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Effects of antidepressant fluoxetine on behavior and gene expression of European sea bass juveniles.

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Resumo

A exposição de organismos aquáticos a vários contaminantes é atualmente visto como um problema. Os fármacos são considerados contaminantes emergentes e a sua presença tem sido documentada mundialmente, numa grande variedade de ecossistemas como rios, estuários e zonas costeiras. Estes compostos não são efecientemente eliminados em estações de tratamento de águas resíduais, chegando assim aos ecossistemas aquáticos. Entre esses compostos, o antidepressivo fluoxetina (FX) e o seu metabólito norfluoxetina são comumente detetados em diversos ecossistemas. A FX é usada no tratamento de várias doenças do foro psicológico (ex: depressões, ansiedade, distúrbio obcessivo-compulsivo e bulímia nervosa), nomeadamente em Portugal onde a prevalência de doenças mentais é elevada. A FX é um inibidor seletivo de recaptação de serotonina (ISRS) que atua ao nível do sistema serotoninérgico do sistema nervoso central, inibindo a recaptação da monoamina serotonina (5-HT) ao nível dos seus transportadores (SERT) da membrana pré-sináptica, aumentando assim a sua concentração nas fendas sinápticas, levando a um aumento da neurotransmissão serotonérgica. Este sistema de neurotransmissão é bastante conservado a nível evolutivo e portanto é expectável que a FX possa causar efeitos em organismos aquáticos expostos. Assim, o estudo tem como objetivo avaliar os efeitos da FX em robalos (*Dicentrarchus labrax*), tanto a nível comportamental como de expressão génica. Os juvenis desta espécie ocupam potenciais habitats contaminados como estuários e portanto estão sujeitos a exposição. Uma vez que o primeiro ano de vida é crucial para as espécies em termos de sobrevivência, é importante avaliar as implicações da FX em juvenis de robalo. Para isso foi realizado um ensaio de 28 dias: 21 dias de exposição a concentrações de 0.5, 5 e 50 µg/ L de FX; mais 7 dias de recuperação. Comportamentos considerados relevantes para a espécie como formação de cardume, alimentação, velocidade de natação e ansiedade foram avaliados ao longo do ensaio. A alimentação foi avaliada através: i) do comportamento alimentar; e ii) *food intake*. A velocidade de natação foi avaliada e calculada recorrendo a um dispositivo de velocidade, registando-se o tempo que cada

indivíduo demorou a percorrer 3m do dispositivo. A ansiedade foi avaliada a partir de: i) posição no aquário; e ii) preferência pela zona escura/ clara (*Scototaxis*) de um dado aquário. O índice hepatossomático (IHS) foi também calculado. A expressão de três genes alvo do cérebro (*sert*; *5-ht$_{3B}$* e *mao*) foi avaliada através do processo *real-time quantitative* PCR, a partir de amostras de cérebro recolhidas ao 21º e 28º dia do ensaio. Os resultados mostram que não houve efeito da FX na formação de cardume e na alimentação. O número de indivíduos que nadou foi bastante reduzido e portanto não foi possível calcular a velocidade de natação, contudo foi observada resistência ao fluxo de água o que poderá indicar que os robalos não tinham condição física para nadar. A ansiedade verificada a partir da posição no aquário não parece ter sido afetada pela FX, no entanto foram observados decréscimos dos níveis de ansiedade por *scototaxis* em todos os grupos expostos o que poderá indiciar efeitos ansiolíticos de todas as concentrações de FX. Alterações na expressão do gene *sert* foram observadas no grupo exposto a 50 µg/ L de FX, contudo a expressão dos genes *5-ht$_{3B}$* e *mao* não foi alterada ao longo do ensaio. Em conclusão, este estudo demonstrou que a FX pode efetivamente ter efeitos tanto a nível comportamental como na expressão génica, e portanto tornasse importante verificar até que ponto estas alterações podem por em causa a espécie e o seu papel nos ecossistemas que ocupam.

Palavras-chave

Antidepressivo; fluoxetina; robalo; comportamento; expressão génica.

Abstract

The exposure of aquatic organisms to several contaminants is a problem and a concern to society. Pharmaceuticals are considered emergent contaminants and their presence is well documented worldwide, in a wide variety of ecosystems such as rivers, estuaries and coastal areas. Their removal in wastewater treatment plants is not fully efficient, thus this compounds can reach aquatic ecosystems. Fluoxetine (FX) and their metabolite norfluoxetine are antidepressants commonly detected in several ecosystems. They are used in the treatment of several mental psychiatric disorders (e.g. depressions, anxiety, obsessive compulsive disorders and nervous bulimic), namely in Portugal where the prevalence of mental diseases is high. FX is a selective serotonin reuptake inhibitor (SSRI) that operates in serotonergic system of central nervous system, inhibiting the reuptake of serotonin (5-HT) by their transporters (SERT) located in pre-synaptic membrane, leading to an increase of the concentration of this monoamine in the synaptic cleft, consequently increasing serotonergic neurotransmission. This neurotransmission system is well conserved during the evolution so it is expected that FX can exert effects on aquatic organisms that are exposed. This study aims to evaluate the effects of antidepressant FX in European sea bass (*Dicentrarchus labrax*) behavior and also at the expression of associated specific genes in brain (*sert*; *5-ht*$_{3B}$ e *mao*). Juveniles of this species tend to occupy potential contaminated habitats such as estuaries and therefore are subject to exposure. Since the first year is the most important in terms of survival, it is important to assess the implications that FX might have on the species early life. For that purpose a 28 days assay was conducted: i) 21 days of exposure to concentrations of 0.5, 5 and 50 µg/ L of FX; plus 7 days of recovery. Relevant behaviors such as shoaling, feeding, swimming velocity and anxiety were evaluated during the assay. Feeding was evaluated by: i) feeding behavior; and ii) food intake. Swimming velocity was evaluated and calculated using a raceway, registering the time each individual took to swim through 3m of the device. Anxiety was evaluated by: i) position on the aquarium; and ii) dark/ light compartment preference (scototaxis). Hepatosomatic index (HSI)

was also calculated. Gene expression was evaluated with brain samples from day 21 and 28, through real-time quantitative PCR. Results showed that FX did not exert any effect on shoaling and feeding. Swimming velocity was not calculated due to the small number of fishes that swam, however flow resistance was observed which can indicate that sea bass juveniles were not in good shape to swim. Anxiety through position on the aquarium wasn't affected by FX, nonetheless a decrease in anxiety levels of all exposed groups was observed by scototaxis, which can indicate potential anxiolytic effects of FX. Alterations on the expression of gene *sert* were registered at the concentration of 50 µg/ L of FX, however the expression of genes *5-ht$_{3B}$* and *mao* wasn't altered during the assay. In conclusion, this study demonstrated that FX can affect the behavior and gene expression of European sea bass, so it is important to evaluate the extension of this problem to the species and their specific role in their ecosystems.

Keywords

Antidepressant; fluoxetine; European sea bass; behavior; gene expression.

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List of Abbreviations

5-Hydroxytryptamine (5-HT)

Analysis of variance (ANOVA)

Central nervous system (CNS)

Control (Ct)

Control solvent (CtS)

Hepatosomatic index (HSI)

Fluoxetine (FX)

Monoamine oxidase (MAO)

Norfluoxetine (NFX)

Quantitative real-time polymerase chain reaction (qRT-PCR)

Sertraline (SER)

Serotonin transporter (SERT)

Selective serotonin reuptake inhibitor (SSRI)

Melting temperature (T_M)

Wastewater treatment plant (WWTP)

Chapter 1. Introduction

1. Fluoxetine

Recent studies have shown the presence of pharmaceuticals and personal care products in the environment (Daughton and Ternes, 1999; Boyd *et al*., 2003; Blair *et al*., 2013). Pharmaceuticals, which are used for human and veterinary purposes are considered emerging contaminants in the environment (Fent *et al*., 2006). Several pharmaceuticals and their metabolites are found in aquatic ecosystems (Boyd *et al*., 2003; Santos *et al*., 2013; Lajeunesse *et al*., 2011), such as rivers (Fent *et al*., 2006; Alonso *et al*., 2010), estuaries (Calamari *et al*., 2003; Thomas and Hilton, 2004; Fent *et al*., 2006; Birch *et al*., 2015), lakes (Blair *et al*., 2013) and coastal areas (Munaron *et al*., 2012). Pharmaceuticals and their metabolites are excreted after their utilization, reaching aquatic ecosystems by several pathways, like urban sewages or hospital wastewaters. Their removal in wastewater treatment plants (WWTPs) is not fully efficient, making them together with effluents, one of the main pathways of contamination of the environment (Fent *et al*., 2006; Kreke and Dietrich, 2008; Santos *et al*., 2013). Some of these compounds remain bioactive in the environment, liable to bioaccumulate and exert toxic effects on aquatic and terrestrial organisms, highlighting their role as contaminants with potential risks at short and long-term to the aquatic ecosystems (Fent *et al*., 2006; Kreke and Dietrich, 2008; Corcoran *et al*., 2010). Within the pharmaceuticals found in the environment there are several groups such as analgesics, psychotropic drugs, antibiotics, β-blockers and anti-inflammatory (Fent *et al*., 2006; Santos *et al*., 2013). Pharmaceutical targets such as receptors, neurotransmission systems, biochemical pathways and enzymes are conserved in evolutionary terms, therefore it is expected that their presence might affect organisms exposed reinforcing the need to study their effects and impacts to the non-target species (Fent *et al*., 2006; Kostich and Lazorchak, 2007; Santos *et al*., 2013).

