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Biomarker and behavioural responses of an estuarine fish following acute exposure to fluoxetine

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ABSTRACT

Antidepressants such as fluoxetine are frequently detected in estuaries and can have profound effects on non-target organisms by interfering with the neural system and affecting essential physiological processes and behaviours. In this context, short-term effects of fluoxetine exposure were analysed in the common goby *Pomatoschistus microps*, an estuarine resident fish species. Two experiments were conducted with fish exposed to: i) fluoxetine concentrations within the μg/L range for 96 h (0.1, 0.5, 10 and 100 μg/L) and ii) fluoxetine concentrations within the mg/L range for 1 h (1, 5 and 10 mg/L). Acute toxicity was assessed via multiple biomarker responses, namely: activity levels of antioxidant (superoxide dismutase and catalase) and detoxification enzymes (ethoxyresorufin O-deethylase and glutathione S-transferase); and biomarkers of effects (lipid peroxidation and DNA damage) and of neurotoxicity (acetylcholinesterase inhibition). Furthermore, behavioural responses concerning activity (active time, movement delay and number of active individuals) and feeding (number of feeding individuals) were also recorded and analysed. Acute fluoxetine exposure for 96 h (in the μg/L range) reduced antioxidant CAT activity with increasing concentrations but had no significant effect on SOD activity. Biotransformation enzymes showed bell-shaped response curves, suggesting efficient fluoxetine metabolism at concentrations up to 10 μg/L. No significant damage (LPO and DNAd) was observed at both concentration ranges (μg/L and mg/L), yet 1 h exposure to higher fluoxetine concentrations (mg/L range) inhibited acetylcholinesterase activity (up to 37%). Fluoxetine (at mg/L) also decreased the number of both feeding and active individuals (by 67%), decreased fish active time (up to 93%) and increased movement delay almost 3-fold (274%). Overall, acutely exposed *P. microps* were able to cope with fluoxetine toxicity at the μg/L range but higher concentrations (mg/L) affected fish cholinergic system and behavioural responses.

1. Introduction

Pharmaceuticals are continuously released to aquatic environments via multiple routes such as household, hospital and industrial wastewater effluents, aquaculture or animal husbandry, resulting in their ubiquitous presence in freshwater and coastal environments worldwide (Caldwell, 2016; Kümmerer, 2009). Consequently, a wide range of concentrations have been reported, usually within ng/L to μg/L range (Mezzelani et al., 2018), yet much higher concentrations, in the mg/L range, have also been reported in surface waters, chiefly associated with effluents from aquacultures and pharmaceutical manufacturing plants (Fick et al., 2009; Larsson et al., 2007; Le and Munekage, 2004). As pharmaceuticals are designed to produce effects at very low concentrations, their frequent detection in the aquatic environment raises concern over putative deleterious effects in non-target organisms.

Antidepressants and its metabolites have been frequently detected in the environment, with concentrations up to 1 μg/L in seawater, 8 μg/L in surface and groundwaters and up to 32 μg/L in waste water treatment plants (Mezzelani et al., 2018). Among these are selective serotonin reuptake inhibitors (SSRIs), which are a group of pharmaceutical compounds used to treat depression and other psychiatric disorders. SSRIs act by blocking the reuptake of serotonin neurotransmitter from the synaptic cleft, increasing serotonin concentrations and consequently affecting neuronal signal transmission (Hiemke and Härtter, 2000). Allied to neuronal function, serotonin is also involved in other physiological mechanisms, such as those related to immune and

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endocrine systems or behavioural responses (Corcoran et al., 2010; Fent et al., 2006). Serotonin and its transporters are highly conserved in many species, particularly among vertebrates (Kreke and Dietrich, 2008; Mennigen et al., 2011), which implies SSRIs may elicit deleterious physiological and neuronal effects in a large number of species.

The antidepressant fluoxetine is one of the most prescribed SSRIs and is frequently detected in surface waters of estuarine and coastal areas (Mezzelani et al., 2018; Silva et al., 2012). Fluoxetine is considered highly toxic to various organisms (Corcoran et al., 2010; Fent et al., 2006), and even though there are inconsistencies across studies (Sumpter et al., 2014), detrimental effects of fluoxetine exposure have been observed in invertebrate and vertebrate species (Sehonova et al., 2018; Silva et al., 2015), and at very short timeframes (i.e. within minutes to hours of exposure) (Ford and Fong, 2016). In fish, fluoxetine has been found to modulate gene transcription and enzymatic activities related to detoxification pathways, to alter endocrine and reproductive processes (e.g. reduce hormone production; fecundity and sexual development), as well as to alter behaviour (e.g. decreased feeding rates and locomotion) (e.g. Cunha et al., 2016; Giacomini et al., 2016; Henry and Black, 2008; Lister et al., 2009; Saaristo et al., 2017). Moreover, fluoxetine uptake and metabolism in fish is known to occur over a short timeframe (Paterson and Metcalfe, 2008) and it has been shown to accumulate in fish tissues (Brooks et al., 2005; Schultz et al., 2011). Yet, a considerable knowledge gap still exists concerning exposure effects on wildlife, particularly in marine and coastal environments (Gaw et al., 2014).

