# Environmental concentration of fluoxetine disturbs larvae behavior and increased defense response at molecular level in zebrafish (*Danio rerio*)

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## ABSTRACT

Fluoxetine (FLX) is one of the main antidepressants used worldwide. After human use FLX enters the aquatic ecosystems, where it has commonly detected in the high ng/L concentration range. Several investigations have shown that exposure to different concentrations of FLX caused different adverse effects towards a number of aquatic species. However, the information on the onset and the relationship between molecular and behavioral FLX-induced effects remains scant. The aim of this study was to assess the effects induced by two FLX concentrations, namely 50 ng/L and 500 ng/L, on swimming activity of zebrafish (*Danio rerio*) larvae at 96 hours post fertilization (hpf) and to investigate if such behavioral effects were related to modulation of the expression of oxidative stress-related (*sod1, sod2, cat, gpxa* and *gst*), stress and anxiety-related (*oxtl, prl2, npy* and *ucn3l*) genes, and genes encoding for the transporters of the main neurotransmitters (*slc6a3, slc6a4a, slc6a4b, slc6a11*). Fluoxetine exposure altered the swimming behavior of larvae, as shown by the reduction of the distance traveled by treated larvae in response to an external stimulus. Such behavioral change was related, at molecular level, to an enhanced expression of *sod1*, *cat* and *gpxa*, suggesting an overproduction of pro-oxidant molecules. In addition, FLX modulated the expression of *oxtl*, *slc6a4a*, *slc6a4b* and *slc6a11,* suggesting its capability to affect anxiety- and neurotransmitter-related genes.



**Keywords:** Antidepressant, gene expression, behavior, zebrafish larvae

# 1. INTRODUCTION

The release of human pharmaceuticals and personal care products (PPCPs) into the aquatic ecosystems persists as a serious environmental problem. Hundreds of human and veterinary therapeutics are excreted in their native form and/or as active metabolites and enter the aquatic environment, where they can affect the physiology of non-target organisms interacting with evolutionarily well conserved biological pathways (Abreu et al. 2015). Among the human pharmaceuticals, psychotropic drugs, including antidepressants, antipsychotics and anxiolytics, are commonly used worldwide (Bocquier et al. 2008). According to the increase in medical prescription in the last two decades (la Poza et al. 2013), a notable increase of the presence of psychotropic drugs in aquatic ecosystems was noted (Santos et al. 2010; Calisto et al. 2011; Al Aukidy et al. 2012). According to an increase of their use, the improper disposal of expired pharmaceutical, the limited biodegradability and the partial removal efficiency of traditional wastewater treatment plants (Palmer et al. 2008; Silva et al. 2011), whereby they are commonly detected in the high ng/L to low µg/L concentration range (Demeestere et al. 2010; Santos et al. 2010). In addition, since 30–90% of ingested drugs are excreted unaltered and released in aquatic environment in an active form (Kashiyama et al. 2010), antidepressants may potentially affect the health status of diverse non-target organisms. Selective serotonin reuptake inhibitors (SSRIs) are the first-line antidepressants prescribed to alleviate depression disorders in humans (Westenberg 2009). SSRIs, including fluoxetine, citalopram and sertraline, block the serotonin transporter inhibiting the serotonin re-uptake into the presynaptic membrane, causing an increase of serotonin levels in the synaptic cleft and, consequently, of neurostimulation in the postsynaptic receptor sites (Stahl 1998). Among SSRIs, fluoxetine (FLX) is one of the most prescribed antidepressants worldwide and is included in the top five psychiatric drugs prescribed in 2011, after citalopram and sertraline (Grohol 2011). Fluoxetine is the active principle of the therapeutic drug Prozac®, primarily prescribed for depression but also to treat compulsive behavior, social anxiety, panic and personality disorders (AHFS 2013). After human

consumption, because of its peculiar chemico-physical features (chemical formula: $C_{17}H_{18}F_3NO$; molar mass = 309.33 g/mol; half-life (*t*1/2) = 2-6 hours, solubility in water = 14 mg/mL at 20 °C, log $K_{ow}$ = 3.93; $pK_a$ = 10.1; Brooks 2014), FLX is excreted and enter the aquatic ecosystems, where it has been detected in both wastewater influents and effluents at levels up to 0.93 µg/L and 0.54 µg/L, respectively (Metcalfe et al. 2010; Styrishave et al. 2011; Silva et al. 2012), while the estimated maximum concentrations in surface waters was 0.012 µg/L (Kolpin et al. 2002). Although the FLX concentrations in aquatic environments are low, aquatic non-target organisms are exposed for their whole life-span to variable concentrations of this antidepressant, which can lead to a series of side effects. Several studies have demonstrated that FLX exposure modulated the levels of serotonin and induced morphological, physiological, neuroendocrine, reproductive, motor and behavioral changes in both invertebrate and vertebrate aquatic species (e.g., Airhart et al. 2007; Park et al. 2012; Prieto et al. 2012; Fong and Ford 2014; Abreu et al. 2014; 2016). In spite of these findings, the information regarding FLX molecular mechanisms of action and behavioral consequences on aquatic organism is scant. Fluoxetine exposure inhibited the serotonin transporter (Pinna et al. 2004), regulated the production of neurosteroids acting as modulators of GABA receptors (Pinna et al. 2004; 2006; Longone et al. 2011) and altered the expression of diverse neuropeptides, including neuropeptide Y, oxytocin and arginine vasopressin, which independently affect both stress and anxiety levels (Landgraf 2005). In addition, FLX induced anxiolytic effects in rodents and fish (Dulawa et al. 2004; Egan et al. 2009). Although zebrafish larvae treated with FLX showed changes in molecular pathways related to stress response (Park et al. 2012) and a study employing adult zebrafish has shown that FLX exposure modulated the expression of genes associated with stress and anxiety, reducing anxiety-related behaviors (Wong et al. 2013), the information regarding the molecular and behavioral consequences caused by FLX exposure during early development in fish is scant. Thus, the aim of the present study was to assess the effects caused by the exposure to two FLX concentrations (50 ng/L and 500 ng/L) on the expression of genes related to oxidative stress response (*sod1*, *sod2*, *cat*, *gpxa* and *gst*), stress and anxiety (*oxtl*, *prl2, npy* and *ucn3l*), and to the transporters of the main