Within the psychotropic drugs antidepressants are used worldwide, particularly in OECD countries of which Portugal is a member (OECD health data, 2011; Infarmed, 2013). Portugal has the highest rates of use of

antidepressants in the European Union, where the prevalence of use is the double of the average of EU (Eurobarometer, 2010). Antidepressants are prescribed for the treatment of depressions and other psychiatric disorders such as anxiety, eating and obsessive-compulsive disorders, and others (Brooks *et al*., 2003; Kreke and Dietrich, 2008; Valenti *et al*., 2012; Auclair *et al*., 2013). Selective serotonin reuptake inhibitors (SSRI) are a group of antidepressants that act in the serotoninergic system of the central nervous system (CNS) by inhibiting the reuptake of serotonin (5-HT) by their transporters (SERT) in the presynaptic membrane, thereby increasing the concentration of 5-HT in the synaptic clefts, therefore increasing serotoninergic neurotransmission (Nutt *et al*., 1999; Kreke and Dietrich, 2008; Valenti *et al*., 2012) (Figure 1). In order to achieve the desired pharmacological effects of SSRI in human patients it is necessary a chronic treatment. Likely, this may occur due to alterations in SERT gene expression levels and not to a direct inhibition of SERT action (Kreke and Dietrich, 2008). There are some evidences that SSRI have potential drug-drug interaction with other classes of pharmaceuticals (Hiemke and Hartter, 2000). Differences in pharmacology of SSRI in terms of selectivity, chemical structure, receptor binding and pharmacokinetic properties should also be considered in the treatment of psychiatric disorders (Nutt *et al*., 1999; Carrasco and Sandner, 2005; Kreke and Dietrich, 2008).

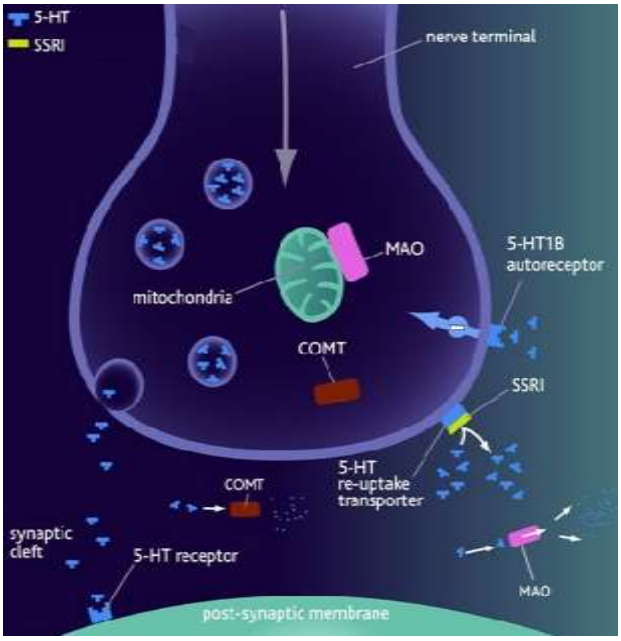

Figure 1: Pharmacological mode of action of the SSRI (Adapted from Pharmacology corner, 2014).

Recently, the organization of 5-HT neuronal network in fishes has been investigated and several proteins (e.g. enzymes, metabolites, receptors, transporters) involved in serotoninergic system, already known in mammals have also been identified in fishes (Kreke and Dietrich, 2008). Catabolic enzyme monoamine oxidase (MAO) has been found in several fish species, such as goldfish (*Carassius auratus)* (Hall and Urueña, 1982), rainbow trout (*Oncorhynchus mykiss*) (Hall *et al*., 1986), pike (*Esox lucius)* (Senatori *et al*., 1990) and zebrafish (*Danio rerio)* (Setini *et al*., 2005; Sallinen *et al*., 2009; Aldeco *et al*., 2011). Contrary to mammals, who have two MAO's (Type A and B), in fishes only one MAO have been detected (Senatori *et al*., 1990; Lillesaar *et al*., 2011). This MAO is described as been different from both MAO A and MAO B (Senatori *et al*., 2003), however some evidences suggested that there are similarities in terms of pharmacology and structure namely MAO A (Setini *et al*., 2005). The presence of 5-HT receptors has also been identified in several teleostei species (Yamaguchi and Brenner, 1997; Clotfelter *et al*., 2007; Kreke and Dietrich, 2008; Maceira *et al*., 2014). 5-HT_1 receptor genes were identified in puffer fish (*Fugu rubripes)*, and when in comparison with the same human gene, some similarities were determined, namely all essential amino acid residues present in their homologues. However, evidence showed the presence of additional phosphorylation sites in puffer 5-HT_1 receptors, which suggests that their regulation could be different from the human gene (Yamaguchi and Brenner, 1997). Experiments in rainbow trout showed that 5-HT_{2C} receptors are involved in the mediation of inhibition of food intake induced by 5-HT. These results are consistent with the fact that the role of this receptor is preserved during the evolution vertebrates. It has been proposed that in mammals the inhibitory effects on food intake induced by the activation of 5-HT_{2C} receptors are mediated by activation of the melanocortin pathway, so if the functions of the receptor are preserved it is hypothesized that in fishes the processes are similar, and also induces anorexigenics effects (Maceira *et al*., 2014). In zebrafish, it was demonstrated that amino acid sequences of transporter *serta* and *sertb* are 66-69% and 75%, respectively, similar to other vertebrates (Wang *et al*., 2006). Hence, there are several evidences about the conservation of SERT in animal phyla (Cavaney *et al*., 2006) nevertheless the regulation and

properties of the transporter protein may be diverse in non target species (Kreke and Dietrich, 2008). Antidepressants are detected in the environment at several concentrations, ranging from ng/ L to µg/ L (Metcalfe *et al*., 2003; Fent *et al*., 2006; Vasskog *et al*., 2006; Kreke and Dietrich, 2008; Schultz and Furlong, 2008; Santos *et al*., 2013; Silva *et al*., 2014). Several SSRIs such as fluoxetine (FX) and sertraline (SER) and their metabolites norfluoxetine (NFX) and desmethylsertraline respectively, citalopram, fluvoxamine (FLV) and paroxetine (PAR) have been documented in the environment and also in animal tissues (Brooks *et al.*, 2003; Metcalfe *et al*., 2003; Brooks *et al*., 2005; Fent *et al*., 2006; Kreke and Dietrich, 2008; Patterson and Metcalfe, 2008; Santos *et al*., 2013; Silva *et al*., 2014). Several studies have already reported the presence of FX in WWTPs. For example, the WWTPs of the lower Great Lakes of Canada with FX concentrations between 0.038 ± 0.003 µg/L and 0.099 ± 0.007 µg/L, (Metcalfe *et al*., 2003). FX was also detected in TronsØ, Norway in SjØlund pump station at 2.3 ng/L, and in three STPs, Lagnes, Breivika and Hamna at 0.4, 125 ± 0.07 and 1.85 ± 0.78 ng/L (Vasskog *et al*., 2006). FX and its metabolite NFX were detected at ranges of 33 - 49 and 3-7 ng/L respectively, in a downstream from the Pecan Creek Water Reclamation Plant (Denton, Texas, United States of America) (Schultz and Furlong, 2008). In Portugal, FX was also detected during the autumn in 15 WWTPs of the North, Center, Lisbon and Tagus Valley, Alentejo and Algarve, at ranges of 105.80 - 157.40 ng/L in wastewater influents (Silva *et al*., 2014). Contrary to others SSRIs, FX is a potent inhibitor of 5-HT_{2A} and 5-HT_{2C}, modulating norepinephrine and dopamine systems in brain, consequently causing weight loss and activation. When compared with other SSRIs, FX has a largest volume of distribution (up to 100 L/kg body weight) which indicates extensive tissue accumulation. Pharmacological half-life ($t_{1/2}$) of FX ranges 1-4 days. Due to FX high volume of distribution together with their high $t_{1/2}$, treatments of 1-22 months are required in order to achieve steady-state conditions in human patients (Kreke and Dietrich, 2008).

Alterations in the neuro-endocrine system (Menningen *et al*., 2011), glucose metabolism and weight loss (Menningen *et al*., 2010) were registered in goldfish after the exposition to FX. Changes in the egg production were detected in

zebrafish after exposure to FX (Lister *et al*., 2009). Behavioral changes were also reported after exposition to FX, in the decrease of swimming velocity of sheepshead minnow (*Cyprinodon variegates)* (Winder *et al*., 2011) and hybrid striped bass (*Morone saxatilis* x *M. chrysops)* (Gaworecki and Klaine, 2008). Anxiety changes were also reported in zebrafish chronically exposed to FX (Egan *et al*., 2009). Mianserin, another antidepressant acts as neuroendocrine disruptor in zebrafish (van der Ven *et al*., 2006).

2. European sea bass

The European sea bass *Dicentrarchus labrax* (Figure 2) belongs to the order of Perciformes and the family Moronidae and has a distribution range along the northeast coast of Atlantic ocean, from Norway to Senegal and from Mediterranean sea to Black sea (Kottelat and Freyhof, 2007) (Figure 3). In Portugal, this species is commonly observed throughout the Portuguese coast (Vasconcelos *et al*., 2010) and a typical user. European sea bass is a predator and their diet varies throughout its ontogeny, being in the juvenile phase essentially composed by benthic macroinvertebrates and gradually being replaced by major invertebrates and other fishes (Kottelat and Freyhof, 2010). Juveniles tend to shoal and habitat estuarine ecosystems that function as nursery habitats (Vasconcelos *et al*., 2010). European sea bass is a species of human consumption, being captured by professional fisheries and also cultivated in aquaculture systems. In Portugal, it is not amongst the most fished species, however is one of the most produced species in aquaculture (INE, 2015), making the species a source of socio-economical value. The occurrence of this species in habitats potentially contaminated by antidepressants as estuaries, make them vulnerable to antidepressants and other pharmaceuticals, what can ultimately lead to exposure of human to these pollutants (Fent *et al*., 2006; Kreke and Dietrich, 2008; Lajeunesse *et al*., 2011; De Domenico, *et al*., 2013). Thus, it is urgent to assess the impacts of such contaminants in European Sea Bass, assessing not only the environmental but also the human risks associated.