Biomarkers are sensitive measurements of biochemical, cellular or molecular interactions, that can signal early-on effects of exposure to xenobiotic compounds at the sub-individual level, and are therefore frequently used as indicators of exposure to and of effects in ecotoxicology studies (van der Oost et al., 2003). Recent studies have reported different effects of fluoxetine on biomarker responses in various aquatic organisms, albeit only a few evaluated *in vivo* fish exposures (e.g. Chen et al., 2018; Ding et al., 2016; Pan et al., 2018). At the individual level, behaviour is an ecologically relevant indicator of exposure to neuroactive compounds, as it may directly impact fitness and survival of aquatic organisms (Brodin et al., 2014).

In this context, the toxicity potential and effects of fluoxetine allied to its pervasive presence in the aquatic environment merits further exploration. Notably, analysing sub-lethal biological responses and behaviour changes of organisms exposed to a wide range of environmental concentrations of this neuroactive compound is of high ecological relevance, and should contribute to improve our understanding of its potential impact on estuarine biota. Accordingly, the aim of this study was to assess the effects of fluoxetine waterborne exposure on key biomarker and behavioural responses of *Pomatoschistus microps* (Krøyer, 1838), an estuarine resident fish species, pivotal to community functioning in temperate estuaries, and frequently used in ecotoxicology and biomonitoring studies (e.g. Fonseca et al., 2011; Oliveira et al., 2013). We conducted two independent short-term exposure experiments where fish were exposed to: i) fluoxetine concentrations within the μg/L range for 4 days (0.1, 0.5, 10 and 100 μg/L), covering the range of environmental concentrations reported for antidepressants and its metabolites; and ii) higher concentrations of fluoxetine for 1 h (1, 5 and 10 mg/L), simulating acute exposure to point source contamination.

Accordingly, multiple biomarker responses were assessed in *P. microps*, namely: the activity levels of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), responsible for protecting cells from reactive oxygen species (ROS) and thus for reducing oxidative stress; the activity of detoxification enzymes ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST), responsible for the metabolism of xenobiotic compounds, including pharmaceuticals; the levels of lipid peroxidation (LPO) and DNA damage (DNAd); and the activity of acetylcholinesterase (AChE) activity as an indicator of neurotoxicity. Concerning behavioural endpoints, we hypothesised that waterborn exposure to fluoxetine may alter locomotory and feeding behaviours in *P. microps*, and thus compromise individual fitness (e.g. by affecting fish ability to capture prey, avoid predatory attacks or to successfully reproduce), which would in the long-term reduce fish survival (Gerhardt, 2007). Ultimately, by combining sub-individual and individual responses, we intend to attain a more comprehensive assessment of fluoxetine toxicity on an estuarine fish species, a group which has seldom been evaluated.

2. Materials and methods

2.1. Fish sampling and acclimatization

P. microps individuals (length 3.01 ± 0.25 cm) were collected at low tide in the Tejo estuary natural reserve, near Alcochete (mean and standard deviation of water salinity and temperature were 19.2 ± 0.10 and 20.9 ± 0.26, respectively), using a hand net, and transported to the laboratory in a common tank (approx. 80 L) with continuous aeration. Upon arrival, fish were divided randomly among three 80 L tanks, equipped with aeration and filtration systems. Throughout the day, a gradual shift to exposure water conditions was performed, with target values for temperature (ca. 20 °C) and salinity (18) similar to field water measurements. Fish were fed daily with newly hatched *Artemia* nauplii and worms (*Hediste diversicolor*). All procedures took place in a controlled temperature room, and water at 18 salinity was prepared with synthetic marine salt dissolved in filtered dechlorinated tap water.

2.2. Experimental design

Fish were allocated randomly among 15 experimental tanks, with 12 individuals per tank, and acclimated to exposure conditions for one week. The acute semi-static toxicity test was performed according to OECD guidelines (test no. 203) for 96 h in 18 L aerated glass tanks with natural photoperiod and no filtration. Four concentrations of the antidepressant fluoxetine and a control treatment were used (0, 0.1, 0.5, 10 and 100 μg/L), with three replicate tanks per concentration. Concentrations used in this trial cover the range of reported environmental concentrations for antidepressants and its metabolites (Mezzelani et al., 2018). Fluoxetine stock solutions were prepared with milliQ-grade water and stored at −20 °C. Daily water renewals were performed, and fluoxetine concentrations appropriately restored to maintain fluoxetine concentrations in the tanks. Water parameters, namely temperature, salinity, pH and ammonia, as well as fish mortalities were recorded daily. Feeding was suspended 24 h before the beginning of the exposure test.

After 96 h exposure, fish were transferred to individual behavioural observation tanks and rested for 10 min in the new environment before each trial, to avoid handling stress interference. All tanks were covered throughout the experimental trials and observations were made through recorded high definition video, to minimize any potential stress or bias caused by visual contact/human presence. In feeding trials, 10 *Artemia* nauplii were released per tank, marking the beginning of a 5-min observation period for feeding and locomotory activities. Analysed behavioural endpoints included the percentage of active and feeding individuals, the overall time individual fish spent moving and the time individual fish took to make the first movement (i.e. movement delay). After behavioural trials, fish were immediately sacrificed, and tissues stored at −80 °C until further analysis.