neurotransmitters (*slc6a3*, *slc6a4a*, *slc6a4b*, *slc6a11*) on zebrafish (*Danio rerio*) larvae at 96 hours post fertilization (hpf). Moreover, as the deregulation of at least one of the main neurotransmitters is associated with anxiety and anxiety-related behaviors (Durant et al. 2010; Cryan and Sweeney 2011), we investigated if changes in the expression of genes encoding for neurotransmitter transporters might alter the swimming behavior of larvae through a touch-evoked response assay.

## 2. MATERIALS AND METHODS

### *2.1 Selection of Fluoxetine concentrations*

Fluoxetine (FLX) standard (Fluoxetine hydrochloride solution; CAS number 59333-67-4) was purchased from Sigma-Aldrich (Steinheim, Germany). Fluoxetine standard (1 g/L in methanol) was diluted in ultrapure water to obtain a 1 mg/L stock solution used for experiments. The concentration of FLX in the stock solution was checked in a previous experiment by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) and was 1.01 ± 0.02 mg/L (Magni et al., 2017). Then, we prepared two working solutions (5 µg/L and 500 µg/L) by diluting the stock solution. Each working solution was then diluted in an appropriate volume of fish water into 12-multiwell plates to expose zebrafish embryos and, after hatching, larvae to selected FLX concentrations. Fertilized eggs were exposed to 50 ng/L and 500 ng/L of FLX and the exposure lasted up to 96 hpf stage of larvae. The lowest tested concentration was similar to FLX concentration found in surface waters worldwide (e.g. Santos et al. 2010; Fong and Ford 2014), while the highest one was similar to that used in a previous experiment investigating the adverse effects of FLX on the freshwater bivalve *Dreissena polymorpha* (Magni et al. 2017). Because of the limited volume of exposure medium (3 mL) and the low tested concentrations, we could not measure the FLX levels in the exposure wells. However, since no degradation of FLX in the stock solution was noted for 14 days (Magni et al. 2017), we are confident that the daily renewal of the exposure medium applied in this study provides constant exposure concentration over a 24-h period.

*2.2 Experimental procedure*

Breeding wild-type zebrafish was maintained into a thermostatic chamber (pH 7.5; 28 °C on a 14-hr light/10-hr dark cycle) placed in the zebrafish facility of the University of Milan, which strictly adheres to the relevant Italian laws, rules and regulations (D.to L.vo 116/92; Autorizzazione Comunale of the city of Milan - D.Lvo 27.1.1992 n° 116, art.10). Breeding pairs were chosen within a number of adults that were born and grown in the zebrafish facility. Fertilized eggs were collected by natural spawning and sampled from different male and female pairs. After fertilization, the eggs were all mixed, seeded and raised at 28 °C into 12-well plates filled with 3 mL of fish water (0.1 g/L Instant Ocean, 0.1% methylene blue). One-cell embryos were immediately exposed to selected FLX concentrations under semi-static conditions up to 96 hpf of the larvae. The chorion membrane was not removed and larvae hatched naturally. Ten fertilized eggs were seeded in each well. The experimental design followed the scheme reported by Taylor et al. (2010). According to Udvardi et al. (2008), three independent biological replicates of each treatment were performed within a 12-well plate. The experiments were replicated twice (two independent plates for a total of 6 independent biological replicates per experimental group). Thirty µL of the prepared working solutions were added to each exposure well (3 mL final volume), while the same volume of fish water alone was added to control wells. Although FLX standard solution was in methanol, we did not perform a solvent control group because the amount of methanol in the exposure wells was absolutely negligible. The average of exposure was renewed every single day up to 96 hpf. Water parameters, embryo (up to 72 hpf) and larvae viability were checked daily. No variation in pH and temperature of the average of exposure was found over the exposure period (pH 7.5; T = 28 °C). A low mortality was noticed during the exposures in all the independent experiments (see *Results* section), and died embryos/larvae were immediately removed from the wells. Experiments were performed by using two different batches of larvae, one for the first plate and one for the second one. A third batch was used to behavioral analyses. As quality control for each batch of larvae, we simultaneously monitored

the survival and the development of some other larvae from each batch into a Petri dish maintained at the same conditions described above. As the larvae survival was always higher than 80%, the batches we used were considered as reliable for experimental testing. At 96 hpf, larvae were collected, washed twice with PBS to remove possible residuals of FLX, euthanized by freezing and stored at –80 °C up to molecular analyses.

