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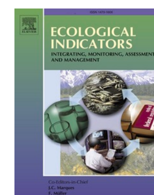

Integrated biological response to environmentally-relevant concentration of amitriptyline in *Sparus aurata*

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ABSTRACT

Amitriptyline (AMI) is a commonly tricyclic antidepressant to treat depression, anxiety, and other conditions. Like many other pharmaceuticals, AMI and its by-products are incompletely removed during wastewater treatment and therefore they are released to rivers, estuaries and coastal waters. The presence of this kind of compounds in the water environment may involve a negative impact on non-target aquatic organisms at relatively low concentrations. However, the knowledge of AMI effects on aquatic organisms in the environment is scarce. Thus, the objective of this work is to determine the effects of environmentally-relevant concentrations of AMI on biological responses at biochemical and cellular levels in marine teleost. Gilt-head seabream (*S. aurata*) were exposed to AMI for 7 days at 0.2 μg/L in an open flow system and a battery of biomarkers were investigated: acetylcholinesterase, catalase, superoxide dismutase, glutathione S-transferase, cytochrome *C* oxidase, P450 CYP1A1 ethoxyresorufin (O) dealkylation, and lysosomal biomarkers. Biomarkers were integrated as IBR/n (biological response index). Overall, it can be concluded that AMI exposure at environmentally-relevant concentration induces significant biological responses to stress in marine teleost, especially in lysosomal biomarkers. However, further research is needed about the effects of AMI and other pharmaceuticals on biomarkers in non-targeted species, to raise the knowledge about the toxicity of this type of emerging pollutants.

1. Introduction

Amitriptyline (AMI) is a commonly tricyclic antidepressant (TCA) to treat anxiety, depression, and other conditions (including diabetic neuropathy, neuralgia, and anorexia) (Bautista-Ferrufino et al., 2011; Moore et al., 2012). TCAs act as an inhibitor of noradrenaline and serotonin reuptake in the central nervous system and in consequence, the concentration of both neurotransmitters increases in the brain (Breyer-Pfaff et al., 2004; Lajeunesse et al., 2008). From the toxicokinetic point of view, AMI is readily absorbed from the gastrointestinal tract, it undergoes extensive metabolism in the liver and is excreted in the urine, mainly in form of metabolites (Mylan, 2020). Like many other pharmaceuticals, AMI and its by-products are incompletely removed during wastewater treatment (Lajeunesse et al., 2008). In fact, AMI highest concentration detected in the environment have been 0.227 μg/L in wastewater treatment plant effluents (Baker and Kasprzyk-Hordern, 2011). The presence of this kind of compounds in the water environment involves a negative impact on non-target aquatic organisms, even at relatively low concentrations (Yang et al., 2018; Ziarrusta et al., 2019).

The knowledge of AMI effects on aquatic organisms in the environment is scarce (Sehonova et al., 2018). Among those studies, Yang et al. (2014) found effects on adrenocorticotropic hormone (ACTH) level, oxidative stress, and antioxidant parameters in freshwater zebrafish (*Danio rerio*) embryos exposed to AMI. Moreover, TCAs long-term exposure affects to early-life stages of freshwater common carp (*Cyprinus carpio*), changing superoxide dismutase and catalase activities, increasing mortality, morphological anomalies, developmental retardation, and produce pathological changes in heart, brain, and cranial and caudal kidney (Sehonova et al., 2017). Ziarrusta et al. (2017) determined that AMI is mainly accumulated in brain (800 ng/g in tissue), gills (600 ng/g in tissue), and liver (250 ng/g in tissue) *Sparus*

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aurata, after exposure at an environmentally-relevant concentration for 7 days. Moreover, Ziarrusta et al. (2019), under the same experimental conditions, determined that fish showed changes in brain and liver metabolome after that exposure. Nonetheless, the effects of AMI on early warning biological responses or biomarkers are still unknown in marine fish, such as gilt-head bream (*Sparus aurata*). This teleost fish is one of the most relevant fish species in fishery and aquaculture industries and it is widespread in Mediterranean and Atlantic coastal waters (FAO, 2020). Additionally, *S. aurata* is a very used model teleost in laboratory toxicological experiments (Souid et al., 2013; Rodrigues et al., 2018; Solomando et al., 2020).

To understand the toxic effect of a long exposure of fishes to AMI, we carried out an experiment combining the metabolomic profiling variation in plasma, liver and brain (Ziarrusta et al., 2019) with the assessment of a battery of exposure biomarkers described herein. To reach this aim, gilt-head breams (*S. aurata*) were exposed to AMI for 7 days at 0.2 μg/L, the highest AMI concentration detected in the environment (Baker and Kasprzyk-Hordern, 2011), previously described by Ziarrusta et al. (2017). After the exposure, a battery of biomarkers was investigated: acetylcholinesterase activity (AChE, neurotoxicity marker); catalase (CAT) and superoxide dismutase (SOD) activities (oxidative stress markers); glutathione S-transferase activity (GST; biotransformation marker) activity; cytochrome *C* oxidase activity (COX; mitochondrial aerobic capacity marker); P450 CYP1A1 ethoxyresorufin (O) dealkylation (EROD; detoxification marker) and lysosomal biomarkers (lysosomal membrane labilization period, lysosomal volume density, lysosomal surface density, lysosomal numerical density; neutral lipid volume density; general stress markers). The biological responses selected are widely used fish biomarkers in toxicological studies to pointed out the presence of pollutants and/or the magnitude of its response at biochemical, cell, or tissue-level (White et al., 2003; ICES, 2012; Briaudeau et al., 2020). It is highly recommended to use a battery of biomarkers in the aforementioned experiments, to understand as much as possible of the whole picture of biological responses generated by a stressor (UNEP/RAMOGE, 1999; Rodrigues et al., 2018; Briaudeau et al., 2020). Moreover, biological responses were integrated calculating IBR/n (biological response index).