Figure 2: European sea bass juvenile specimen.

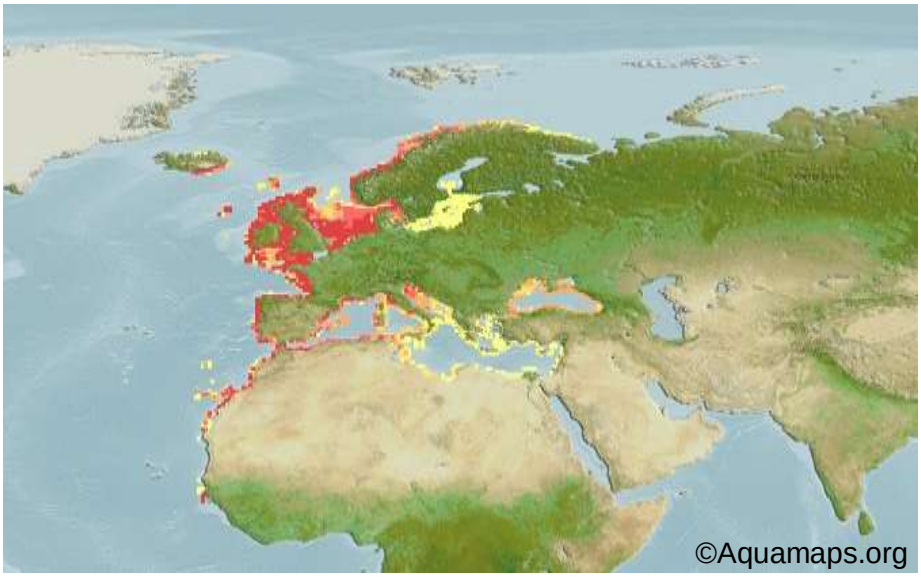

Figure 3: Geographical distribution of European sea bass.

3. Objectives

This study aims to evaluate the effect, at the behavioural level and neuroendocrine disruption, of the exposure to an SSRI (FX) on juvenile European sea bass. Behavioral alterations are important as they can interfere with several actions of the individuals (alimentation, reproduction, refugee, movement and migrations), consequently affecting the population dynamics. Behavioural associated effects will be evaluated based on swimming velocity, anxiety and food intake. Alterations in gene expression of components of the serotinegic system can provide further information on the neuroendocrine disruption potential of SSRI and also explain physiological alterations leading to behavioral changes.

Chapter 2. Effects of antidepressant fluoxetine on behavior and gene expression of European sea bass juveniles[1]

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1. Abstract

Recently, the presence of several pharmaceuticals as antidepressant (FX) and their metabolites has been documented in aquatic ecosystems. FX is a selective serotonin reuptake inhibitor (SSRI) that operates in the serotonergic system of the central nervous system. These compounds are not always eliminated in wastewater treatment plants, reaching aquatic ecosystems as rivers and estuaries. This study aims to evaluate the effect of FX exposure on European sea bass (*Dicentrarchus labrax*) juveniles at behavior level and also at the expression of associated specific brain genes. For this purpose, an exposure assay of 21 days plus 7 days of recovery was conducted. Several behaviors such as shoaling, feeding (behavior and food intake), swimming velocity and anxiety (position on the aquarium and scototaxis), were evaluated during the assay to assess deviants to the normal parameters. The expression of three associated genes was also evaluated with brain tissue samples collected at 21th and 28th day. Results showed that FX did not exert any effect on shoaling and in feeding. FX effects on swimming velocity were not able to assess due the small number of individuals that swam, nonetheless flow resistance behavior was observed however with no apparent pattern. Anxiolytic effects of FX were observed on scototaxis tests, since there was a decrease of anxiety levels in exposed fishes, nonetheless no effects were observed in the preference for a specific position on the aquarium. The expression of gene *sert* appears to be altered by the exposition to high concentrations of FX, however no large differences were observed in the expression of the other two genes, *5-ht$_{3B}$* and *mao*.

2. Introduction

Recent studies demonstrate the presence of pharmaceutical and personal care products in the environment (Boyd *et al*., 2003; Ellis, 2006; Salgado *et al*., 2010; Blair *et al*., 2013). Several pharmaceuticals, as antidepressants and their metabolites are found in aquatic ecosystems (Boyd *et al*., 2003; Santos *et al*., 2013; Lajeunesse *et al*., 2011) and wastewater treatment plants (WWTPs) at several concentrations, ranging from ng/ L to µg/ L (Fent *et al*., 2006; Kreke and

Dietrich, 2008; Santos *et al*., 2013). WWTPs are not efficient in the removal of pharmaceuticals, which makes them together with effluents one of the main pathways of contamination of the environment (Fent *et al*., 2006; Kreke and Dietrich, 2008; Santos *et al*., 2013). The targets sites of these compounds are evolutionary well conserved (e.g. metabolic pathways, neurotransmission systems, etc). Moreover, some of these compounds remain bioactive in the environment, liable to bioaccumulate and exert toxic effects on ecosystems, therefore they considered emerging contaminants with potential risks to aquatic ecosystems not only on a short but also long-term scale (Fent *et al*., 2006; Kreke and Dietrich, 2008; Corcoran *et al*., 2010).

FX is a SSRI antidepressant considered an emergent contaminant, and its presence is well documented in several ecosystems (Metcalfe *et al*., 2003; Schultz and Furlong, 2008) and WWTPs (Silva *et al*., 2014). FX acts in the serotonergic system of the central nervous system by inhibiting the reuptake of serotonin (5-HT) by their transporters (SERT) in the pre-synaptic membrane, thereby increasing the concentration of 5-HT in the synaptic clefts and therefore increasing serotonergic neurotransmission (Nutt *et al*., 1999; Kreke and Dietrich, 2008; Valenti *et al*., 2012). FX can also modulate norepinephrine and dopamine systems in brain by inhibiting 5-HT_{2A} and 5-HT_{2C} (Nutt *et al*., 1999; Carrasco and Sandner, 2005).) Several studies have been conducted to assess the effects of these compounds in different fish species. Anxiety changes were reported in zebrafish *Danio rerio* after chronic exposure to FX (Egan *et al*., 2009). Sheepshead minnow *Cyprinodon variegatus* (Winder *et al*., 2011) and hybrid striped bass *Morone saxatilis* x *M. chrysops* (Gaworecki and Klaine, 2008) when exposed to FX decreased their swimming velocity. Also, neuro-endocrine alterations (Menningen *et al*., 2011), glucose metabolism changes and weight loss (Menningen *et al*,, 2010) were reported in goldfish *Carassius auratus*.

Estuaries are worldwide acknowledged as important aquatic ecosystems, suffering the impacts of the many human activities that can compromise their ecological function and threat their long-term viability. Estuaries have become reservoirs of emergent contaminants that can threat not only the individual

organisms exposed but also the ecological functioning of the ecosystem. European sea bass (*Dicentrarchus labrax*) is a marine species that commonly uses estuaries as feeding areas and also as nursery habitat for juveniles (Vasconcelos *et al*., 2010). This species with high socio-economic value can be endangered by the exposition to emerging contaminants as antidepressant fluoxetine. Thus, this species was selected as the model to evaluate the environmental risk of antidepressant pharmaceutical pollutants, due to their important role in their ecosystems low maintenance cost in the laboratory and the good response in behavioral terms when exposed to pollutants (Almeida *et al*., 2010).

The present study aims to evaluate the effect of the exposure to FX on juvenile European seabass by assessing (i) behavior (swimming velocity, anxiety and food intake) and (ii) the expression of genes codifying for components of the serotonergic system in brain. The behavioral alterations are important as they can interfere with several actions of the individuals (feeding, reproduction, refugee, movement and migrations), consequently affecting the population dynamics, what is particularly important for commercially exploited species as the European sea bass.

3. Material and methods

3.1. Animals used

European sea bass juveniles were provided by Tinamenor aquaculture, Santander, Spain. The animals were maintained under conditions of water recirculation in a 2200L tank and a photoperiod of 12: 12 hours (light: dark). The tank was regularly monitored in order to maintain constant seawater levels of 35 ppm), 13-14 ºC, 7.5-8.0 pH, and ammonia and nitrites levels under 0.5 mg/ mL. Water exchanges were made in order to avoid water quality deterioration. Gradually, the water salinity was being decreased until 25 ppm, in order to cope with the assay requirements. Animals were fed three days a week with commercial pellets of granulometry 4mm.

3.2. Experimental assay

The experimental assay included five groups in triplicate namely: control (Ct), control solvent (0.01% dimethyl sulfoxide) (CtS) and three concentrations of FX (0.5, 5 and 50 µg/L) (Figure 4). A total of 120 individuals (11.11 ± 0.67 cm total length and 11.05 ± 2.38 g total weight) were used and distributed by 15 aquariums of 30 L (with 23 L of water), in a total of 8 individuals per aquarium. The experimental assay was made in a semi-static system with daily 80% of water changed durating the 21 days of exposure to FX plus 7 days of recovery with no chemicals added. The aquariums were kept under conditions of salinity of 25 ppm, temperature range of 17-19º C, pH ≈ 6 and photoperiod of 12:12 hours (light: dark).