Fluoxetine uptake and metabolism in fish is known to occur over a short timeframe, within 5 h of exposure to low concentrations (0.55 μg/L) (Paterson and Metcalfe, 2008). Hence, we hypothesised that 1 h of exposure to a higher range of concentrations (mg/L) would allow for fluoxetine uptake and metabolism and would suffice to generate biological or behavioural effects in *P. microps*. Accordingly, an acute static toxicity test was conducted, where fish were individually exposed to

three fluoxetine concentrations and a control treatment (0, 1, 5 and 10 mg/L) for 1 h, in 1 L glass beakers with water also at 20 °C and 18 salinity. Concentrations used in this trial were chosen to mimic acute exposure to point source contamination (Fick et al., 2009; Larsson et al., 2007). Twelve fish were exposed per treatment and post-exposure procedures were performed as described above.

All experimental procedures were performed in accordance with animal testing guidelines and licenced by university animal welfare committee and national authorities.

2.3. Biomarkers quantification

For biomarkers' quantification different fish tissues were dissected, namely liver, head and gills. 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. Four individuals were pooled for liver samples and a 1:5 (w/v) tissue:buffer ratio was used in homogenization, whilst head and gills were individually homogenized in 1 and 0.5 mL of the same buffer at pH 7.2, respectively.

Aliquots of liver homogenate were separated for lipid peroxidation (LPO), to which BHT (butylated hydroxytoluene) at 4% was added (1:15 v/v sample) to prevent further lipid peroxidation. The remaining liver homogenate was centrifuged at 12000 g for 20 min at 4 °C, and aliquoted for superoxide dismutase (SOD), catalase (CAT), ethoxyresorufin-*O*-deethylase (EROD) and glutathione *S*-transferase (GST) determination. Gills homogenates were aliquoted for DNA damage quantification, while head homogenates were centrifuged at 11000 g for 3 min at 4 °C and used in acetylcholinesterase (AChE) activity analysis.

All biomarker responses were determined in a microplate reader (Biotek Synergy HT) and each reading was done in triplicate. Protein content was adjusted to 0.5–0.7 mg mL^{-1} for biomarker determinations, except for AChE assays, for which protein content was adjusted to 0.3 mg mL^{-1}.

Superoxide dismutase (SOD) was measured according to Marklund and Marklund (1974), based on its ability to inhibit pyrogallol autoxidation, with few adaptations. Briefly, increase in absorbance was followed for 5 min at 325 nm, after incubation of 5 µL of homogenate with 265 µL of 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, and 30 µL of a 30 mM pyrogallol solution in 10 mM HCl. Control assays were performed in the absence of homogenate samples to determine pyrogallol autoxidation. SOD activity was expressed as U min^{-1} mg^{-1} of total protein concentration, with one unit of SOD as the amount of enzyme that inhibits the reduction of pyrogallol by 50% per minute of reaction.

Catalase (CAT) activity was determined according to Aebi (1974), by measuring the decrease in absorbance at 240 nm, caused by substrate consumption. Briefly, 130 µL of 50 mM phosphate buffer were added to 20 µL of sample, and the reaction was started with the addition of 150 µL of substrate (30 mM H_2O_2 in 50 mM phosphate buffer, pH 7). 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). The reaction was initiated with the addition of 10 µL of NADPH (8.33 mg mL^{-1}) to 190 µL of 7-ethoxyresorufin solution (0.1 mg mL^{-1} in 100 mM phosphate buffer (pH 7.4)) and 100 µL of sample, at 30 °C for 20 min. Fluorescence from 7-hydroxyresorufin was measured at 537/583 nm excitation/emission wavelengths, and resorufin sodium salt was used as standard. 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). Briefly, the conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) by GST was measured through changes in absorbance at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$), for 3 min. The assay was started with the addition of 250 µL of a final reaction mixture containing 100 mM phosphate buffer (pH 6.5), 20 mM CDNB and 20 mM reduced glutathione, to 50 µL of sample. GST 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). The reaction of thiobarbituric acid reactive substances (TBARS) with 2-thiobarbituric acid (TBA) occurred after incubation of 500 µL of TCA 12%, 450 µL of 60 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA and 500 µL of TBA 0.73% with 50 µL of sample for 60 min, at 97 °C. Samples were cooled on ice and centrifuged at 13400 g for 3 min, and absorbance was measured at 535 nm ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$). LPO was expressed as nmol of TBARS formed per mg of total protein.

DNA damage (DNAd) was determined in gills following the DNA alkaline precipitation assay by Olive (1988). Samples (50 µL) were first mixed with 250 µL of a 2% SDS solution containing 10 mM EDTA, 10 mM Trisbase (pH 12.4) and 50 mM NaOH. Then, 250 µL of a 0.12 M KCl solution were added and the mixture was incubated at 60 °C for 10 min. After cooling down on ice for 15 min, the mixture was centrifuged at 8000 *g* for 5 min, at 4 °C. Following the addition of 200 µL of Hoechst dye (1 µg mL^{-1} in 0.1 M K-phosphate buffer, pH 7.4) to 50 µL of the mixture, DNA concentration in the supernatant was determined at 360 nm/460 nm of excitation/emission wavelengths. Fluorescence values were compared to a DNA standard curve and DNAd was expressed as µg DNA per mg of total protein.