2. Materials and methods

2.1. Reagents

Amitriptilyne hydrochloride (AMI) (98%) was used to prepare stock ethanolic solutions (5000 mg/L) and then diluted with MilliQ water to the dosing solutions (85.2 ug/L). The final concentration of ethanol in the dosing solution was < 0.1%. All stock solutions were stored at − 20 °C before use. Additional information regarding the used reagents and materials is provided in Ziarrusta et al. (2017).

2.2. Experimental design

Juvenile *Sparus aurata* (~40 g weigh and ~13 cm length) were acquired from Groupe Aqualande (Roquefort, France) and transported to Research Centre for Experimental Marine Biology and Biotechnology (Plentzia Marine Station; PiE-UPV/EHU, Basque Country, Spain), where the exposure experiment was carried out. Then, fish were acclimatized for 14 days (18 °C air temperature, 14:10 h light: dark cycle), and they were stabilized for an additional 2 days in their respective experimental tanks before starting the exposure. Fish were fed with 0.10 g pellets/fish (EFICO YM 868, 3 mm; BioMar) daily and the water was continuously oxygenated. Water temperature (13.5 °C) and pH (7.3 ± 0.3) were constant during the whole experiment. Dissolved oxygen, ammonium, nitrite, and nitrate levels were measured periodically during the experiment to check the maintenance of water quality.

Exposure experiments were run in parallel, one for the control tank (no AMI) and the other for the exposed tank (nominal concentration AMI = 0.2 μg/L) for 7 days. During the exposure period, two polypropylene tanks (one control, one exposure), each containing 250 L of seawater, were supplied with 145 gilt-head breams per tank. Additional information regarding the performance of the experiment is provided in Ziarrusta et al. (2017).

Five fish were sampled from each experimental group after 2, 4, and 7 days of experimentation. The fish were immediately anesthetized in a tank containing 200 mg/L $NaHCO_3$ and 200 mg/L tricaine diluted in 10 L of seawater. Then, fish were processed by measuring, weighing, and dissecting (brain, liver, and gills). Tissue samples were snap frozen in liquid nitrogen and then stored in − 80 °C freezer until analysis.

This study was carried out in parallel with bioaccumulation, biotransformation, and metabolites experiments (Ziarrusta et al. 2017, 2019). Moreover, water samples from the exposure tank were collected to analyse AMI concentration, when fish were removed (Ziarrusta et al., 2017). The time-weighted average concentration was determined (0.12 ± 0.02 μg/L), as the mean concentration of the 3 sampling days (Ziarrusta et al., 2017). Besides, the concentrations of AMI and its metabolites in the control group samples were lower than the detection limit (<1 ng/L). No mortality was detected during the experiment, and there were no significant differences in the hepatosomatic index ((liver weight × 100)/fish weight) and in general condition ((fish weight × 100)/length) between control and exposure groups, as a sing of fish health maintaining throughout the experiment (Ziarrusta et al., 2017).

Fish processing described herein was evaluated by the Bioethics Committee of the University of the Basque Country, and it was accepted according to the current regulations (procedure approval CEEA/380/2014/ETXEBARRIA LOIZATE) by the local authority.

2.3. Enzyme activities

Acetylcholinesterase (AChE) activity was determined spectrophotometrically in brain, while catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), and cytochrome *C* oxidase (COX) activities were measured in gills and liver. Besides, P450 CYP1A1 ethoxyresorufin (O) dealkylation (EROD) was analyzed in liver. The tissue sample was homogenized using Precellys homogenizer equipped with Cryolys system. Then, the homogenates were centrifuged (10000 xg) at 4 °C for 20 min. The supernatant was gathered and it was used for enzyme determination, which was stored at − 80 °C before activity assays.

Enzyme activities were represented as nmol of substrate converted per min per mg of protein (AChE, CAT, SOD, GST, and COX). EROD activity was represented as pmol resorufin/min/mg. The protein concentration in the tissue was measured in triplicate following the Bradford's method (Guilhermino et al., 1996). All enzyme assays were carried out at 25 °C.

AChE activity was analysed by measuring the 5-thio-2-nitobenzoic acid formation at 412 nm (Guilhermino et al., 1996; ICES, 2012). CAT activity was defined by measuring the H_2O_2 degradation at 240 nm (Claiborne, 1985). SOD activity was defined by a colorimetric method using a SOD Assay Kit (Sigma). The assay is based on the production of formazan dye recorded at 450 nm. GST activity was defined by measuring the thioether creation at 340 nm (Guilhermino et al., 1996). COX activity was defined using a microplate spectrophotometer at 550 nm (Blanco-Rayón et al., 2019). EROD was determined by a fluorimetric method using a CYP1A1 EROD activity kit (Ikzus Environment). Emission and excitation wavelengths set at nm 583 and 537 nm, respectively, were applied to measure the activity.

2.4. Lysosomal biomarkers

Lysosomal membrane stability (LMS) in liver was determined by cytochemical expression of acid phosphatase activity in serial cryotome sections (10 μm thick; Leica CM 3000 cryotome), following a standardized procedure (UNEP/RAMOGE, 1999). The time of acid

labilisation treatment needed to produce the maximum staining intensity was determined as the Labilisation Period (LP; in min) using a light microscope (UNEP/RAMOGE, 1999; ICES, 2004).