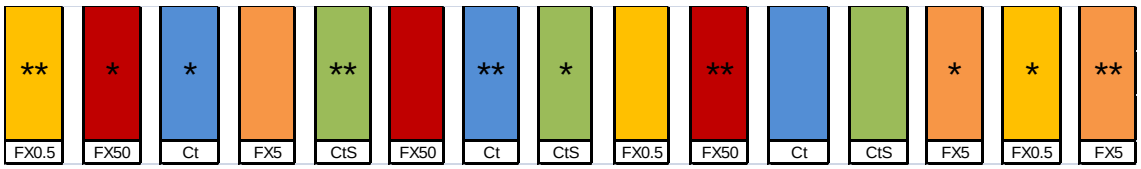


Figure 4: Scheme of the experimental assay. * Aquariums used for the raceway tests; ** aquariums used for scototaxis tests.

3.3. Behavior evaluation

Sea bass behavior evaluation wasn't uniform. Shoaling and position on aquarium was observed twice a day (10-12 a.m. and 15-17 p.m.). Feeding behavior and food intake were evaluated every two days, according to the days where fishes were fed. Swimming velocity and scototaxis tests were done at several points of interest during the assay: i) exposure period (day 1, 3, 7, 15 and 21); and ii) recovery (day 28).

Shoaling is considered the regular behavior of European sea bass juveniles (Vasconcelos *et al*., 2010) and in order to evaluate potential alterations, the number of isolated fishes was registered. For this behavior the following criteria was used: 1 - less than 3 fishes (<37.5%); 2 - three to five fishes (37.5% - 62.5%); and 3 - more than five fishes (>67.5%), considering a total of 8 fishes per aquarium.

Feeding started at the third day of the assay. Feeding behavior was observed during the moments where sea bass juveniles were fed. For that purpose the following criteria was used in order qualify the reaction to pellets: 1 - indifference (when fishes don't react to pellet), 2 - timidity (fishes react cautiously to pellet); and 3 - voracious (fishes reacts immediately to pellet). Food intake was also evaluated at the same periods of time. Animals were provided with 2% of the total fish weight in each aquarium. Half an hour after feeding, the remaining pellet was collected and dried in a oven until the weight remained stable. This weight permits to calculate the exact amount of food that was not eaten, and therefore quantify the food intake of each aquarium.

A 4 m raceway device (Figure I in Annex) was used to assess alterations of the swimming velocity in European sea bass exposed to FX. Prior to the beginning of the assay, all conditions of the raceway were tested and calibrated in order to establish the best conditions for the test (Protocol I in Annex). Each animal was placed in the same position of the raceway device to swim against a counter current flow of 6 L/ min. The time (in seconds) that each individual took to swam 3 m of the raceway was registered and the swimming velocity was calculated as the quotient of the distance and the time to reach 3m race. When fishes did not swam and stayed in the initial position resisting the water flow, this time was also registered for a maximum of sixty seconds (Gravato and Guilhermino, 2009; Almeida *et al*., 2010; Almeida *et al*., 2012) and use to estimate the flow resistance as indicator of a deviant behavior. Handling of fish can cause stress and therefore physiological alterations (Pickering *et al*., 1982; Barton, 2000), however it is unavoidable in this test. Giving the short time of handling process possible effects will be acute (Pickering *et al*., 1982); so in order to minimize stress, fishes only relayed a maximum of sixty seconds in the raceway device.

The number of individuals that occupied the top and bottom of the aquarium were observed separately, following the same criteria used for shoaling observations. European sea bass juveniles exhibit demersal behavior (Barnabé, 1989) and therefore tend to occupy the center and the bottom of the aquarium to imitate their natural environment. So, the preference for upper

regions of the aquarium was used as proxy of low anxiety levels (Gaworecki and Klane, 2008; Egan *et al*., 2009). Scototaxis (dark/ light preference) was used to assess the anxiety of European sea bass juveniles. For that purpose a specific device was mounted using an aquarium of 70L divided in three zones, colorless central zone, a black and a white compartment and filled with 30L of water from the system (Figure II in Annex). Similarly to raceway experiment, previous tests were made in order to test the best conditions to perform the experiment (Protocol II in Annex). Each individual was placed in the central zone of the aquarium and its preference was registered (Maximino *et al*., 2010; Maximino *et al*., 2011; Brandão *et al*., 2013), considering the preference for black compartment as proxy of anxiety.

3.4. Biological samplings

Biological sampling was done at 21th and 28th day of the assay. Animals were anesthetized with MS222 (0.25%) and sacrificed according to the 3R's policy, and weight and length (total and standard) were measured. Liver was also removed and weighted in order to calculate hepatosomatic index (liver weight (g)/ fish weight (g) x100). Brain samples were collected and stored in RNAlater at -80º C, in order preserve the tissues for molecular analysis.

3.5. RNA extraction and cDNA synthesis

Total RNA was extracted from the RNAlater stored brain samples using the Illustra RNAspin Mini Kit (GE Healthcare) according to the manufacturer protocol. Them, RNA was purified with DNase I from the same kit in order to clean DNA remains that could possible contaminate our samples. RNA quality was verified by electrophoresis in agarose by gel red stains and by the measurement of the ratio of optical density $\lambda 260/ \lambda 280_{nm}$. RNA was quantified and diluted to 1 µg (Pfaffl, 2001). After, RNA was used as template to synthesize cDNA with qScript cDNA SuperMix (Quanta Biosciences).

3.6. Quantitative real-time PCR

Gene expression of *sert*, *5-ht$_{3B}$*, *mao*, *ef1*, *l17* and *18s* was assessed by means of quantitative real time PCR (qRT-PCR) (Table 1). qRT-PCR was performed in a Eppendorf Realplex 4, and for the purpose a mix contain 10 µl of SYBR Green fluorescence Supermix (Quanta Biosciences), 2 µl of each primer reverse and forward (Concentration of 3 nM) and 2 µl of cDNA in a total volume of 20 µl in duplicate. Reactions for qRT-PCR were conducted following protocol: denaturation program (94ºC for 2min), amplification and quantification program repeated 40 times (94ºC for 30s, 55ºC for 30s, 72ºC for 30s and 72ºC for 10min)and a melting curve program (from 55 to 95º C)to determine the formation of the specific products. When the reaction was over, we proceed to the analysis and the values of crossing points (CP) and melting temperature (T_M) were recorded in a database intended for statistical analysis. CP is defined as the point at which the fluorescence rises appreciably above the background. The formation of PCR products is responsible for this increase and therefore it's important to know the cycle where this begins (Pfaffl, 2001). Gene expression was quantified by normalization with multiple references genes using Normfinder algorithm (Urbatzka *et al*., 2013) For characterization of the transcription profile elongation *18s* and *ef1* were used as reference genes. For the determination of the relative quantification of the target genes in comparison to reference genes we used the relative expression ratio equation (Pfaffl, 2001):

$$\text{ratio}=\frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\textit{control-sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control-sample})}}$$

This equation is a mathematical model of relative expression ratio in qRT-PCR, where the ratio of the target genes is expressed in a sample versus a control in comparison to reference genes. Ratio allows to assess physiological changes in gene expression (Pfaffl, 2001). The variables of this equation are: E_{target} is the qRT-PCR efficiency of the target gene transcript; E_{ref} is the qRT-PCR efficiency of the reference genes transcript; $^{\Delta CP}{}_{\text{target}}$ is the CP deviation of control-sample of the target gene transcript and $^{\Delta CP}{}_{\text{ref}}$ is the CP deviation of control-sample of the reference genes transcript. As reference genes we used

housekeeping genes due to their stability and secure unregulated transcript (Pfaffl, 2001).

Table 1: Selected genes for the RT-qPCR. Product (bp) and Efficiency of PCR reaction.

Gene (Acession number)		Primer sequence (5'-3')	Product (bp)	Efficiency (%)
sert (002060)	F	GTTGATGGCAGTGTTTGGTG	109	92
	R	GAAGATGGGGCAGATGTGTT		
5-ht$_{3B}$ (00046560)	F	TCATCTGGCTGAATGTGTGC	91	100
	R	AACTCCGCAGTCGTATTTCC		
mao (00018080)	F	GCCAATCACCTCAACCAAAC	100	104
	R	AGGGACAAACCAAACACTGG		

3.7. Data analysis

All data was averaged by day and group (mean ± standard deviation) with the exception of anxiety, where results are given in frequencies. Shoaling, feeding and position on the aquarium data were aggregated into three different periods of the assay: i) short exposure (day 2 to 9); ii) long exposure (day 10 to day 20); and recovery (day 22 to 28). Flow resistance and anxiety were analyzed in several days of two periods: i) exposure (day 1, 3, 7, 15 and 21); and ii) recovery (day 28). HSI and gene expression data was analyzed in two different periods: 21th day (end of exposure); and 28th day (end of recovery).

Differences in food intake, HSI and three target genes expression between concentration groups and during the time of the assay were investigated by two.way analysis of variance (ANOVA) with concentration and time as fixed factors (Zar, 1996). Gene expression data were log transformed in order to stabilize the variance and to fit data to a normal distribution, fulfilling one of the ANOVA assumptions. Homogeneity of variance was tested with a Cochran test and whenever variance was still heterogeneous, conclusions from ANOVA results were only accepted for those cases where significance levels were less than 0.01. Furthermore, in the event of significance, an a posteriori Tukey HSD for unequal sample sizes was used to determine which means were significantly

different at a 0.05 level of probability (Spjotvoll and Stoline, 1973). All analyses were performed using the software STATISTICA version 12.

3.8. Ethical statement

The animals were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei no 197/96) approved by the Portuguese Parliament in 1996 and with the European directive 2010/63/UE approved by the European Parliament in 2010.

4. Results

4.1. Behavior results

According to the shoaling results, in average less than 3 fishes in a total of 8 fishes per aquarium (criteria 1) were isolated in the aquarium (Figure 5). Such pattern was systematically registered for all five groups of treatments and throughout the duration of the assay, without alterations in the recuperation period. Results showed that there were no alterations to the typical shoaling behavior of juvenile sea bass during the assay.