P. microps' head homogenates (cleaned of gills) were used for determination of acetylcholinesterase (AChE) activity, which has been described as the main cholinesterase form in this species' head tissues, and a proxy of brain AChE (Monteiro et al., 2005). Acetylcholinesterase (AChE) was determined according to Ellman et al. (1961), adapted to microplate (Guilhermino et al., 1996). Briefly, 250 µL of a final reaction mixture containing 100 mM phosphate buffer (pH 7.2), 75 mM acetylthiocholine and 10 mM DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) were added to 50 µL of sample (protein adjusted to 0.3 mg mL^{-1}). The reaction of thiocholine with DTNB to produce the yellow anion TNB, was followed at 412 nm ($\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$), every 20 s for 10 min. The enzymatic activity was expressed in nmol of substrate hydrolysed per minute per mg of total protein.

Protein content was quantified following Bradford's method, adapted to microplate: 250 µL of Sigma Bradford solution is added to each replicate of sample (10 µL) and incubated for a 15 min period (light protected and at room temperature) after which absorbance is measured at 595 nm. Bovine serum albumin solution (1 mg mL^{-1}) was used as protein standard.

2.4. Data analyses

Data was first checked for normality and homogeneity of variances and transformed when necessary in order to meet these assumptions (using Shapiro-Wilk and Levene tests, respectively). In the 96 h experiment, differences in biomarker responses among replicate tanks per treatment (n = 3) were first tested through analysis of variance. No differences were found among replicate tanks, except for one control tank in one biomarker response (DNAd). Since statistical results did not differ for DNAd analysis when considering tank and individual responses, for consistency with other biomarker responses, we present DNAd results based on all measurements in the following analyses. Accordingly, differences in biomarker responses among treatments in both experiments were tested through analyses of variance (ANOVA), followed by post-hoc Tukey tests. Number of replicates per treatment in the 96 h experiment were n = 9 for SOD, CAT, EROD, GST and LPO and

n = 12 for DNAd and AChE; whilst in 1 h experiment the number of replicates were n = 3 for SOD, CAT, EROD, GST and LPO, n = 4 for DNAd and n = 6 for AChE. To test for independence of behavioural responses to treatment we used Kruskal-Wallis test (active time and movement delay, n = 12) and Fisher's exact test of independence (number of active and feeding individuals, n = 12), followed by post-hoc tests. According to data normality assumptions, Pearson product moment correlation (r_p) analysis was used to test for correlations among biomarker responses (parametric data), and Spearman rank correlation coefficients (r_s) to test for correlations between biomarkers and behavioural responses (non-parametric data). All analyses were performed in R software (RStudio Team, 2016), and a significance level of 0.05 was considered for all statistical tests used.

3. Results

Water parameters were constant across tanks and exposure days. Temperature (20.7 °C ± 0.2 °C), salinity (18.1 ± 0.1), pH (7.3 ± 0.2), and conductivity (26.8 uS ± 0.2 uS) were measured daily, and ammonia levels were maintained under 0.2 mg/L. Three individuals from three different tanks died over the 96 h experiment (one in the control, one in the 10 µg/L and one in the 100 µg/L tank), thus mortality did not exceed 10%, as recommended by OECD guidelines.

Following 96 h of exposure to fluoxetine concentrations within the µg/L range (0.1, 0.5, 10 and 100 µg/L), dose-dependent inhibition of catalase activity with fluoxetine was observed, with significant differences from the control group at 10 and 100 µg/L ($F = 3.95$, p-value < 0.01, Fig. 1b). Concerning biotransformation enzymes, bell-shaped response curves were observed for both GST and EROD activity ($F > 4.8$, p-value < 0.01, Fig. 1c and d). No significant effects were observed in SOD activity, in LPO and DNAd levels, or in AChE activities ($F > 0.36$, p-value > 0.05, Fig. 1a, e, f and g). Concerning behaviour, no significant effects of fluoxetine were found after 96 h ($\chi^2 > 11.77$, Fig. 2a and b; $H > 2.80$, Fig. 2c and d; p-values > 0.05). Few correlations were found among *P. microps* responses in the 96 h experiment. Specifically, GST activity was positively correlated with EROD ($r_p = 0.64$, p-value < 0.001) and SOD ($r_p = 0.60$, p-value < 0.001) activities. EROD activity was positively correlated to SOD activity ($r_p = 0.38$, p-value < 0.01) and negatively to LPO levels ($r_p = -0.31$, p-value < 0.05).