*2.3 Touch-evoked response assay*

Changes in swimming behavior induced by FLX on zebrafish larvae were assessed by a touch-evoked response assay (Sztal et al. 2016). The touch-evoked response assay is a standardized method to assess swimming performance and skeletal muscle function in zebrafish larvae (Sztal et al., 2016). We used larvae from a different batch with respect to those used for gene expression analyses. Ten fertilized eggs were seeded into a 12-multiwell plate (three replicates per experimental group, including control, for a total of 30 larvae per treatment) and exposed to the selected FLX concentration up to 96 hpf at the condition described above. At the end of the exposure, each larvae was transferred singularly to a Petri dish (Ø = 4 cm) filled with exposure medium. Petri dish was placed under an illuminated stage. At 25 cm height, an IPhone7 was mounted and used to record the swimming activity of larvae after solicitation. A single larvae was placed in the middle of the Petri dish and left 1 min for acclimation. Then, a mechano-sensory stimulus was delivered by gently touching it with a blunt needle on the top of the head. We recorded the movement of each larvae until it stopped. The stimulus was delivered three times, 30 seconds last between the stimuli, to check for consistency of the response. The three 1080p FullHD videos acquired for each specimens were analyzed by using MTrackJ plugin working on the freeware ImageJ software, returning the mean distance travelled by each larvae after solicitation.

*2.4 RNA extraction and quantitative RT-qPCR*

Total RNA was extracted from pools of all the alive larvae at 96 hpf (9-10 per each biological replicate; three replicates per plate, two plates, for a total of six biological replicates per treatment)

using SV Total RNA Isolation System (Promega, Madison, Wisconsin) according to the manufacturer's procedure. Residues of genomic DNA were eliminated by incubating samples with DNasel. First-strand cDNAs were synthesized with the ImProm-II Reverse Transcription System (Promega), using random oligonucleotides to prime the reverse transcription of 1 μg of total RNA. Quantitative RT-qPCR was performed to investigate variations of genes involved in oxidative stress response (*sod1*, *sod2*, *cat*, *gpx1a* and *gst*), stress and anxiety (*oxtl*, *npy*, *prl2* and *ucn3l*), as well as in neurotransmitter transport (*slc6a3*, *slc6a4a*, *slc6a4b*, *slc6a11*). Specific primers were used to evaluate gene expression level, while the amount of 18S ribosomal RNA was tested in all the samples for normalization purposes. Sequences of primers of genes involved in stress and anxiety and neurotransmitter transporters are reported in Wong et al. (2013), while sequences of primers of genes involved in antioxidant responses are reported in Parolini et al. (2017). The sequences of all the primers used in the present study are reported in Supplementary materials (Table S2). Efficiency and specificity of all the primers were checked before the analyses. Reactions were performed in a 96-well format iQ5TM Multicolor Real-Time PCR Detection System (Biorad) using the iQTM SYBR® Green Supermix (Bio-rad). Three independent RT-qPCR experiments from the same reverse transcribed sample were performed for each pair of gene-specific primers. We verified the presence of a single PCR product by melting-curve and agarose gel analyses.

*2.5 Statistical analysis*

Linear Mixed Models (LMM) were applied to analyze the effect of FLX treatments on gene expression and behavior (touch evoked response). The FLX concentrations were included in the models as a fixed effect factor, while the plate of exposure as a random effect in order to account for experimental or zebrafish larvae' batch sources of variation. Fisher' LSD *post-hoc* test was used to assess significant differences (* $p < 0.05$; ** $p < 0.01$) between treated and control groups. The likelihood ratio test, comparing the log-likelihood value of the model including or, respectively, excluding the random effect of the exposure plate identity, was applied to check for the effect of the

exposure plate. All the statistical analyses were performed by IBM SPSS Statistic 21 software package.

## 3. RESULTS AND DISCUSSION

Our results showed that the exposure to low concentrations of FLX altered the movement of zebrafish larvae and modulated the expression of genes related to oxidative stress responses, anxiety and neurotransmission.