Lysosomal structural changes (LSC) in liver were determined by cytochemical expression of β-glucuronidase enzyme activity (Alvarado et al., 2005). LSC was measured using a light microscope (100x magnification) and an image analysis (BMS, Sevisan) in five areas per fish, according to Blanco-Rayón et al. (2019). LSC analysis is based in the determination of volume density (Vv_{LYS}), surface density (Sv_{LYS}), surface-to-volume ratio (S/V_{LYS}), and numerical density (Nv_{LYS}). These stereological parameters are calculated in the following way (Lowe et al., 1981): $Vv_{LYS} = V_{LYS}/VC$, $Sv_{LYS} = S_{LYS}/V_C$, $S/V_{LYS} = S_{LYS}/V_{LYS}$, and $Nv_{LYS} = N_{LYS}/V_C$; where LYS = lysosomes, C = cytoplasm, V = volume, S = surface, and N = number.

Changes in levels of intracellular neutral lipids accumulation were evaluated after Oil Red O (ORO) staining, according to Marigómez and Baybay-Villacorta (2003). Five areas were assessed per fish using a 40 × objective lens. Changes in levels of intracellular neutral lipids accumulation are expressed as volume density of neutral lipids ($Vv_{NL} = V_{NL}/V_C$; NL = neutral lipids, V = volume, and C = cytoplasm).

2.5. Integrative biological response (IBR/n) index

IBR index was established on the integration of GST (gills), COX (gills), LP, S/V_{LYS}, and $Vv_{NL,}$ following the selection criteria of Marigómez et al. (2013). The chosen biomarkers represent different endpoints of biological response and they are linked to each other taking into account their biological complexity level. After standardizing the values of each biomarker following the calculations of Beliaeff and Burgeot (2002), those values were showed on a radar chart, in order to visualize easily the responsiveness level of each biomarker. The IBR index was obtained by adding the standardized values of each biomarker (IBR = ΣAi) and dividing by the biomarkers number used (IBR/n) (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006).

2.6. Statistical analysis

SPSS v. 26 software was used for statistical analyses. Homogeneity of variance and normality of data were tested (Levene's test and Kolmogorov-Smirnov's test, respectively). For all biomarkers, differences between the control and exposed group in each day were determined using the Student *t*-test for parametric variables (AChE, GST, EROD, Vv_{LYS}, Nv_{LYS}) and the Mann-Whitney *U* test for nonparametric variables (CAT, SOD, COX, LP, S/V_{LYS}, Vv_{NL}). Moreover, one-way ANOVA (Duncan's post-hoc test) was used for normally distributed variables to determine differences between the different days for the control and exposed fish, while the Kruskal-Wallis test followed by Dunn's test was used for not normally distributed biomarkers. Differences between gills and liver in enzyme activities were determined using Student *t*-test for parametric variables and Mann-Whitney *U* test for nonparametric variables. The Z-score test was employed to assess significant differences in the IBR/n index. For all statistical analyses, a 95% significance level ($p < 0.05$) was used.

3. Results

3.1. Biochemical biomarkers

AChE activity showed no significant differences between control and exposure groups (Fig. 1). In contrast, control groups showed in day 2 significantly higher values in comparison with days 4 and 7.

In the case of CAT activity, significant differences were only found in liver at day 4 between the control and exposure group (~x1.5 higher in the exposure group) (Fig. 2A and E). CAT activity of controls measured in gills and liver was significantly lower on day 4 than on day 2 (Fig. 2A and E). In SOD activity, significant differences were reported in gills on

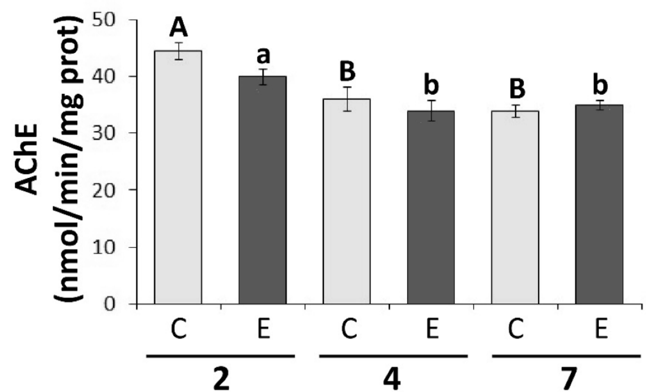

Fig. 1. Acetylcholinesterase (AChE) activity in brain of gilt-head breams exposed for 7 days to two concentrations (0 (C), 0.2 μg/L (E)) of amitriptyline. Intervals indicate standard error. Letters indicate significant differences between the different days for control and exposed fish (control groups, capital letters; exposed groups, lowercase letters).

day 2 (the exposure group tripled the control's values) and in liver on day 7 (the values of the control group double that of the exposed) (Fig. 2B and 2F). About controls, the SOD activity of day 2 in gills was higher compared to days 4 and 7, and in liver, values of day 7 were higher than in day 4 (Fig. 2B and F). Differences in GST activity in gills were observed only on day 4 when the activity of the control group was 1.5 times higher than that of the exposure group (Fig. 2C and G). In gills, GST activity of the control group at day 4 was higher than the rest controls, and in the liver, however, the value of day 2 was higher than in the other two days (Fig. 2C and G). In the case of COX activity, significant differences appeared only in gills, showing the exposure group higher activity (~x1.5 than the control group) on day 7 (Fig. 2D and H). Regarding the differences between controls the value of day 7 was significantly lower than day 2 in gills and liver (Fig. 2D and H). Significant differences in EROD activity were observed on day 2, in which the control group activity was 1.5 times higher than the exposed one (Fig. 3).