After 1 h of exposure to 1, 5 and 10 mg/L concentrations of fluoxetine, AChE activity was significantly inhibited by 27 and 37%, at 5 and 10 µg/L, respectively ($F = 5.60$, p-value < 0.01, Fig. 3g). For CAT and LPO, differences among treatments but not to control were observed ($F > 4.80$, p-value < 0.05, Fig. 3b and e). However, no significant changes were observed in SOD, EROD and GST enzymes activities nor in DNAd ($F > 0.05$, p-value > 0.05, Fig. 3a, c, d and f). Yet, fluoxetine at the highest concentration (10 mg/L) significantly reduced the number of active and feeding individuals, both by 67% ($\chi^2 > 11.77$, p-value < 0.01, Fig. 4a and b). Moreover, the active time of individual fish significantly decreased with exposure to fluoxetine at all concentrations tested ($H = 19$, p-value < 0.001, Fig. 4c). This decrease was concentration-dependent and ranged from 55% at the lowest concentration (1 mg/L) up to 93% at the highest concentration (10 mg/L). Furthermore, significant delays in fish movement were observed after exposure to fluoxetine at all concentrations tested ($H = 16.11$, p-value < 0.001, Fig. 4d), increasing from 69 ss on average in control to 173 ss at 1 and 5 mg/L (152 and 153%, respectively) and to 256 ss on average (274%) at 10 mg/L exposure treatment. Correlations among *P. microps* responses were also observed in the 1 h experiment. GST was positively correlated with EROD ($r = 0.62$, p-value < 0.05), and negatively with fish active time ($r_s = -0.60$, p-value < 0.05). AChE activity was positively correlated with fish active time ($r_s = 0.74$, p-value < 0.001) and negatively with fish movement delay ($r_s = -0.53$, p-value < 0.01).

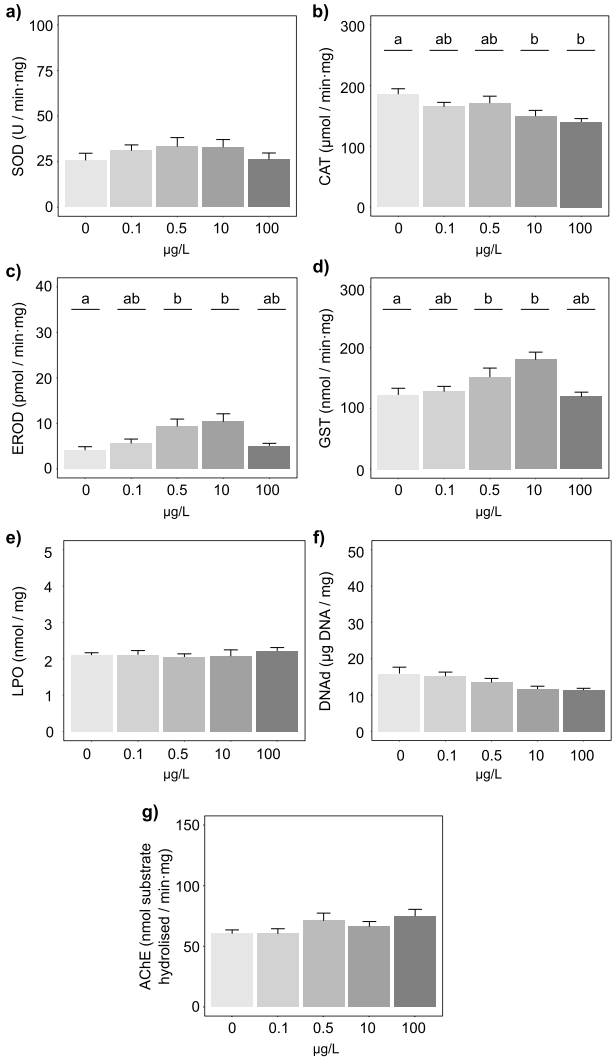

Fig. 1. Biomarker responses of *P. microps* exposed to fluoxetine (µg/L) for 96 h. One control and four fluoxetine treatments were tested (0, 0.1, 0.5, 10 and 100 µg/L). Bar plots with mean and standard deviations of biomarkers responses: **a)** SOD (superoxide dismutase) activity, **b)** CAT (catalase) activity, **c)** EROD (ethoxyresorufin O-deethylase) activity, **d)** GST (glutathione S-transferase) activity, **e)** LPO (lipid peroxidation) levels, **f)** DNAd (DNA damage) and **g)** AChE (acetylcholinesterase) activity. Different letters indicate significant differences from post-hoc comparison Tukey tests, following a one-way analysis of variance for each biomarker response. Number of replicates per treatment: n = 9 for SOD, CAT, EROD, GST and LPO; n = 12 for DNAd and AChE.

4. Discussion

Acute exposure to fluoxetine altered several biomarker responses in *P. microps*, although responses differed between the 96 h exposure trial (from 0.1 to 100 µg/L concentrations) and the 1 h exposure trial at higher concentrations (from 1 to 10 mg/L). Fish behavioural changes (feeding and locomotor activity) and neurotoxicity (acetylcholinesterase activity) were only observed after 1 h exposure to higher concentrations (mg/L).