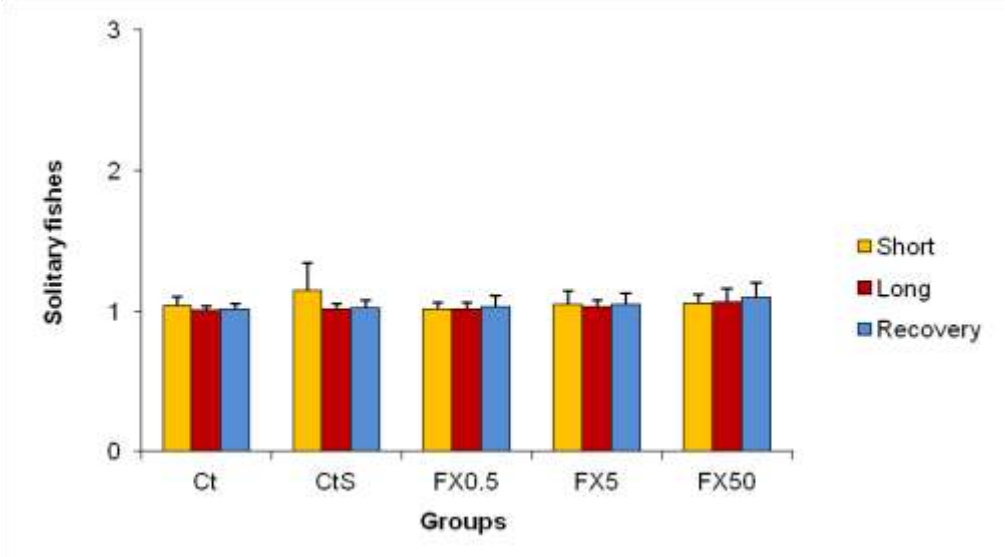
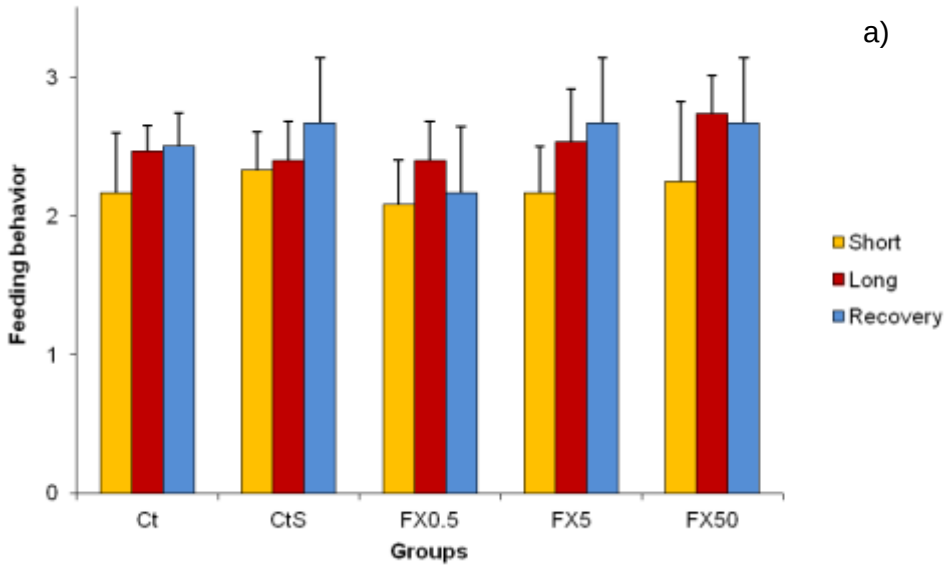


Figure 5: Number of solitary juvenile sea bass observed in each aquarium divided by group during three periods (short, long and recovery) of the assay. Criteria used: 1 - less than three fishes; 2 - three to five fishes; and 3 - more than five fishes were solitary in respective group in that day. Results are given as mean±SD.

According to feeding behavior observations, timidity was in average the most frequent behavior of sea bass juveniles in all groups during the three periods of the assay (Figure 6a). Since this pattern was observed in all five testing groups, exposure to FX did not exert any effects on the feeding behavior of sea bass juveniles. In average, the intake of food was always above 50 % in the five testing groups, and during the three periods of the assay (short and long exposure and recovery). During the assay the percentage of food intake by sea bass juveniles varied significantly between testing groups ($F=3.28$; $p<0.05$) and between periods of the assay ($F=10.34$; $p<0.05$). There was a trend to increase the food intake throughout time and in all groups food intake was always higher than in previous period (Figure 6b). On short period of exposure, food intake levels were low in groups exposed to 5 and 50 µg/ L of FX when compared to the other groups, nonetheless no significant differences were detected. On long period of exposure, there was a increase in food intake in all five testing groups, namely in the group exposed to 50 µg/L of FX. During the recovery period, the tendency of the previous period was also observed, with CtS and FX50 groups reaching the highest percentages of food intake.



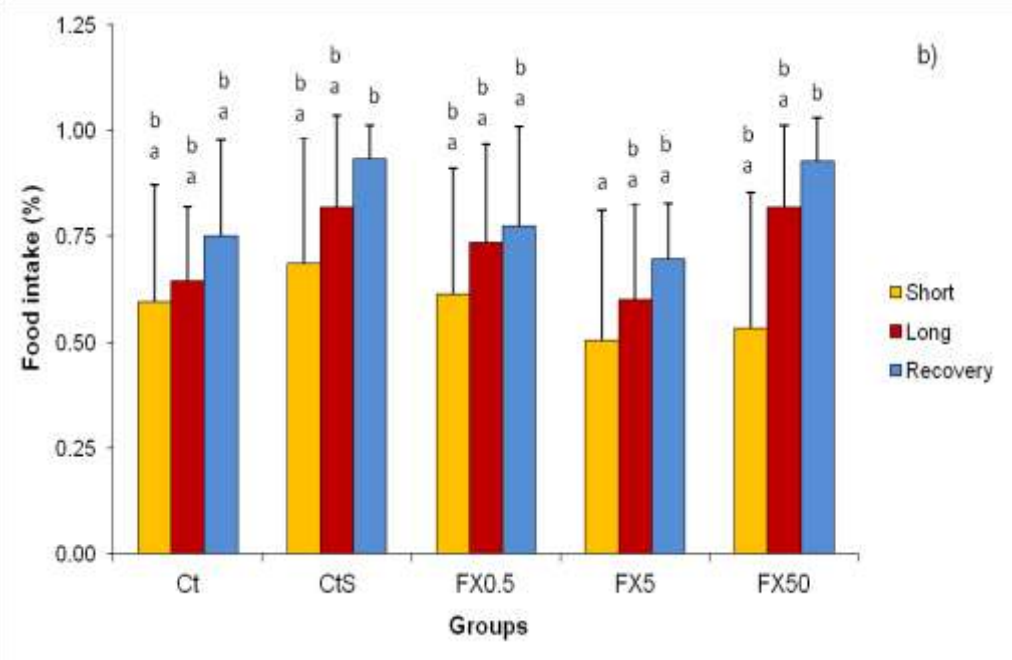


Figure 6: Feeding results as: a) feeding behavior (Criteria used: 1 - indifference, 2 - timidity; and 3 - voracious); and b) food intake (in percentage) of juvenile sea bass of five testing groups during three periods of the assay (short, long and recovery). Results are given as mean ± SD. Legend: Ct - control; CtS - solvent control; FX0.5 - concentration of 0.5 µg/L of FX; FX5 - concentration of 5 µg/L of FX; and FX50 - concentration of 50 µg/L of FX. Different letters denote significant differences (p<0.05) between groups.

The raceway experiment showed that only 14 juveniles swam the 3 m distance of the raceway (Table II in Annex). Nonetheless, swimming velocity of sea bass juveniles was calculated with the few data obtained, and ranged from 0.03 to 0.32 m/s in exposure period and 0.16 to 0.50 m/s in recovery period (Table 2). Also, during the first 15 days of the assay, juveniles tended to resist the counter current of the raceway and during this period, flow resistance was observed in all groups (Figure 7). Also, the majority of juveniles could resist for at least sixty seconds to the counter current (Figure 7). The frequency of individuals that swam and that presented flow resistance behavior are summarized in Table II in Annex.

Table 2: Swimming velocity results in m/s (mean ± SD). Results are distributed by two periods. Number (n) of fishes that swam in each period of time.