3.2. Lysosomal biomarkers

Regarding lysosomal biomarkers, lysosomal membrane stability was different between control and exposed fish in all sampled days, being more stable in control groups than in exposed ones (Fig. 4A). Differences between the control and exposed group were observed in lysosomal structural changes after 4 days of exposure (Fig. 4B and 4C). In exposed fish lysosomal enlargement was observed, S/V_{LYS} (inverse to size) was lower (~x1.5) and Vv_{LYS} was higher (~x3.5) comparing to control fish (Fig. 4B and 4C). No significant differences were observed in the number of lysosomes (Nv_{LYS}) between the control and exposed groups at any day (Fig. 4D). Moreover, significant accumulation of neutral lipids was observed in exposed fish after day 4 (~x1.5) and was maintained higher in the exposed group than in the control group on day 7 (Fig. 4E).

3.3. Integrative biological response (IBR/n) index

The integration of the selected biomarkers (GST in gills, COX in gills, LP, S/V_{LYS}, and Vv_{NL}) using IBR/n index showed that AMI caused stress after 2 days of continuous exposure. This stress becomes significant at days 4 and 7 (Fig. 5), mainly, due to, the increase of S/V_{LYS} and Vv_{NL}, as shown in the radar charts. Regarding controls, slight distress was observed on day 2, but they recovered on day 7 of the experiment because of due to the decrease of GST and COX, as explained in the radar chart.

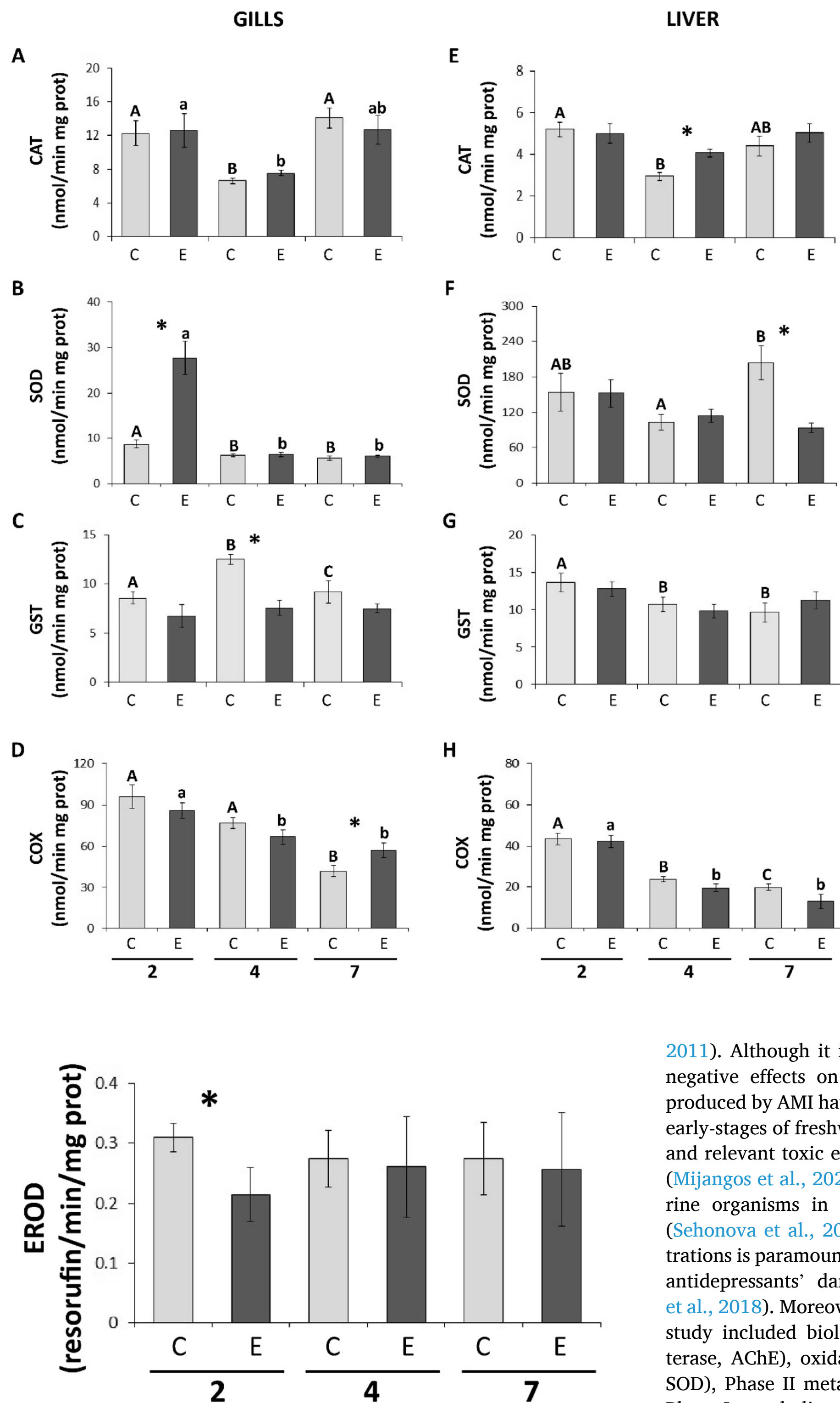

Fig. 2. Enzyme activities in gills and liver of gilt-head breams exposed for 7 days to two concentrations (0 (C), 0.2 μg/L (E)) of amitriptyline. Intervals indicate standard error. An asterisk represents the difference between the control and exposed groups in each day of exposure. Letters indicate significant differences between the different days for control and exposed fish (control groups, capital letters; exposed groups, lowercase letters). CAT: catalase, SOD: superoxide dismutase, GST: glutathione S-transferase, COX: cytochrome *C* oxidase.