Biotransformation enzymes are responsible for the metabolism of different xenobiotic compounds, including pharmaceuticals. Induction of biotransformation enzymes following fluoxetine exposure has been reported *in vitro* (Thibaut and Porte, 2008) and *in vivo* in fish (e.g. Chen et al., 2018). However, fluoxetine, and its metabolite norfluoxetine, have been found to accumulate in fish tissues (Brooks et al., 2005; Paterson and Metcalfe, 2008), as well as to inhibit different CYP isoforms at high concentrations (mg/L range) (Smith et al., 2012; Thibaut

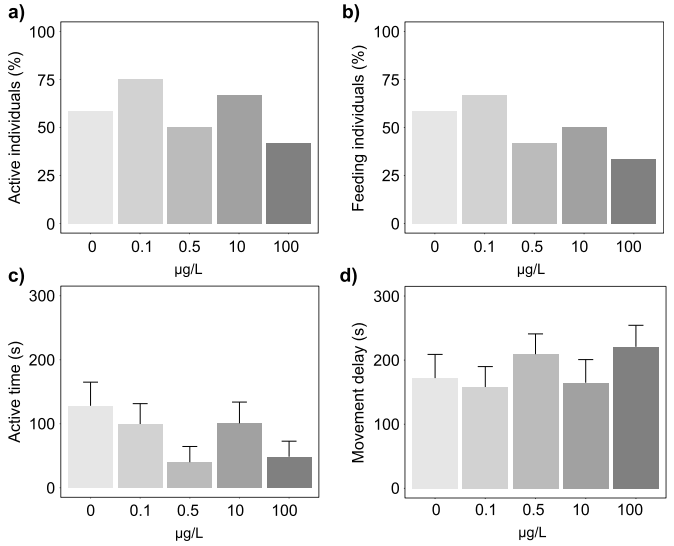

Fig. 2. Behavioural responses of *P. microps* exposed to fluoxetine (µg/L) for 96 h. One control and four fluoxetine treatments were tested (0, 0.1, 0.5, 10 and 100 µg/L). Bar plots of the percentage of **a)** active individuals and **b)** feeding individuals, and of mean and standard deviations of **c)** fish active time and **d)** movement delay. Different letters indicate significant differences from post-hoc analysis, following Fisher's exact test of independence (number of active and feeding individuals) and Kruskal-Wallis test (active time and movement delay), with n = 12 replicates per treatment for each behaviour endpoint.

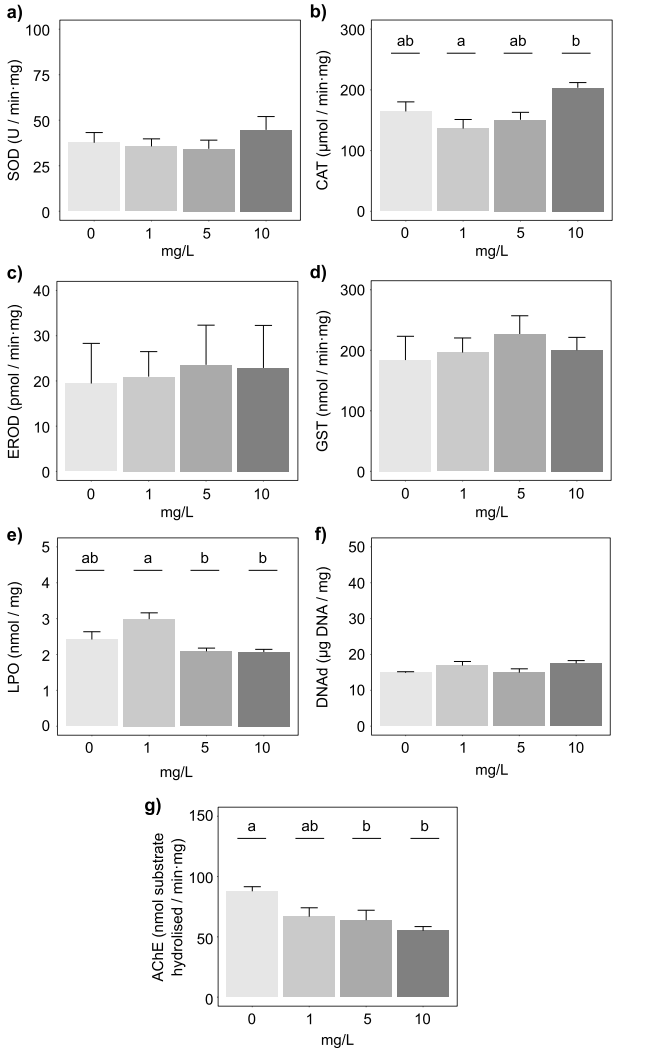

Fig. 3. Biomarker responses of *P. microps* exposed to fluoxetine (mg/L) for 1 h. One control and three fluoxetine treatments were tested (0, 1, 5 and 10 mg/L). Bar plots with mean and standard deviations of biomarkers responses: **a)** SOD (superoxide dismutase) activity, **b)** CAT (catalase) activity, **c)** EROD (ethoxyresorufin O-deethylase) activity, **d)** GST (glutathione S-transferase) activity, **e)** LPO (lipid peroxidation) levels, **f)** DNAd (DNA damage) and **g)** AChE (acetylcholinesterase) activity. Different letters indicate significant differences, from post-hoc comparison Tukey tests, following a one-way analysis of variance for each biomarker response. Number of replicates per treatment: n = 3 for SOD, CAT, EROD, GST and LPO; n = 4 for DNAd and n = 6 for AChE.

et al., 2006), including EROD (Laville et al., 2004). In this study, positive correlations between biotransformation enzymes in both exposure trials points to fluoxetine metabolism in *P. microps'* liver, although significant differences were only evident in the 96 h trial. Fluoxetine modelled EROD and GST activities the same way (in the µg/L range), with increasing enzymatic activity up to 10 µg/L then returning to basal levels at higher concentrations (100 µg/L). This follows the hormetic model, which describes low-dosage induction of enzymatic activity followed by inhibition at higher dosages, resulting in a bell-shaped response curve (Calabrese and Baldwin, 2003). The reduction in activity of biotransformation enzymes at higher concentrations can result from downregulation of genes involved in detoxification pathways, as observed by Cunha et al. (2016), or from direct enzyme inhibition by fluoxetine and/or its metabolites.