*3.1 Fluoxetine-induced effects on larvae swimming behavior*

The mean survival of zebrafish larvae at 96 hpf exposed to 50 ng/L and 500 ng/L of FLX was 85 ± 7 % and 94 ± 14 %, respectively, and was similar to the value of the control group (94 ± 7%). All the larvae were at the same developmental stage as confirmed by a preliminary microscopy analysis before their euthanasia. A significant decrease of the larvae swimming behavior, in terms of distance travelled after receiving an external stimulus, was found ($F = 10.257$; $p < 0.001$; Figure 1). Larvae treated with both the FLX tested concentrations travelled a shorter distance compared to the control group, accounting a decrease of 30% at 50 ng/L and more than halved at 500 ng/L of exposure. Diverse studies have demonstrated that swimming behavior in both larvae and adult zebrafish induced by FLX exposure could be related to perturbation of the main neurotransmitters, whose expression was altered by this antidepressant (e.g., Airhart et al. 2007; Njagi et al. 2010; Wong et al. 2013). For instance, short-term exposure to high FLX concentration (4.6 μM) increased extracellular serotonin levels in zebrafish larvae and larvae resulting in a brief episode of hyper-swimming activity followed by a sustained decrease in spontaneous swimming activity that lasted up to 14 days (Airhart et al. 2007). Moreover, adult zebrafish chronically treated with FLX showed a decreased horizontal activity in term of reduced distance traveled in the open-field test. Similarly, decreased horizontal activity was also found in juveniles of the Chinook salmon (Clements and Schreck 2007) and in the sheepshead minnow (Winder et al. 2012) after FLX exposure. As an alternative mechanism of action,

FLX might increase the production of neurosteroids (e.g., allopregnanolone) as previously showed in mammals (e.g., Pinna et al. 2006). According to previous literature findings, we might suppose that swimming alterations of zebrafish larvae induced by FLX exposure should be cause by changes of both serotonin and neurosteroid levels. In fact, neuropeptides have modulatory roles in a variety of behaviors, including stress and anxiety. This hypothesis was supported by the results of a previous work of RNA-sequencing performed by Wong et al. (2013), which has demonstrated that diverse neuropeptides associated with stress and anxiety were regulated by FLX exposure. Thus, the significant reduction in distance travelled by FLX-treated larvae compared to controls might be related to the modulation of the neuropeptide and neurotransmitter transporter gene expression, suggesting an anxiolytic effect of FLX.

*3.2 Modulation of anxiety-related and neurotransmitter transporter gene expression*

The expression of anxiety-related behavior genes is shown in Figure 2. Despite a statistically ($F = 11.599$; $p = 0.004$) over expression of *oxtl* gene at both the tested FLX concentrations, no significant modulation of *prl2* and *ucn3l* genes was found ($F < 1.270$; $p > 0.311$ for both the genes). Fluoxetine is mainly designed to exert an anxiolytic effect, as demonstrated in previous studies of murine models (Lillesaar 2011) and fish (Gould et al. 2007; Jacobson and Ctyan 2010). Fluoxetine exerts such effect by modulating neuropeptides, neurosteroids, and principally serotonin and GABA transporters (Griebel et al. 2000). Fluoxetine exposure significantly overexpressed the gene *oxtl* encoding for the isotocin. Although isotocin is homologous to the mammalian oxytocin, which affects diverse social behaviors in humans having anxiolytic effects (e.g., Meyer-Lindenberg et al. 2011), the role of isotocin in behavioral functions in fish needs to be elucidated (Godwin and Thompson 2012). A study of wild-type adult zebrafish has shown that a peripheral injection of isotocin induced a dose-dependent decrease of the level of fear in response to a predator (Braida et al. 2012), suggesting an anxiolytic effect of this neuropeptide hormone. Thus, as the FLX-induced over expression of *oxtl* implies a potential increase in isotocin levels, our data suggest an anxiolytic effect of this drug on

larvae. In murine models, studies of pharmacological manipulations, gene expression, and transgenic animals have demonstrated that also the increase of NPY levels is associated with a reduction of anxiety (Thorsell 2008; Missing et al. 2010; Trent and Menard 2011; Onaka et al. 2012). In contrast to results on zebrafish adults (Wong et al., 2013), FLX treatment did not modulate the expression of the *npy* gene, suggesting an anxiolytic effect of this drug. Anxiety-related behavior are often associated with a physiological stress response, which in teleosts primarily induces neuroendocrine activation, including the release of catecholamines from chromaffin tissue, and the stimulation of the hypothalamic-pituitary-interrenal axis culminating in the release of corticosteroid hormones into circulation (e.g., Barton 2002). At the molecular level, changes in the expression of urocortin 3 (*ucn3l*) and prolactin (*prl2*) have been related to the onset of stress and anxiety situations. The lack of over expression of both *ucn3l* and *prl2* stress markers might suggest that larvae did not experience stress and anxiety, and FLX acts to prevent the onset of these adverse situations. Lastly, many pharmacological studies have shown that anxiety and anxiety-related behaviors are associated with the deregulation of at least one of the main neurotransmitter systems in the brain, namely serotonergic, GABAergic, catecholaminergic and glutamatergic systems (Durant et al. 2010; Cryan and Sweeney 2011). Although the binding of FLX with neurotransmitter transporters is well known (Sghendo and Mifsud 2011), studies investigating the modulation of transporter gene expression have returned contrasting results. In fact, whilst some studies on fish and murine models showed changes in the expression of serotonin transporters genes following chronic FLX administration (Mennigen et al. 2008; Lee et al. 2010; Benton et al. 2012; Huang et al. 2012; Park et al. 2012), others did not show significant modulations of these genes (Airhart et al. 2007; Wong et al. 2013). Changes in the expression of genes related to transporters of the main neurotransmitters (*slc6a3*, *slc6a4a*, *slc6a4b*, *slc6a11*) are reported in Figure 3. Our results showed that FLX treatments significantly modulated the expression of both orthologues genes encoding for the serotonin transporters (*slc6a4a* - $F = 20.195$; $p < 0.001$ and *slc6a4b* - $F = 11.734$; $p < 0.001$) and GABA transporters (*slc6a11* - $F = 13.016$; $p = 0.001$), pointing out their significant overexpression at both tested concentrations. In contrast, as