Fig. 3. P450 CYP1A1 ethoxyresorufin (O) dealkylation (EROD) activity in liver of gilt-head breams exposed for 7 days to two concentrations (0 (C), 0.2 μg/L (E)) of amitriptyline. Intervals indicate standard error. An asterisk represents the difference between the control and exposed group in each day of exposure.

4. Discussion

AMI, like other pharmaceuticals, is present in surface waters, due to its rising consumption and its incomplete elimination during wastewater treatments (Lajeunesse et al., 2008; Baker and Kasprzyk-Hordern, 2011). Although it is known that this kind of compounds could have negative effects on non-target aquatic organisms, biological effects produced by AMI have been mainly studied on human cells cultures and early-stages of freshwater fish (Yang et al., 2018; Ziarrusta et al., 2019) and relevant toxic effects have been also found in sea urchin embryos (Mijangos et al., 2020). Thus, the knowledge about AMI effects on marine organisms in the environment is scarce, especially in teleost (Sehonova et al., 2018). The use of environmentally-relevant concentrations is paramount, in order to ensure a realistic understanding of the antidepressants' damaging effects on aquatic organisms (Sehonova et al., 2018). Moreover, the battery of biomarkers applied in the present study included biological responses of neurotoxicity (acetylcholinesterase, AChE), oxidative stress (catalase, CAT; superoxide dismutase, SOD), Phase II metabolism enzymes (glutathione S-transferase, GST), Phase I metabolism enzyme (P450 CYP1A1 ethoxyresorufin (O) dealkylation, EROD), mitochondrial aerobic capacity (activities cytochrome *C* oxidase activity, COX), and general stress (LMS, LSC, accumulation of neutral lipids), attaining an integrated view of the degree of biological effects on the organism.

The general condition of fish throughout the experiment was properly maintained, as showed by the data about mortality, condition index, and hepatosomatic index reported for the same experiment elsewhere (Ziarrusta et al., 2017).

AChE is answerable for the signal termination at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine, avoiding

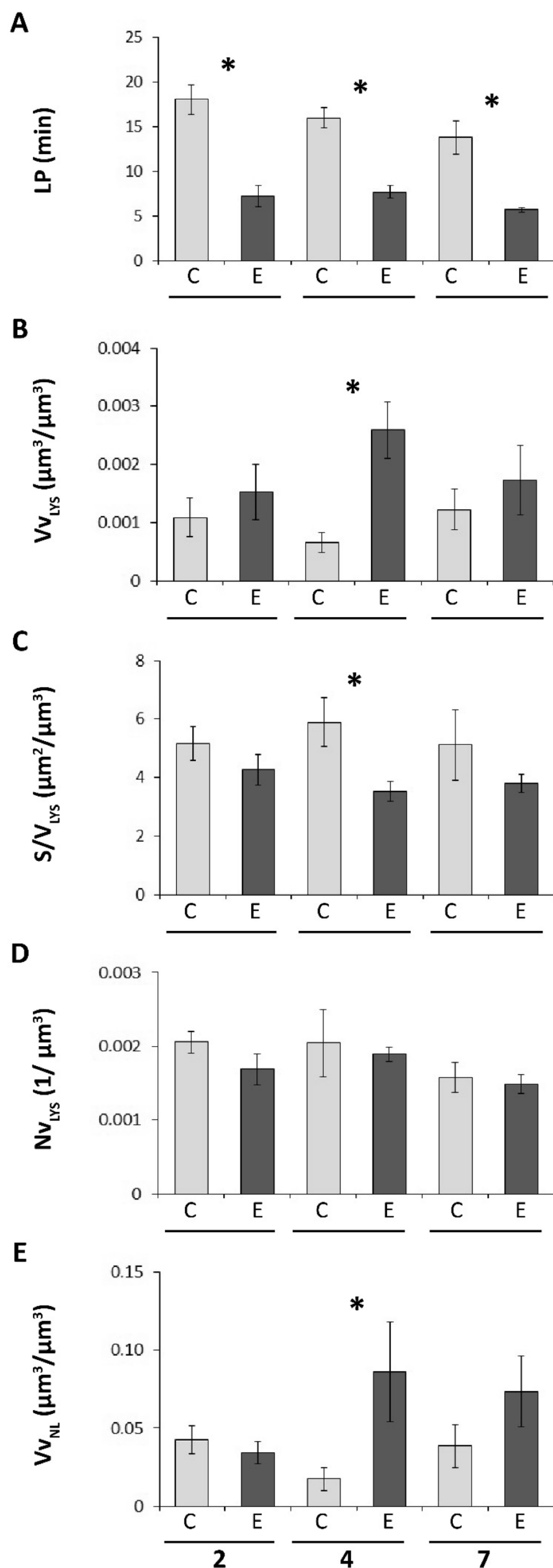

Fig. 4. Lysosomal biomarkers in liver of gilt-head breams exposed for 7 days to two concentrations (0 (C), 0.2 μg/L (E)) of amitriptyline. Intervals indicate standard error. An asterisk represents the difference between the control and exposed group in each day of exposure. LP: lysosomal membrane labilization period, Vv_{LYS}: lysosomal volume density, S/V_{LYS}: lysosomal surface density, Nv_{LYS}: lysosomal numerical density; Vv_{NL}: neutral lipid volume density.