Antioxidant enzymes, such as SOD and CAT, are the primary defence mechanisms against reactive oxygen species (ROS), which may be a product of chemicals exposure and uptake (van der Oost et al., 2003). Fluoxetine cytotoxicity at high concentrations (mg/L) has been linked to increased ROS production in fish hepatocyte cells (Laville et al., 2004), and only a few studies have explored fluoxetine effects on antioxidant enzymes' activity in fish *in vivo*, yet with varying responses. For instance, Pan et al. (2018) found total antioxidant capacity (T-AOC), and CAT and SOD activities significantly increased after 3 day exposure to fluoxetine at 0.1 µg/L in the goldfish (*Carassius auratus*). On the other hand, Ding et al. (2016) reported that a 7 day exposure to higher concentrations of fluoxetine (4–100 µg/L) caused a significant reduction in SOD activity in the same species. Cunha et al. (2016) also reported SOD inhibition, yet increased CAT activity in zebrafish embryos exposed to fluoxetine (0.4–247.5 µg/L) for 80 h.

In this study, catalase activity was the only biomarker to vary in both exposure trials, with a decreasing trend in activity with increasing fluoxetine concentrations after 96 h at low concentrations (µg/L). On the other hand, no significant fluoxetine effects were observed in SOD activity. Contrary to previous findings, our results suggest that acute exposure to both fluoxetine concentration ranges (µg/L and mg/L) does not generate overt oxidative stress in *P. microps*, which is further supported by the lack of oxidative damage in lipids and DNA. LPO levels and DNA damage showed no significant alterations in comparison to control treatments, even at higher concentrations (mg/L). Yet, Ding et al. (2016) and Chen et al. (2018) have previously reported increased levels of lipid peroxidation in fish exposed to fluoxetine at µg/L concentrations after 7 and 42 days exposure, respectively. To the best of our knowledge, this is the first study to assess DNA damage in fish exposed to fluoxetine, yet *in vitro* studies with invertebrate species have shown fluoxetine genotoxicity and DNA damage at concentrations as low as ng/L (e.g. Gagné et al., 2006; Lacaze et al., 2015), whilst others have reported decreased or no DNAd *in vivo* (e.g. Franzellitti et al., 2015; Magni et al., 2017; Maranho et al., 2014).

Differences in antioxidant responses among studies may be related to different experimental settings, such as exposure time and concentrations tested, as well as to different life-stages and species-specific responses. Smith et al. (2010) described high intra-species variability in *in vitro* hepatic fluoxetine metabolism in four fish species, which hindered inter-species comparisons. Noteworthy, in *P. microps,* the reduced

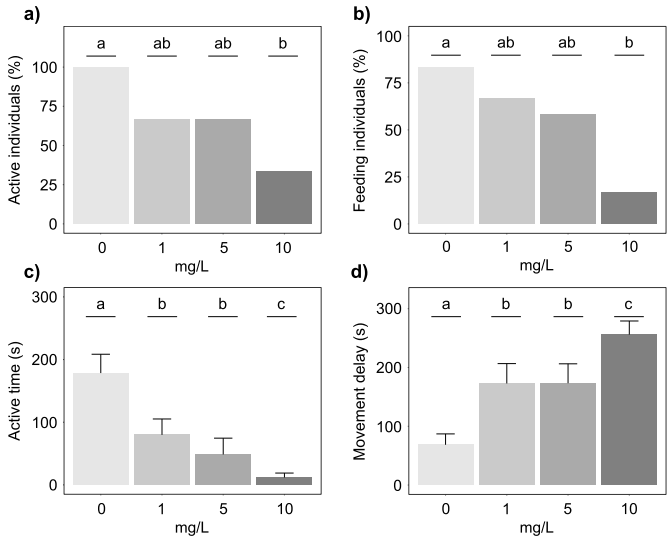

Fig. 4. Behavioural responses of *P. microps* exposed to fluoxetine (mg/L) for 1 h. One control and three fluoxetine treatments were tested (0, 1, 5 and 10 mg/L). Bar plots of the percentage of **a)** active individuals and **b)** feeding individuals, and of mean and standard deviations of **c)** fish active time and **d)** movement delay. Different letters indicate significant differences from post-hoc analysis, following Fisher's exact test of independence (number of active and feeding individuals) and Kruskal-Wallis test (active time and movement delay), with n = 12 replicates per treatment for each behaviour endpoint.