expected, FLX did not statistically modulated the expression of the dopamine transporter *slc6a3* ($F = 3.012$; $p > 0.05$). We may speculate that short-term exposure to low FLX concentrations can increase extracellular neurotransmitters levels and stimulate the organism to synthetize new transporters according to feedback mechanisms. In addition, a previous study of mammals showed that a change in the expression of *slc6a4* was preceded by changes in *oxtl* encoding for the receptor of oxytocin (Parker et al. 2014), facilitating serotonin release (Yoshida et al. 2009). Thus, since FLX exposure increases the expression of *oxtl* and consequently the synthesis of isotocin, we can speculate that the increase of extracellular serotonin levels may be due not exclusively to the exposure to FLX but also to the isotocin increase. This hypothesis should explain the over expression of serotonin transporter *slc6a4a* and *slc6a4b*, which might be due to a homeostatic upregulation of these genes due to the great amount of extracellular serotonin. As FLX also modulated the expression of GABA receptors genes in mouse (Pinna et al. 2004; 2006; Longone et al. 2011) and reduced the activity of *slc6a11* in adult zebrafish, we can suppose that the over expression of *slc6a11* we found in larvae might be due to similar mechanisms of homeostatic adjustment we hypothesized for serotonin transporters. Although we did not measure serotonin and GABA concentrations before and after FLX treatment to support our hypothesis, a number of studies have shown that perturbations in serotonin levels can alter the RNA messages of serotonin receptor subtypes and serotonin transporter protein (Airhart et al. 2007). Considering these results, the inhibitory effect of FLX on the synthesis of neurotransmitter transporters in larvae may be expected at higher concentrations and/or prolonged exposures. In fact, the human FLX therapeutic concentration ranges between 20 and 38 µg/L (Margiotta-Casaluci et al. 2014) and the inhibitory effects on neurotransmitter transporter expression in teleosts and rodents were noted only at higher concentrations compared to those tested in the present study (Mennigen et al. 2008; Lee et al. 2010; Benton et al. 2012; Huang et al. 2012; Park et al. 2012). In addition, since therapeutic effect of FLX appears and persists for some days after the administration, we may expect similar trend in zebrafish (i.e. later larval stage or adult fish). Despite of our findings, considering that the expression of anxiety-related genes did not completely agree with previous studies, further

investigations should be necessary to shed light on the molecular mechanisms of FLX towards early-life zebrafish stages and on the relationships between gene expression and the behavior of larvae.

*3.3 Modulation of antioxidant enzymes encoding genes*

Results of the expression of genes encoding for antioxidant (*sod1*, *cat*, and *gpxa*) and detoxifying (*gst*) enzymes are shown in Figure 4. Interestingly, a statistically significant effect of FLX treatment on *sod1* ($F = 14.024$; $p < 0.001$), *sod2* ($F = 5.141$; $p = 0.021$) and *cat* ($F = 10.534$; $p = 0.002$) was found. In contrast, the expression of *gpxa* was marginally non-significantly modulated by FLX treatment ($F = 3.596$; $p = 0.053$). Conversely, FLX did not affect *gst* expression statistically ($F = 0.07$; $p = 0.932$). An over expression of genes encoding antioxidant enzymes was noted, mainly at 500 ng/L. The significant increase of *sod1* expression suggested that both the FLX concentrations induced the production of superoxide anion, whose toxicity has to be counterbalanced by the activity of SOD enzyme. In contrast, *sod2* was over expressed only in response to the highest tested concentration. These findings agreed a previous study investigating the expression of antioxidant-related genes in zebrafish larvae after the exposure to cocaine and its main metabolites (Parolini et al. 2017). Our results suggest that the defense against superoxide anion during early developmental stages is mainly mediated by cytosolic SOD (encoded by *sod1*) and not by mitochondrial SOD (encoded by *sod2*), which is activated only at high concentrations. These findings were in accordance with previous *in-vitro* investigation performed on rat pheochromocytoma (PC12) cells showing that the administration of FLX lead to an over expression of *sod1* gene (Li et al. 2000). As SOD activity prevents superoxide anion toxicity by its reduction to hydrogen peroxide, as expected, *cat* was overexpressed at the end of the treatment to 500 ng/L of FLX, while *gpxa* showed a significant modulation of its expression at both the treatments. These results indicated that both enzymes contribute to the cellular defense against $H_2O_2$ produced by the activation of *sod1*. However, further investigations should elucidate the mechanisms by which different doses and exposure times of FLX initiate antioxidant response and the potential protective action of FLX against oxidative stress-related damage.