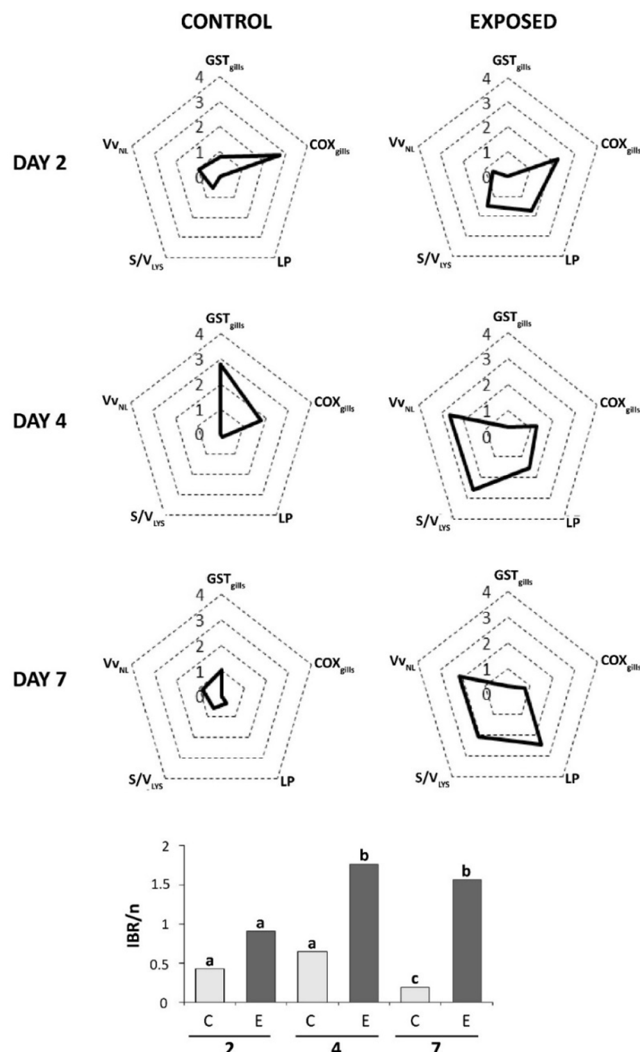

Fig. 5. Radar charts for five selected biomarkers (GST in gills, COX in gills, LP, S/V_{LYS}, and Vv_{NL}) and the corresponding IBR/n index for gilt-head breams exposed for 7 days to two concentrations (0 (C), 0.2 μg/L (E)) of amitriptyline. Letters indicate significant differences between the experimental groups, according to the Z-score test ($p < 0.05$).

continuous nerve firings, which is essential for the proper functioning of neuromuscular and sensory systems (Murphy, 1986). To detect neurotoxicity, the AChE enzyme activity measurement is widely used as biomarker, because the inhibition of AChE is linked with the mechanism of toxic action of carbamate and organophosphorus insecticides that bind to the catalytic site of the enzyme (ICES, 2012). In AMI bioaccumulation study of samples from the experiment herein (Ziarrusta et al., 2017), the highest concentration of AMI (up to ~ 15 ng/g) was found in brain tissue, because it is the main target organ of psychoactive drugs. Its hydrophobicity gives it the ability to cross the blood–brain barrier by passive diffusion (Ziarrusta et al., 2017). Due to the accumulation of AMI in brain tissue, an effect on AChE enzyme activity was expected. However, AChE activity did not show differences between the control and exposed groups, indicating that AMI has not neurotoxic effect at the used concentration (0.2 μg/L). A study with sertralin (another antidepressant that inhibits serotonin reuptake) showed an increase in AChE activity after 1, 2, 4, and 7 days of exposure (>4.46 μg/L) (Xie et al., 2015). In addition, Nunes-Tavares et al. (2002) demonstrated that amitriptyline-like TCAs inhibited AChE in *Electrophorus electricus* fish by fluorimetric methods, but the estimated TCA concentration (IC_{50}) required to inhibit 50% of activity was ~ 30 mg/L. A similar effect

would be expected, being AMI, sertraline, and TCAs similar compounds. Nonetheless, the antidepressant concentrations used in both cases were much higher than the used in our experiment (0.2 μg/L) and considerably higher than those found in the environment (Baker and Kasprzyk-Hordern, 2011), which may explain why AMI did not affect AChE activity.

Most pollutants are known to alter the prooxidant challenge/antioxidant defences balance in the cell, depressing or increasing antioxidant capacity (Di Giulio et al., 1989; Regoli and Giuliani, 2014). Marine organisms, such as fish, possess high responsiveness in enzymatic antioxidant defences after exposure to multiple stress and thus, antioxidant enzyme activities are widely applied as biomarkers of oxidative stress (Valavanidis et al., 2006; Solomando et al., 2020). Enzymes participating in antioxidant defenses are a coordinated system and include superoxide dismutase (SOD), which catabolizes superoxide radicals, and catalase (CAT), which degrade hydrogen peroxide and hydroperoxides. Second-line enzymes in antioxidant defense include those of glutathione metabolism. Glutathione S-transferase (GST), as part of the phase II metabolic reactions, catalyzes the reduced glutathione conjugation to nucleophilic xenobiotics or cellular components damaged by ROS attack, which leads to their detoxification. Under stress conditions, ROS concentrations are increased, which results in an increased capacity of antioxidant systems (Paris-Palacios et al., 2013). Therefore, changes in CAT, SOD, and GST enzyme activities were analyzed to study whether AMI can induce a relevant oxidative effect. The analysis of the metabolomics profiles along the exposure (Ziarrusta et al., 2019) showed the accumulation of longer chain acyl carnitines pinpointing lipid storage disorders as a negative effect of SSRIs that may be associated with oxidative stress usually produced by xenobiotics (Gómez-Canela et al., 2017).