	Period	Swimming velocity	n
Ct	Exposure	0.03	1
	Recovery	0.5	1
CtS	Exposure	0.13	1
	Recovery	0.21 ± 0.08	2
FX0.5	Exposure	-	-
	Recovery	-	-
FX5	Exposure	0.20 ± 0.15	6
	Recovery	-	-
FX50	Exposure	0.32 ± 0.26	2
	Recovery	0.16	1
			14

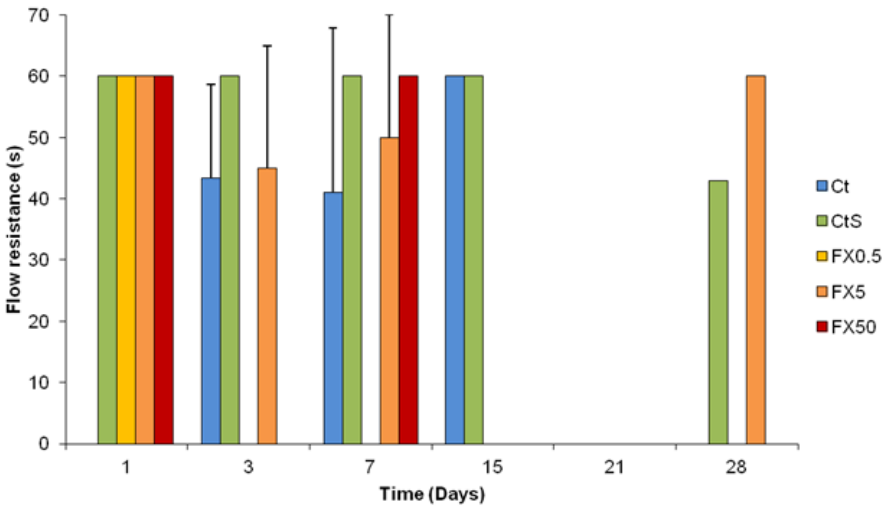
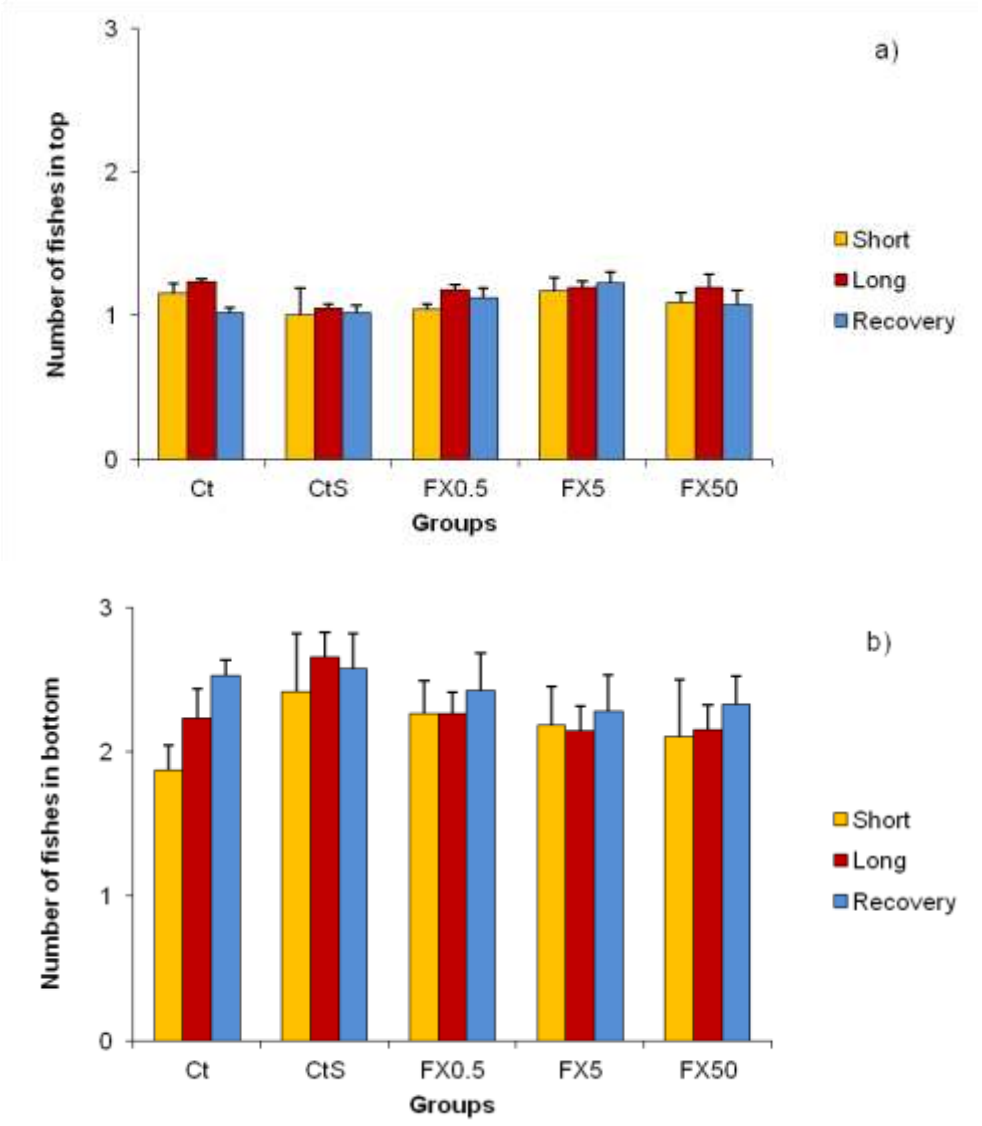


Figure 7: Flow resistance in time (s) of the five testing groups for each day of observation. Results are given as mean ± SD. Legend: Ct - control; CtS - solvent control; FX0.5 - concentration of 0.5 µg/L of FX; FX5 - concentration of 5 µg/L of FX; and FX50 - concentration of 50 µg/L of FX. Results are given as mean± SD.

Regarding to location of fishes in the aquarium, results showed no alterations to the typical behavior of the sea bass juveniles. The number of fishes located at the top of water column was always less than 25%, with an average of less than three fishes observed in all treatment groups and during the assay (Figure 8a). In contrast, the location of fishes at the bottom of the aquarium revealed a different pattern, since in average, 3 to 5 fishes prefer the bottom of the aquarium (Figure 8b). This pattern maintained during the three periods of the

assay in all treatment groups. Preference for upper regions was not observed, so no anxiolytic effects of FX were observed on the position. However, scototaxis results showed a decrease in anxiety levels in fishes exposed to the three concentrations of FX, in contrast to control groups where the levels always above 60% (Figure 8c). From day 7 to the end of exposure period, the percentage of individuals with preference for the black compartment (anxiety) decreased in FX0.5, FX5 and F50. In the first day of recovery (day 21) levels of anxiety were still low, however by the end of the recovery period the percentage of individuals with preference for the black compartment on FX5 increases to initial levels (100%). Even low FX concentrations led to a decrease of anxiety in sea bass juveniles.



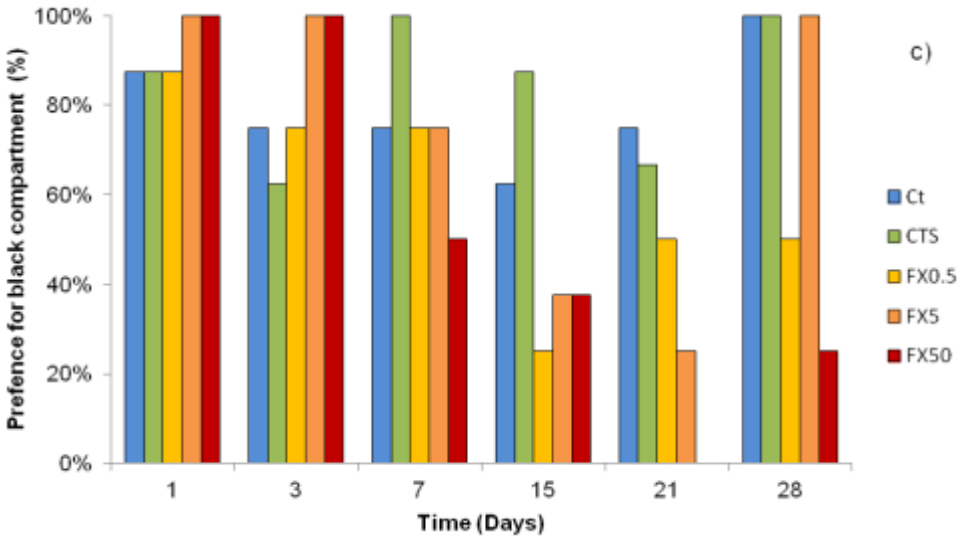


Figure 8: Anxiety results of sea bass juveniles as: preference for the top (a) and bottom (b) of the aquarium (Criteria used: 1 - less than three fishes; 2 - three to five fishes; and 3 - more than five fishes) for three periods of the assay; and c) scototaxis (in percentage). Results of a) and b) are given as mean ± SD and c) in frequency. Legend: Ct - control; CtS - solvent control; FX0.5 - concentration of 0.5 µg/L of FX; FX5 - concentration of 5 µg/L of FX; and FX50 - concentration of 50 µg/L of FX.

4.2. Hepatosomatic index

HSI varied between 2.27±0.93% after 21 days of assay, and 2.13±0.71% after seven days of recovery. HSI results did not varied significantly between groups (F=1.87; p>0.05) or between periods of exposure (F=0.65; p>0.05) (Figure 9), indicating the lack of effect of FX on the fish condition.