## 4. CONCLUSION

Our findings showed that the exposure to low FLX concentration altered the swimming activity of zebrafish larvae at 96 hpf and modulated the expression of genes related to antioxidant defense, stress and anxiety and neurotransmitter transporters. In further studies it should be interesting to in-depth investigate the relationships occurring between behavioral alteration and modulation of gene expression in order to shed light on the molecular mechanism(s) of action of FLX in zebrafish and other non-target aquatic organisms. Moreover, as FLX persists in the organisms for a long time negative consequences might occur also after 96 hpfin later developmental stages, whichneed to be investigated in more in-depth studies. Moreover, the continuative and increasing use of FLX may lead to a consequent increase of its concentrations in aquatic ecosystems, conferring to this molecule a sort of pseudo-persistence that, coupled with its high stability in water, can result in long-lasting exposures to higher concentrations and by extension to more hazardous consequences. Coupling our data with those from previous studies on FLX, the exposure to this antidepressant, also at environmentally relevant concentrations, may cause detrimental consequences at individual and population levels, affecting swimming activity, feeding, predation and reproductive associated behaviors. For this reason, studies on the toxicity of FLX on aquatic organisms should have to be a priority for a correct environmental risk assessment of this antidepressant.

## 5. REFERENCES


Abreu MS, Giacomini ACV, Gusso D, Rosa JGS, Koakoski G, Kalichak F, Idalêncio R, Oliveira TA, Barcellos HHA, Bonan CD, Barcellos LJG (2016) Acute exposure to waterborne psychoactive drugs attract zebrafish. Comp Biochem Physiol C Toxicol Pharmacol 179:37-43

Abreu MS, Giacomini ACV, Koakoski G, Oliveira TA, Gusso D, Baldisserotto B, Barcellos LJG (2015) Effects of waterborne fluoxetine on stress response and osmoregulation in zebrafish Environ Toxicol Pharmacol 40:704-707

Abreu MS, Koakoski G, Ferreira D, Oliveira TA, Rosa JGS, Gusso D, Giacomini ACV, Piato AL, Barcellos LJG (2014) Diazepam and fluoxetine decrease the stress response in zebrafish. PLoS One 9 doi:10.1371/journal.pone.0103232

AHFS, 2013. AHFS Di Monographs. Drugscom http://www.drugs.com/monograph

Airhart MJ, Lee DH, Wilson TD, Miller BE, Miller MN, Skalko RG (2007) Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC) Neurotoxicol Teratol 29:652–664

Al Aukidy M, Verlicchi P, Jelic A, Petrovic M, Barcelo D (2012) Monitoring the release of pharmaceutical compounds: occurrence and environmental risk assessment of two WWTP effluents and their receiving bodies in the Po Valley, Italy. Sci Total Environ 438:15–25

Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integ and Comp Biol 42:517-525

Benton CS, Miller BH, Skwerer S, Suzuki O, Schultz LE, Cameron MD, Marron JS, Pletcher MT, Wiltshire T (2012) Evaluating genetic markers and neurobiochemical analytes for fluoxetine response using a panel of mouse inbred strains. Psychopharmacology 221:297–315

Bocquier A, Bezzou K, Nauleau S, Verger P (2008) Dispensing of anxiolytics and hypnotics in southeastern France: demographic factors and determinants of geographic variations. Fundam Clin Pharmacol 22:323–333

Braida D, Donzelli A, Martucci R, Capurro V, Busnelli M, Chini B, Sala M (2012) Neurohypophyseal hormones manipulation modulate social and anxiety related behavior in zebrafish. Psychopharmacology 220:319–330

Brooks BW (2014) Fish on Prozac (and Zoloft): Ten years later. Aquatic Toxicology 151:61-67

Calisto V, Domingues MRM, Esteves VI (2011) Photodegradation of psychiatric pharmaceuticals in aquatic environments — kinetics and photodegradation products. Water Res 45:6097–6106

Clements S, Schreck CB (2007) Chronic administration of fluoxetine alters locomotor behavior, but does not potentiate the locomotor stimulating effects of CRH in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Comp Biochem Physiol A Mol Integr Physiol 147:43-49

Cryan JF, Sweeney FF (2011) The age of anxiety: role of animal models of anxiolytic action in drug discovery. Br J Pharmacol 164:1129–1161

Demeestere K, Petrović M, Gros M, Dewulf J, Van Langenhove H, Barceló D (2010) Trace analysis of antidepressants in environmental waters by molecularly imprinted polymer-based solid-phase extraction followed by ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry. Anal Bioanal Chem 396:825-837

Dulawa SC, Holick KA, Gundersen B, Hen R (2004). Effects of Chronic Fluoxetine in Animal Models of Anxiety and Depression. Neuropsychopharmacology 29:1321–1330

Durant C, Christmas D, Nutt D (2010) The pharmacology of anxiety. In: Stein M, Steckler T (ed) Behavioral Neurobiology of Anxiety and Its Treatment. Curr Top Behav Neurosci, vol 2. Springer, Berlin, Heidelberg, pp.303–330

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV (2009) Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 205:38–44

Fong PP, Ford AT (2014) The biological effects of antidepressants on the molluscs and crustaceans: A review. Aquat Toxicol 151:4-13

Godwin J, Thompson R (2012) Nonapeptides and social behavior in fishes. Horm Behav 61:230–238

Gould GG, Brooks BW, Frazer A (2007) [$^3$H] citalopram binding to serotonin transporter sites in minnow brains. Basic Clin Pharmacol Toxicol 101:203–210