Regarding CAT, significant differences between control and exposed groups were found only in the liver on day 4. An increase in CAT activity has been observed to occur in the presence of various pollutants; among others, in studies with model contaminants such as cadmium (Souid et al., 2013), or with emergent pollutants, for example, microplastics (Solomando et al., 2020) or drugs, as the antidepressant sertralin (Xie et al., 2015). This increase in CAT activity would be an attempt to adjust the antioxidant defense against an increase in oxidative stress, in which the enzyme would increase the rate of H_2O_2 degradation to lower ROS levels (Xie et al., 2015). This increase in activity would therefore reflect an increase in ROS caused by AMI. Human fibroblast cell culture studies treated with AMI showed a gradual increase in SOD activity at short time (24 and 48 h) (Moreno-Fernández et al., 2008). In the present study, however, at day 7, a marked decrease in SOD activity was observed in liver of exposed fish, compared with control. Other pollutants could provoke declines in SOD activity in fish, among others, copper (Vutukuru et al., 2006). Decreased activity of antioxidant enzymes could be due to excessive ROS level (excessive substrate) or damage caused by oxidants (Vutukuru et al., 2006; Sehonova et al., 2017). CAT and SOD enzymes work in a coordinated manner in the antioxidant system because H_2O_2 produced by SOD is degraded by CAT, making the activities of these enzymes often related (Souid et al., 2013). The decrease of SOD activity in liver observed after 7 days of exposure could be related to the transient increase of the activity of CAT on day 4. The reduction in SOD activity could mean less H_2O_2 generation and consequently reduced CAT activity. Besides, a decrease in SOD leads to an increase in the level of radical superoxide, a radical capable of inducing CAT inhibition (Kono and Fridovich, 1982), and in consequence, that could provoke oxidative stress. In fact, the metabolomic study of samples from the experiment herein suggested hepatic oxidative stress produced by AMI, due to accumulation of longer chain acyl carnitines in the liver (Ziarrusta et al., 2019).

SOD activity in gills, at day 2 there was an increase in the exposed group, but instead of a gradual increase in activity over time observed in experiments with cells (Žikić et al., 2001; Moreno-Fernández et al., 2008), on day 4 and 7 the activity was equalized with control. Inhibition of SOD activity in gills may lead to the generation of excess radicals exceeded the capacity of SOD, causing a decrease in the activity, as explained before in the case of SOD in liver (Vutukuru et al., 2006; Sehonova et al., 2017).

In gills, a significant difference was also observed in GST activity between control and exposed groups on day 4, with lower activity in the exposed group. Schmidt et al. (2008) observed a reduction in GST mRNA levels when treating human cells with imipramine, an antidepressant that inhibits serotonin and norepinephrine reabsorption. These antidepressants can inhibit gene expression and therefore activity level could be reduced as well. The inhibition of GST could cause serious damage to organisms, due to the important role that GST plays in the homeostasis of redox potential, carrying out the conjugation of GSH and xenobiotics or oxidized components (Vlahogianni et al., 2007). Unlikely, in the liver, GST activity did not show significant differences between the control and exposed groups on different days. A study with sertralin (Xie et al., 2015) showed an increase in GST activity in liver of *Carassius auratus* after 1, 2, 4, and 7 days of exposure. As explained with AChE, being a compound with a similar effect, close effects were expected with AMI. However, as mentioned earlier, the differences in antidepressant concentrations used (compared to 0.2 μg/L of AMI; > 4.46 μg/L of sertralin) may explain the different observed responses in both studies.

Energy metabolism regulation is an essential issue of the stress response, and therefore, energy-related biomarkers could be proper tools to determine physiological stress (Kültz, 2005; Sokolova et al., 2012). Energetic metabolism can be affected directly and/or indirectly by pollution via disruption of ATP-producing pathways and increased metabolic costs (Sokolova et al., 2012). Cytochrome *c* oxidase (COX) regulates ATP production, proton pumping, and oxidative phosphorylation (Arnold, 2012) and thus, COX is considered a bioenergetic marker (Lucassen et al., 2003; Morley et al., 2009). In gills, the exposed group showed higher activity than control at day 7, reflecting a higher level of oxygen consumption and could reflect the effect of AMI on energy metabolism. Higher COX activity has been observed in mussels under hypoxia condition, suggesting that the increase of this enzyme activity acts as a mechanism for oxygen lack compensation (Ivanina et al., 2011). This observed effect of AMI on energy metabolism is in agreement with other antidepressant studies (Webhofer et al., 2011) and with metabolomics study in liver of Ziarrusta et al. (2019), where a glutamate levels variation was observed. The observed alteration in glutamate concentration in fish liver could be associated with energy metabolism because a glutamate-derived product, α-ketoglutarate, plays as intermediate in the Krebs Cycle (Ziarrusta et al., 2019).