antioxidant responses we observed following acute fluoxetine exposure could result from expedite fluoxetine biotransformation and excretion, which would minimize antioxidative response and prevent oxidative damage in this species. Alternatively, other antioxidant mechanisms not measured in this study could be contributing to low oxidative stress levels. Furthermore, at the mg/L range, an increasing trend in antioxidant enzymes with increasing concentrations could be observed, with consequent reduction of LPO levels. Although Paterson and Metcalfe (2008) found rapid uptake and metabolism of fluoxetine in fish within 5 h of exposure to 0.55 µg/L, our results suggest that the activation of antioxidant defences in *P. microps* may require exposure periods longer than 1 h or much higher concentrations (5 or 10 mg/L). In this context, studies of short-term (hourly) exposures to enhanced concentrations of pharmaceuticals in biota are paramount to screen for affected mechanisms (e.g. Hamilton et al., 2016; Magno et al., 2015). Furthermore, identification of fluoxetine metabolic pathways, biotransformation efficiency and tissue bioaccumulation across fish species warrants further investigation.

Only recently has AChE activity been measured in fish brains and shown to increase at concentrations ranging from 0.1 to 200 µg/L in acute and chronic exposures (Chen et al., 2018; Pan et al., 2018). In this study, no significant effects were observed in *P. microps* AChE activity in the 96 h (exposure in µg/L) trial, although an increasing trend in activities could be observed, and is in line with the previous studies. However, in the 1 h exposure trial (exposure in mg/L), AChE activity was significantly inhibited at 5 and 10 mg/L concentrations. In human serum, cholinesterase inhibition also occurred at high fluoxetine concentrations, in the mg/L range (ca. 0.9–18 mg/L) (Müller et al., 2002). Fluoxetine and other SSRIs also evidenced a dose-dependent inhibitory effect on zebrafish embryos cholinesterases (Farias et al., 2019; Yang et al., 2018). Accordingly, high fluoxetine concentrations and rapid uptake and accumulation in fish brain (Paterson and Metcalfe, 2008; Schultz et al., 2011) likely lead to the prompt AChE inhibition even after only 1 h of exposure.

Decreased locomotor activity and latency in movement have been described in different fish species after short and long-term exposures to fluoxetine at both ng/L and µg/L range (e.g. Meijide et al., 2018; Saaristo et al., 2018; Winder et al., 2012). Fluoxetine has also been associated to decreased feeding rates in fish at these concentration ranges (e.g. Mennigen et al., 2010; Weinberger and Klaper, 2014), which could be linked to either reduced appetite (due to serotonin modulation) or indirect effects on activity (e.g. reduced locomotion and stimuli response) (McDonald, 2017). At higher concentrations, in the mg/L range, hourly exposures have induced changes in behaviour in fish and invertebrates (Hamilton et al., 2016; Magno et al., 2015). In our study, only exposure to higher fluoxetine concentrations (mg/L over an hour) caused adverse effects on feeding and activity patterns of *P. microps*. Specifically, individual fish were less active and movement delay was increased at all concentrations in the mg/L range. The number of active and feeding individuals was also significantly reduced at 10 mg/L. However, *P. microps* behaviour was not affected after 96 h exposure to concentrations of up to 100 µg/L, suggesting that this species behavioural responses were less sensitive to fluoxetine exposure in comparison to previous studies. Typically, estuarine species are well adapted to the high natural variability of these environments, which allows them to tolerate stressful conditions, including of anthropogenic origin (Elliott and Quintino, 2007). Albeit fish used in this study were collected at a natural reserve site, we cannot exclude prior exposure (and consequent conditioning) of fish to fluoxetine and other SSRIs, which have been identified in the area, although at very low concentrations (< 10 ng/L, Reis-Santos et al., 2018). Furthermore, the majority of previous studies were performed in freshwater fish species and laboratory reared individuals (e.g. zebrafish, minnow, goldfish), thus different responses to fluoxetine toxicity could be linked to interspecies evolutionary differences. Brown et al. (2014) highlighted differential susceptibility of fish to pharmaceuticals, based on evolutionary divergence in species drug-target activation, physiology, behaviour and ecology.

Given fluoxetine's mode of action, behavioural changes have been associated with modulation of the serotonergic system. The strong correlations between *P. microps* AChE activity and fish movement delay (negative correlation) and active time (positive correlation) further suggests that changes in fish activity could also be linked to alterations in the cholinergic system. This hypothesis has also been suggested by other authors as a possible route of behaviour modulation (e.g. Farias et al., 2019; Winder et al., 2012). Therefore, AChE activity could be a suitable biomarker of fluoxetine toxicity in behavioural studies, yet links between serotonergic and cholinergic pathways and fish behaviour need to be further resolved. A metabolomics approach could provide valuable insights into metabolic pathways and interactions between these two systems and behaviour responses following exposure to SSRIs.

In conclusion, acute exposure to fluoxetine induced hepatic biotransformation enzymes in *P. microps*, yet no significant oxidative stress responses were observed. Behavioural and neurotoxic effects were only observed at higher concentrations (mg/L). Nonetheless, further insights into the variability of inter-specific responses, as well as into chronic exposure effects at environmental relevant concentrations in non-model species, are needed to improve environmental risk assessment of fluoxetine and other SSRIs.

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