Griebel G, Belzung C, Perrault G, Sanger DJ (2000) Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology 148:164–170

Grohol J (2012) Top 25 Psychiatric Medication Prescriptions for 2011. Psych Central http://psychcentral.com/lib/top-25-psychiatric-medication-prescriptions-for2011

Huang GJ, Ben-David E, Tort Piella A, Edwards A, Flint J, Shifman S (2012) Neurogenomic evidence for a shared mechanism of the antidepressant effects of exercise and chronic fluoxetine in mice. PLoS One, 7:e35901, doi: 10.1371/journal.pone.0035901

Jacobson LH, Cryan JF (2010) Genetic approaches to modeling anxiety in animals. In: Stein M, Steckler T (ed), Behavioral Neurobiology of Anxiety and Its Treatment. Curr Top Behav Neurosci, vol 2. Springer, Berlin, Heidelberg, pp.161–201

Kashiyama K, Ito C, Numata H, Goto SG (2010) Spectral sensitivity of light induced hatching and expression of genes mediating photoreception in eggs of the Asian tadpole shrimp *Triops granarius*. Comp Biochem Physiol A: Mol Integr Physiol 156:416–421

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LD, Buxton HT (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: a national reconnaissance. Environ Sci Tech 36:1202-1211

la Poza E, Guadalajara N, Jódar L, Merello P (2013) Modeling Spanish anxiolytic consumption: economic, demographic and behavioral influences. Math Comput Model 57:1619–1624

Landgraf R (2005) Neuropeptides in anxiety modulation, in: Holsboer F, Ströhle A (ed) Anxiety and Anxiolytic Drugs. Handbook of Experimental Pharmacology, vol 169. Springer, Berlin, Heidelberg, pp.335–369

Lee JH, Ko E, Kim YE, Min JY, Liu J, Kim Y, Shin M, Hong M, Bae H (2010) Gene expression profile analysis of genes in rat hippocampus from antidepressant treated rats using DNA microarray. BMC Neurosci 11:152

Li XM, Chlan-Fourney J, Juorio AV, Bennett VL, Shrikhande S, Bowen RC (2000) Antidepressants upregulate messenger RNA levels of the neuroprotective enzyme superoxide dismutase (SOD1). J Psychiatry Neurosci 25:43-47

Lillesaar C (2011) The serotonergic system in fish. J Chem Neuroanat 4:294–308

Longone P, Di Michele F, D'Agati E, Romeo E, Pasini A, Rupprecht R (2011) Neurosteroids as neuromodulators in the treatment of anxiety disorders. Front Endocrinol 2:55

Magni S, Parolini M, Della Torre C, de Oliveira LF, Catani M, Guzzinati R, Cavazzini A, Binelli A (2017) Multi-biomarker investigation to assess toxicity induced by two antidepressants on *Dreissena polymorpha*. Sci Tot Env 578:452-459

Margiotta-Casaluci M, Owen SF, Cumming RI, de Polo A, Winter MJ, Panter GH, Rand-Weaver M, Sumpter JP (2014) Quantitative cross-species extrapolation between humans and fish: the case of the anti-depressant fluoxetine PLoS One doi: 10.1371/journal.pone.0110467

Mennigen JA, Martyniuk CJ, Crump K, Xiong H, Zhao E, Popesku J, Anisman H, Cossins AR, Xia X, Trudeau VL (2008) Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). Physiol Genomics 35:273–282

Metcalfe CD, Chu S, Judt C, Li H, Oakes KD, Servos MR, Andrews DM (2010) Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ Toxicol Chem 29:79–89

Meyer-Lindenberg A, Domes G, Kirsch P, Heinrichs M (2011) Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. Nat Rev Neurosci 12:524–538

Njagi, J., Ball, M., Best, M., Wallace, K. N., Andreescu, S., 2010. Electrochemical Quantification of Serotonin in the Live Embryonic Zebrafish Intestine. Anal. Chem. 82, 1822-1830

Onaka T, Takayanagi Y, Yoshida M (2012) Roles of oxytocin neurones in the control of stress, energy metabolism, and social behaviour. J Neuroendocrinol 24:587–598

Palmer PM, Wilson LR, O'Keefe P, Sheridan R, King T, Chen CY (2008) Sources of pharmaceutical pollution in the New York City watershed. Sci Total Environ 394:90–102

Park JW, Heah TP, Gouffon JS, Henry TB, Sayler GS (2012) Global gene expression in larval zebrafish (*Danio rerio*) exposed to selective serotonin reuptake inhibitors (fluoxetine and sertraline) reveals unique expression profiles and potential biomarkers of exposure. Environ Pollut 167:163–170

Parker MO, Annan LV, Kanellopoulos AH, Brock AJ, Combe FJ, Baiamonte M, Teh MT, Brennan CH (2014) The utility of zebrafish to study the mechanisms by which ethanol affects social behavior and anxiety during early brain development. Prog Neuropsychopharmacol Biol Psychiatry 55:94-100

Parolini M, Ghilardi A, Della Torre C, Magni S, Prosperi L, Calvagno M, Del Giacco L, Binelli A (2017) Environmental concentrations of cocaine and its main metabolites modulated antioxidant response and caused cyto-genotoxic effects in zebrafish embryo cells. Env Poll 226:504-514

