous study (Duarte et al., 2019), with no changes in DNA damage reported for *Pomatoschistus microps* after 4 days of exposure to μg/L concentrations and 1 h exposures to mg/L range concentrations. Ultimately, LPO and DNA damage likely occur after longer exposure periods, supporting the potential for FLX to promote oxidative and genotoxic effects in the long term, even at low concentrations.

FLX can modulate fish cholinesterase activity and ultimately alter fish behaviour, with AChE activity pointed out as a valuable biomarker to study FLX neurotoxicity even if the mechanisms of this interaction are still unclear (e.g. Duarte et al., 2019; Farias et al., 2019). No significant differences in AChE activity were observed in *A. regius* juveniles, yet a slight increase at high FLX treatment could be perceived, which is in agreement with previous studies (Chen et al., 2018; Pan et al., 2018). However, inhibition or no effects in acute exposures have also been reported (Duarte et al., 2019; Farias et al., 2019). Differences in exposure duration, concentrations tested, as well as species and life-stages considered are likely the cause for such varying responses, and evidence the need for further investigation on the impact of FLX on fish neurological pathways.

Environmental DCF concentrations did not generate obvious oxidative stress in *A. regius* juveniles. Similarly, no DNA damage was previously observed in *Rhamdia quelen* liver after acute and chronic exposures to a comparable range of concentrations (Ghelfi et al., 2016; Guiloski et al., 2017), though increased DNA damage in *O. niloticus* juveniles was observed after exposure to much higher concentrations (mg/L) of DCF for 15 days (Pandey et al., 2017). Few studies have measured the long-term effects of waterborne DCF exposure to lipid peroxidation in fish yet these report inexistent (Schwarz et al., 2017; Stancova et al., 2017) or decreased (Guiloski et al., 2017) levels of LPO. After long-term dietary exposure to DCF, Maulvault et al. (2018a) also reported inexistent lipid peroxidation in *Dicentrarchus labrax* juveniles.

Concerning PROP, two studies have measured lipid peroxidation *in vivo* in fish after dietary exposure, where no differences in LPO levels were found (Ding et al., 2016, 2015). Similarly, in this study, waterborne exposure to PROP did not trigger lipid peroxidation in *A. regius*, but increased DNA damage in fish muscle, which, to the best of our knowledge, is the first record on genotoxic damage of this pharmaceutical in fish. Furthermore, only Pereira et al. (2018) have assessed the effects of PROP on cholinesterase activities in freshwater fish *Phalloceros harpagos* and, in agreement with our results, found no significant differences after acute and chronic exposures to a wide range of μg/L concentrations.

4.3.3. Energy metabolism

Disruption of fish energy metabolism by low μg/L FLX concentrations has been previously acknowledged (Mennigen et al., 2010; Mishra et al., 2017). However, no other studies have to date specifically addressed the effects of FLX on LDH and IDH enzymes activities, nor ETS activity in fish. Contrarily to previous findings, fish energy metabolism in *A. regius*

juveniles' muscle and heart was not affected by FLX. Nonetheless, potential links between decreased fish growth and muscle energy metabolism cannot be discarded, especially considering the negative correlations observed between heart and muscle ETS activity and fish length and weight. Although FLX had no impact on energy metabolism at low concentration, the cost of FLX exposure in fish metabolic performance merits further investigation.

DCF clearly affected fish energy metabolism through an increase in muscle ETS activity at both concentrations and via a significant reduction of CEA. As a measure of cellular oxygen consumption and metabolism (King and Packard, 1975), increased ETS levels suggests that even at the lowest concentration tested, DCF significantly increased energy expenditure in *A. regius* juveniles. Interestingly, this increase in energy demand and consequent reduction of net energy budget (CEA) was not sufficient to bring changes in energy reserves (proteins, lipids and carbohydrates). Similarly, no changes in liver LDH activity were reported in *Gasterosteus aculeatus* exposed to 1 μg/L for 14 days (Lubiana et al., 2016), and increased LDH activity in serum and gills of *Clarias gariepinus* juveniles was found only after exposure to concentrations in the mg/L range (Ajima et al., 2015).

On the other hand, PROP exposure increased muscle LDH/IDH ratio, due to a decrease in muscle IDH activity. IDH is involved in cellular energy production via the aerobic pathway but it is also a key enzyme to maintain cellular defense mechanisms (Jo et al., 2001). Since mitochondrial energy production, i.e. ETS activity, was not affected by PROP, this reduction in IDH activity might indicate a decreased capacity to counteract PROP oxidative stress in the muscle, which is further revealed by the increased DNA damage levels at the high concentration. Nonetheless, the slight decrease in growth rate observed at high PROP concentration, might be linked to this shift to anaerobic metabolism, along with the slight increase in ETS, i.e. energy consumption, hence reducing the amount of energy available for somatic growth. Indeed, this interpretation is also supported by the negative correlations observed between heart LDH activity and both fish length and weight. Accordingly, previous studies have reported inhibition of metabolic processes such as glycogen production and glucose release in rainbow trout as a result of PROP binding to hepatic b-adrenoceptors (Fabbri et al., 1998; Gesto et al., 2014), yet further investigation is needed to clarify the impact of PROP in fish metabolism.

5. Conclusions

Overall, pharmaceuticals from different therapeutic classes caused distinct responses in *A. regius* juveniles. FLX was the most toxic pharmaceutical of the three, impairing fish growth and liver biotransformation mechanisms. Additionally, oxidative stress observed in FLX treatment appears to be triggered by the combination of inhibited biotransformation mechanisms and prominent bioconcentration in fish muscle which is further emphasized by the increase in LPO and DNA damage in the liver. No changes in energy metabolism were found at low FLX concentration, yet effects at high concentration evidenced fish growth impairment and should be considered in future studies, given that FLX concentrations in the low μg/L range are environmentally relevant.

PROP and DCF exposure caused no effects on fish growth or condition, yet PROP bioconcentrated in muscle tissues, whilst DCF was not detected. Moreover, DCF did not enhance oxidative stress in fish liver, yet increased fish energy consumption in muscle, although not sufficient to cause changes in metabolic strategy or energy reserves. Interestingly, PROP caused a significant decrease in IDH activity, may be related to its role in cellular antioxidant defense, suggesting increased oxidative stress in muscle, as revealed by DNA damage increase.

Overall, each pharmaceutical generated different individual and biochemical responses. Specifically, FLX higher toxicity was evident in changes at the individual level as well as in biochemical changes in fish liver, whereas PROP and DCF effects were observed on biochemical changes in fish muscle. Although the modes of action of these pharmaceuticals are not fully described in fish, these may be the cause for differences in responses observed in this study. An Omics approach could give further insight into the mechanisms underlying pharmaceuticals' toxicity in fish, including those related to biotransformation, oxidative stress, as well as detrimental effects on fish growth and energy metabolism. Ultimately, future research addressing the impacts of pharmaceuticals in different fish species to evaluate how physiology, behavior and ecology underpin inter specific differences in effects, is key to improve our understanding of the environmental risk posed by pharmaceuticals.

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.

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Appendix. Supplementary data

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