In fish, the liver is the most important organ for detoxification. The cytochrome P450 enzymes (CYPs) play relevant roles in the contaminants and endogenous molecules metabolism in the liver (White et al., 2003; ICES, 2012). CYP1A is the most studied CYP isoform in fish ecotoxicology, which can be assessed by the ethoxyresorufin O-deethylase (EROD) assay (White et al., 2003; ICES, 2012). It is described that AMI is metabolized mainly via CYP2 in human (Dean, 2017), however the CYP2 induction in fish is dissimilar and not fully understood (not induced by phenobarbital-type inducers, like in human) (Sadar et al., 1996; Uno et al. 2012). In fact, EROD (CYP1, P450) was selected as well-known Phase I metabolism biomarker in fish (ICES, 2012). In the present study, a significant decrease of EROD activity in exposed fish was observed in comparison to the control group on day 2. Induction of EROD activity has been observed in the presence of various environmental pollutants (such as polycyclic aromatic hydrocarbons), but there are cases where, as in this work, decreases in EROD activity have also been observed in relation to drugs (e.g., exposure to the antibiotic oxytetracycline) (Rodrigues et al., 2018). Therefore, the obtained data reflects that amitriptyline causes a short-term inhibition in liver transformation reactions, compromising the appropriate metabolic process of detoxification and subsequent excretion of AMI.

Lysosomal responses are widely used as effect biomarkers in fish (Köhler et al., 2002; Alvarado et al., 2005; Briaudeau et al., 2020). Fish

liver lysosomes play an important role in responses to pollutants through the sequestration and detoxification of pollutants (ICES, 2012). Eventually, lysosomal responses to environmental stress could be divided into three main categories (ICES, 2012; Briaudeau et al., 2020): (a) reduction of lysosomal membrane stability, (b) increase of lysosome size, and (c) changes in lysosomal contents such as accumulation of unsaturated neutral lipids. In the current study, lysosomal membrane stability, expressed as Labilisation Period (LP), was always below 10 min in exposed fish liver. Those values indicated that AMI might produce a high degree of liver cell damage, which has been linked with potential pathological alterations at tissue level (Köhler et al., 2002; ICES, 2012).

The increase in size and reduction in numbers of lysosomes (lysosomal enlargement) are considered as early lysosomal responses to pollutants (Lowe et al., 1981; Kohler et al., 1992; Briaudeau et al., 2020). In the present study, Vv_{LYS} was higher and S/V_{LYS} lower in exposed groups than in controls, significantly at day 4. This result reveals that AMI may provoke lysosomal enlargement, even at environmentally-relevant concentration.

Regarding changes in lysosomal contents, intracellular accumulation of neutral lipids is considered as exposure biomarker linked mainly to organic chemical pollution (Marigómez and Baybay-Villacorta, 2003; ICES, 2012). In the current study, neutral lipid accumulation was higher in exposed fish than in control ones at days 4 and 7, significantly at day 4. Lipid storage disorders, induced by cationic amphiphilic drugs such as AMI, have been determined in cell cultures by Xia et al. (2000). Moreover, effects on lipid metabolism were expected due to the changes observed in metabolites belonging to that process (Ziarrusta et al., 2019). In agreement with lysosomal membrane destabilisation and with lysosomal enlargement, lysosomal perturbations in fish liver may indicate early tissue lesions by the intralysosomal accumulation of unsaturated neutral lipids (Kohler et al., 1992). This is the first study that determines the effects of an antidepressant on lysosomal biomarkers in fish. Thus, more studies about antidepressant effects on lysosomal responses are needed, to reinforce the obtained results.

In order to illustrate the overall effect of AMI, five biological responses to stress with high responsiveness were selected: GST in gills and COX in gills, as biochemical markers; LP and S/V_{LYS}, as cell-level markers and Vv_{NL}, as tissue level marker. Then, they were integrated into the Integrative Biological Response (IBR) index. Apart from contributing to an overall evaluation of biological responses to AMI, the use of this index reduce the misgivings produced by the inherent variability of individual biomarkers. Indeed, this index has been previously applied to other marine organisms, such as fish, based on a biomarkers combinations of different biological levels (Broeg and Lehtonen, 2006; Marigómez et al., 2013; Briaudeau et al., 2020). The IBR/n values confirmed that environmentally-relevant concentration of AMI causes a toxic effect on *S. aurata* after 4 days of exposure, even though the highest concentration of AMI in tissues was found on day 7 (Ziarrusta et al., 2017). Moreover, radar charts showed that biochemical markers reacted first to AMI exposure and then, that response was observed at cell and tissue-level, as expected (ICES, 2012).

4.1. Concluding remarks

The most relevant biological responses were expected on day 7 because the highest concentration of AMI in different tissues (brain, gills, and liver) was found on that day (Ziarrusta et al., 2017). In fact, marked stress signals were observed on day 7 in SOD activity and lysosomal membrane stability in liver and COX activity in gills. However, in the rest of biomarkers, concentration-dependent effects were not observed. Thus, the effect of amitriptyline is not entirely dependent on the accumulated concentration. In addition, the metabolism of AMI may also play an important role in changes in activity given on different days.

Overall, the data obtained in the current study showed that AMI exposure at environmentally-relevant concentration induces significant biological responses to stress in marine teleost *S. aurata*, including oxidative stress, alterations in energy and lipid metabolisms, in agreement with the conclusions obtained through metabolomics studies (Ziarrusta et al., 2019). AMI effects were observed markedly on lysosomal biomarkers. Moreover, further research is needed about the effects of AMI on biomarkers in non-targeted species, to raise the knowledge about the toxicity of this type of emerging pollutant.

CRediT authorship contribution statement

Esther Blanco-Rayón: Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Haizea Ziarrusta:** Conceptualization, Investigation, Methodology, Writing - review & editing. **Leire Mijangos:** Investigation, Methodology, Writing - review & editing. **Maitane Olivares:** Conceptualization, Funding acquisition, Resources, Writing - review & editing. **Olatz Zuloaga:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing. **Nestor Etxebarria:** Funding acquisition, Resources, Supervision, Writing - review & editing. **Urtzi Izagirre:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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

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