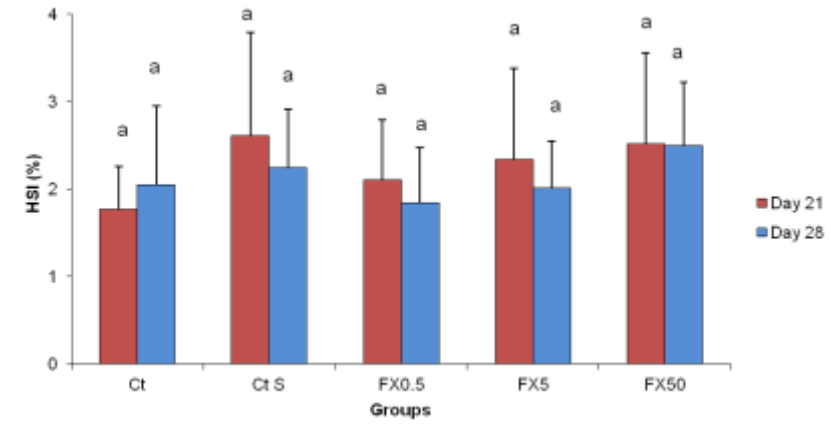
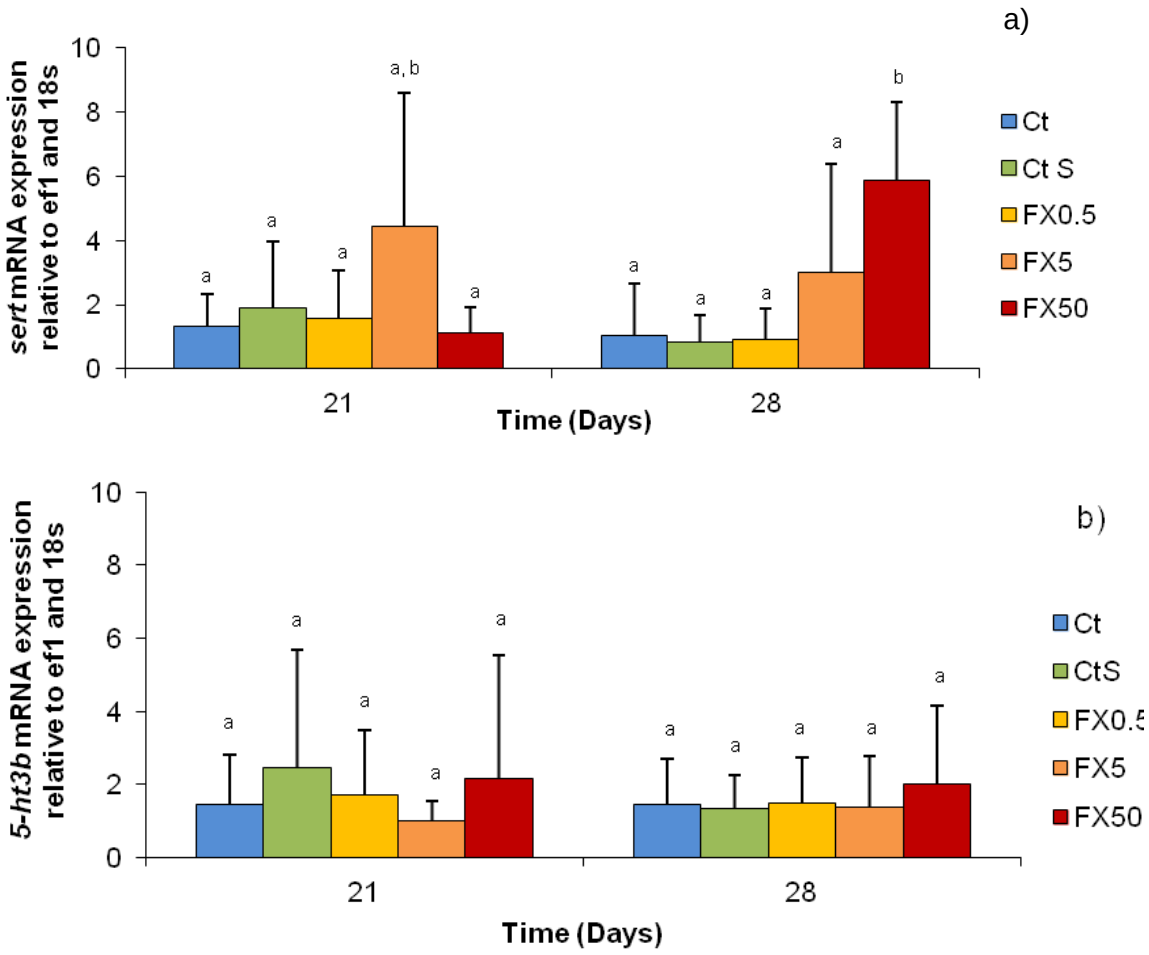


Figure 9: HSI results of the five testing groups at the end of exposure period (day 21) and at the end of recovery period (day 28). Results are given as mean ± SD. Legend: Ct - control; CtS - solvent control; FX0.5 - concentration of 0.5 µg/L of FX; FX5 - concentration of 5 µg/L of FX; and FX50 - concentration of 50 µg/L of FX. Different letters denote significant differences (p<0.05) between groups.

4.3. Brain gene expression

The expression of the target genes *sert*, *5-ht*$_{3b}$ and *mao* was assessed at day 21 and 28 (Figure 10). Two-way ANOVA detected significant differences between groups in different days (F=3.10; p<0.05) for gene *sert* mRNA transcription. A significant transcriptional increase in FX50 from day 21 to day 28 was observed (Figure 10a). No significant differences were detected between groups (F=0.53; p>0.05) and in different days [F=0.41; p>0.05] in mRNA transcription of gene *5-ht*$_{3B}$. In fact, levels were stable from day 21 to day 28 (Figure 10b). For gene *mao*, no significant differences were detected between groups (F=0.58; p>0.05) in different days [F=1.16; p>0.05]. Again, transcription levels were approximately stable from day 21 to 28 (Figure 10c).



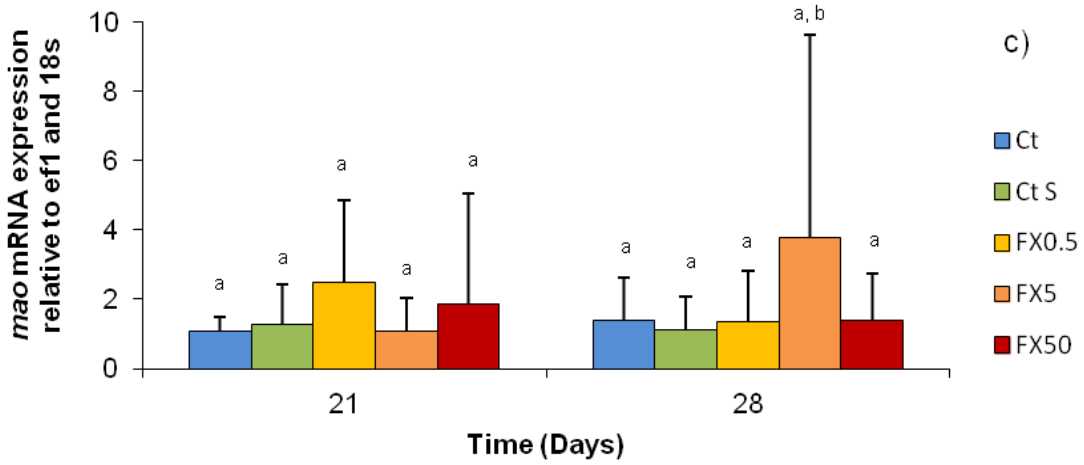


Figure 10: Results of target brain genes for last day of exposure (day 21) and the last day of recovery (day 28) for: a) gene *sert*; b) gene *5-ht3b*; and c) gene *mao*. Results are given as mean ± SD. Legend: Ct - control; Ct S - solvent control; FX0.5 - concentration of 0.5 µg/L of FX; FX5 - concentration of 5 µg/L of FX; and FX50 - concentration of 50 µg/L of FX. Different letters denote significant differences (p<0.05) between groups.

5. Discussion

This study aimed to evaluate the effects of three concentrations of antidepressant FX in European sea bass juveniles at several behavior levels (shoaling, position on the aquarium, feeding, swimming, anxiety and food intake) and also at the expression of target genes on brain (sert, 5ht-$_{3b}$ and mao). This estuarine and coastal species was chosen because is a potential target of this type of contaminants.

5.1. Behavior

Shoaling results were very constant during the assay. In fact, during the three periods less than three fishes were solitary, indicating that exposure to FX did not altered the typical shoaling behavior of sea bass juveniles. Similar results were obtained by Ólsen *et al*. (2014) without differences in shoaling of endler guppy (*Poecilia wingei*) after the two periods of exposure to SSRI Citalopram. However Xie *et al*. (2015) observed that shoaling in crucian carp (*Carassius carassius*) decrease when SER concentrations (4.36, 21.3 and 116 µg/ L) increased. Considering that sea bass juveniles tend to shoal (Vasconcelos *et al*., 2010) and since the number of solitary fishes were low and

constant during the assay, results indicate that FX had no influence in shoaling of this species.

In general, juveniles responded timidly to the presence of food in the aquarium. From short to long period, more individuals started to have voracious behavior that continued to increase in the recovery period. Food intake results showed that this parameter varied largely during the assay, however there was a tendency to increase over the assay. At short period of time, food intake in fishes from the two highest concentrations of FX was lower than control groups. The increase of fishes with voracious behavior complement and confirm that during the assay there was a increase in food intake, however it is not completely in accordance with previous studies where anorexic effects of FX were observed. Low concentrations (at least ≤0.54 µg/ L) of FX have already been described as not causing alterations on food intake. Menningen *et al*. (2010) observed no significant differences in goldfish exposed to 0.54 µg/ L for a period of 28 days when compared to control group. When exposed to 0.12 µg/ L of SSRI SER, Eurasian perch (*Perca fluviatilis*) showed the same pattern, without feeding alterations when compared to control group. Our results showed the same pattern, indicating that low concentrations of FX do not alter food intake of European sea bass. However, when exposed to higher concentrations of FX, fishes appear to have different responses to the antidepressant. Menningen *et al*. (2009) results showed a decrease of food intake exposed to 5 µg/ L of FX for thirteen days. The lowest value of food intake observed was at day 9, and from day 9 to 13 there was a small increase. Menningen *et al*. (2010) observed that 54 µg/ L of FX decreased food intake in goldfish during 28 days of exposure, mainly after 8 days of exposure, continuing lower than control until the end of the assay. Our results for similar concentrations of 5 and 50 µg/ L of FX showed the same pattern as this two studies, however we cannot affirm that FX has anorexic effects in European sea bass juveniles due to no significant differences observed.