Pinna G, Costa E, Guidotti A (2006) Fluoxetine and norfluoxetine stereospecifically and selectively increase brain neurosteroid content at doses that are inactive on 5-HT reuptake. Psychopharmacology 186:362–372

Pinna G, Costa E, Guidotti A (2004) Fluoxetine and norfluoxetine stereospecifically facilitate pentobarbital sedation by increasing neurosteroids. Proc Natl Acad Sci USA 101:6222–6225

Prieto MJ, Gutierrez HC, Arévalo RA, Chiaramoni NS, del Valle Alonso S (2012) Effect of risperidone and fluoxetine on the movement and neurochemical changes of zebrafish. Open J Med Chem 2:129

Sackerman J, Donegan JJ, Cunningham CS, Nguyen NN, Lawless K, Long A, Benno RH, Gould GG (2010) Zebrafish behavior in novel environments: effects of acute exposure to anxiolytic compounds and choice of *Danio rerio* line. Int J Comp Psychol 23:43

Santos LHMLM, Araujo AN, Fachini A, Pena A, Delerue-Matos C, Montenegro MCBSM (2010) Ecotoxicological aspects related to the presence of pharma-ceuticals in the aquatic environment. J Hazard Mater 175:45–95

Sghendo L, Mifsud J (2012) Understanding the molecular pharmacology of the serotonergic system: using fluoxetine as a model. J Pharm Pharmaco. 64:317-25

Silva BF, Jelic A, López-Serna R, Mozeto AA, Petrovic M, Barceló D (2011) Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. Chemosphere 85:1331–1339

Silva LJG, Lino CM, Meisel LM, Pena A (2012) Selective serotonin re-uptake inhibitors (SSRIs) in the aquatic environment: An ecopharmacovigilance approach. Sci Tot Env 437:185-195

Stahl SM (1998) Mechnism of action of serotonin selective reuptake inhibitors: serotonin receptors and pathways mediate therapeutic effects and side effects. J Affect Disord 51:215–235

Styrishave B, Halling-Sørensen B, Ingerslev F (2011) Environmental risk assessment of three selective serotonin reuptake inhibitors in the aquatic environment: a case study including a cocktail scenario. Environ Toxicol Chem 30:254–261

Sztal TE, Ruparelia AA, Williams C, Bryson-Richardson RJ (2016) Using Touch-evoked Response and Locomotion Assays to Assess Muscle Performance and Function in Zebrafish. J Vis Exp 116

Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M (2010) A practical approach to RT-qPCR—Publishing data that conform to the MIQE Guidelines. Methods 50:S1-S5

Thorsell A (2008) Central neuropeptide Y in anxiety- and stress-related behavior and in ethanol intake. Ann N Y Acad Sci 1148:136–140

Trent NL, Menard JL (2011) Infusions of neuropeptide Y into the lateral septum reduce anxiety-related behaviors in the rat. Pharmacol Biochem Behav 99:580–590

Udvardi M, Czechowski T, Scheible WR (2008) Eleven golden rules of quantitative RT-PCR. Plant Cell 20:1736–1737.

Westenberg HG (2009) Recent advances in understanding and treating social anxiety disorder. CNS Spectr 14:24–33

Winder VL, Pennington PL, Hurd MW, Wirth EF (2012) Fluoxetine effects onsheepshead minnow (*Cyprinodon variegatus*) locomotor activity. J Environ Sci Health B 47:51–58

Wong RY, Oxendine SE, Godwin J (2013) Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. Genomics 14:348.

## Figure captions

**Figure 1**: effects on swimming activity of zebrafish larvae in terms of distance travelled after an external stimulus due to the exposure to fluoxetine (50 ng/L and 500 ng/L). Asterisks above histograms show significant differences between treated and control group (** $p$ <0.01).

**Figure 2**: gene expression level of neuropeptide Y (*npy*), isotocin (*oxtl*), prolactine (*prl2*) and urocortine 3 (*urt3l*) measured in zebrafish larvae at 96 hpf after the exposure to fluoxetine (50 ng/L and 500 ng/L; n = 6 replicates). Asterisks above histograms referred to significant differences between treated and control group (* $p$ <0.05; ** $p$ <0.01).

**Figure 3**: gene expression level of transporters of dopamine (*slc6a3*), serotonin (*slc6a4a* and *slc6a4b,* respectively) and GABA (*slc6a11*) measured in zebrafish larvae at 96 hpf after the exposure to fluoxetine (50 ng/L and 500 ng/L; n = 6 replicates). Asterisks above histograms referred to significant differences between treated and control group (* $p$ <0.05; ** $p$ <0.01).

**Figure 4:** gene expression level of superoxide dismutase1 (*sod1*), superoxide dismutase2 (*sod2*), catalase (*cat*) and glutathione peroxidase (*gpxa*) measured in zebrafish larvae at 96 hpf after the exposure to fluoxetine (50 ng/L and 500 ng/L; n = 6 replicates). Asterisks above histograms referred to significant differences between treated and control group (* $p$ <0.05; ** $p$ <0.01).