Since the number of fish that swam during the essay was low it was not possible to assess the influence of FX in the swimming velocity of European sea bass. Prior to the experiments, during the preliminary tests (calibration of

the system) almost all fishes tested swam against the 6 L/m flow, not suggesting the difficulties experienced during the assay. Since individuals of Control group did not swim also, we exclude the hypothesis of FX as responsible for these results. Everything was done according to previous studies for swimming velocity (Gravato and Guilhermino, 2009; Almeida *et al*., 2010; Almeida *et al*., 2012), with the exception of the water flow which is different from those used in these studies. Flow resistance was observed in European sea bass, however when exposed to a different contaminant, polycyclic aromatic hydrocarbons and during a period of 96 hours, where the increase of the concentration leaded to a decrease of flow resistance time (Almeida *et al*., 2012). Such pattern wasn't observed in the present study, however several fishes resisted the water flow in the raceway experiments. Since there was no change on the time of resistance with increase of FX concentration, results do not indicate any relation between FX exposure and flow resistance. Flow resistance may indicate that fishes were not in conditions to swim against the current. The number of fishes that resist the water current was higher than those who swam. Since flow resistance may indicate that fishes were not in conditions to swim, this could explain why the number of fishes that swam was low, however more tests should be made in order to complement our data and further investigate the effect of FX in the swimming velocity of fishes. Swimming capacity is very important in fish performance and survival (Almeida *et al*., 2010; Almeida *et al*. 2012) namely for a predator specie as European sea bass (Pickett and Pawson, 1994). Also, is determinant for a fish to capture prey, avoid other predators, reproduction, migrations and shoal (Weis *et al*., 2001). Alterations in the swimming capacity of mice (Lira *et al*., 2003) and fish (Gaworecki and Klaine, 2008; Winder *et al*., 2011; Barry, 2013) have already been described, so it is important to evaluate if FX can also compromised swimming capacity in European sea bass juveniles. Alterations on swimming capacity can cause difficulties to individuals with repercussions on populations, especially during their first year, when mortality is high.

Relatively to the anxiety results it was shown that the exposure of European sea bass juveniles to antidepressant FX can alter their anxiety. Regarding the

position in the aquarium, results showed that the number of fishes at the bottom was higher than in the top of the aquarium. Since this pattern was observed during the assay and also in control groups we can exclude any effect of FX on the preference for a position on the aquarium. Contrary to our results, previous studies showed anxiolytic effects of FX which leads to increased time that fishes spend in upper regions of the aquariums. Zebrafish exposed for 14 days to 100 µg/ L of FX, increased the time spent in upper regions of the tank in comparison with control group (Egan *et al*., 2009). Gaworecki and Klane (2008) also observed the same tendency in hybrid striped bass, where individuals that were exposed to 150 µg/ L of FX preferred the top of the tank to the bottom or the center. Since the concentrations used in these two studies were superior to the present study, we cannot exclude the possibility that higher FX concentrations can affect the position in aquariums of European sea bass juveniles. Scototaxis results showed a decrease in anxiety levels. During the entire assay the percentage of individuals with preference for the black compartment of the aquarium (anxiety) was never lower than 60% in the control groups. However, during the period of exposure to FX there was a decrease of individuals with preference for the black compartment of the aquarium. Our results indicate that anxiety levels have decreased in exposed fish during the experimental period. Such pattern was particularly evident in fishes from the top concentration, since at the end of exposure period (21 days) no fish preferred the black compartment. Although this concentration is much higher than those found in the environment (Fent *et al*., 2006; Kreke and Dietrich, 2008; Silva *et al*., 2014), this result can predict negative consequences of a possible exposition of European sea bass juveniles to FX. Namely decrease of shelter search or decrease of fear against predators and therefore enhance the mortality during the first year, endangering the natural populations and their dynamics. During the recuperation period, the preference for the black compartment in the exposed fishes tended to return to initial levels, but at different rates. FX anxiolytic effects have already been reported for several species. Maximino *et al*. (2011) used scototaxis to evaluate the anxiety in zebrafish and acute FX effects were not observed, as white avoidance has not occurred, however after chronic exposure (2 weeks) anxiolytic effects were observed. These results are

in concordance with our results where anxiolytic effects were observed at the 15th day. Also in zebrafish, who were exposed to 100 µg/ L during 14 days, a decrease in anxiety was observed when compared with control group (Egan *et al*., 2009). Valenti *et al*., (2012) also observed decrease of anxiety in fathead minnows after exposure to antidepressant SER (3, 10 and 30 µg/ L) for 28 days. In resume, the present results reinforce the anxiolytic effects of FX in fishes.

5.2. Hepatosomatic index

Liver is the major detoxification organ (Kime, 1998), so a altered liver can be a indicator of contamination (Pinkney *et al*., 2001; Sadekarpawar and Parikh, 2013). Following such approach, the study monitored the evolution of HSI during the assay. After exposure period, HSI increase in comparison to control group. On recovery period there was a decrease of HSI in the three FX groups in comparison with control group where there was a increase. Other studies have already described the same pattern of HSI in fishes exposed to antidepressant FX. Menningen *et al*. (2010) results showed that HSI of goldfish increased when exposed to 0.54 and 54 µg/ L of FX when compared to control group. However no significant differences were detected during the assay and control solvent has the same pattern as the FX groups and therefore we cannot affirm that FX has altered this condition factor.

5.2. Gene expression

Sert is a transporter of 5-HT that is inhibited by SSRI like FX, increasing therefore the extracellular levels of 5-HT. During the assay, significant differences were detected in the expression of gene in fishes exposed to 50 µg/ L of FX. The expression of *sert* increased on the recovery period, after the contaminant was removed. FX can act in *sert* at short term, but alterations on gene expression appears after long time exposure (Kreke and Dietrich, 2008). On 21th day, sea bass juveniles exposed to 50 µg/ L of FX have no significant differences between the other groups, however when the antidepressant is removed there is a increase in the expression of this gene, so we can suppose that FX acted somehow on the transcription of *sert*. Few studies have

documented the effects of FX in the expression of serotonin transporters in fishes. Airhart *et al*. (2007) observed that 4.6 µM of FX had no effect on the expression of *sert* on zebrafish larvae (6 days post fertilization) brain (forebrain, midbrain and hindbrain), however a significant decrease of the expression was observed in the spinal cord. Gaworecki and Klaine (2008) observed a decrease in brain levels of 5-HT in hybrid striped bass after exposition to FX, which can indicates that 5-HT transporters in fishes can also respond to FX. Previously, studies in mammals have also showed effects of FX on gene *sert*. Neumaier *et al*. (1996) studied the effects of chronic exposure to FX on mRNA expression of *sert* in rat dorsal raphe nucleus. This specific area of the brain contains numerous fivers that connect to other regions of the brain, and therefore it is very important in the distribution of 5-HT by the brain, also there is a homologous region of brain in fishes called isthmus (Kreke and Dietrich, 2008). Animals were treated with FX, showed a decrease of *sert* mRNA levels, however after long exposure (plus 14 days) levels of *sert* increased to control groups levels, which may indicate a acute effect of FX on *sert* mRNA expression (Neumaier *et al*., 1996). Le Poul *et al*. (2000) results showed no differences in the expression of *sert* mRNA in rat brain (anterior raphe) exposed to 8 mg/ kg FX for 21 days. Benmansour *et al*. (1999) observed that SSRI paroxetine (10 mg/ kg) increased mRNA expression of *sert* in rat brain (dorsal raphe). The importance of gene *sert* in the modulation of several behaviors has been documented. Experiences with null mutant mice's for *sert* demonstrated that this individuals increased anxiety and reduced exploratory locomotion in the light/ dark exploration (Holmes *et al*., 2003) and the responses to different antidepressants (Holmes *et al*., 2002). Other results also in mice, showed latency to feed and more immobility in forced swim (Lira *et al*., 2003). Due to the conservation in vertebrates of target sites of antidepressants (Kreke and Dietrich, 2008) we can assume the importance of gene *sert* in the behavior of fishes. Since our results show anxiolytic effects of FX, we can suppose that this could be related to the modulation of SERT expression.

Our results showed no significant differences in the expression of gene *5-ht$_{3B}$* and maintained expression levels stable. *5-ht$_{3B}$* protein belongs to the

family of *5-ht$_3$* receptors, wherein this family is the only in 5-HT receptors with ligand-gate ion channel as transduction pathway (Kreke and Dietrich, 2008). This protein is identified both on peripheral and CNS of humans and other mammals (Kreke and Dietrich, 2008; Bétri *et al*., 2011), however in fishes little is known about their presence and/or function in this organisms. Lucchelli *et al*. (1995) results showed in guinea pigs that FX inhibits 5-HT release from *5-ht$_3$* receptors in dorsal raphe, and that FX has moderate potency at central *5-ht$_3$* receptors. The inhibitory effects of FX on *5-ht$_3$* receptors were also investigated by Fan (1994) with whole-cell patch-clamp technique, and for that it was used rat nodose ganglia. Results showed that *5-ht$_3$* receptor is a possible acting site of FX, however it was observed that FX effects on the receptor may only have short-life action (Fan, 1994). More studies about this gene in fish brain should be made, in order to increase knowledge about the role of this gene in serotoninergic system of fishes.

Again, no significant differences were observed in our results, towards to the expression of gene *mao*. Enzymes *mao* have catabolic action, and are responsible for degradation and recycle vesicular 5-HT (Kreke and Dietrich, 2008). These enzymes have already been described in several fish species such as goldfish (Hall and Urueña, 1982), rainbow trout (Edwards *et al*., 1986; Nicotra and Senatori, 1989) and zebrafish (Setini *et al*., 2005). However, little is known about the effects of SSRIs in fish *mao* expression. Cusin *et al*. (2002) observed in humans that antidepressants paroxetine and fluvoxamine appears to have no major role in *mao* A. *Mao* in fishes share similarities with *mao* from mammals, especially with *mao* A (Kreke and Dietriech, 2008; Lillesaar *et al*., 2011). Our results showed differences in expression of *mao* mRNA, however not significant and since this enzyme is not the main target of FX, we can expect that no problematic alterations occur after exposure of European sea bass to this antidepressant. Nonetheless, more studies should be made in order to assess the role of *mao* in fishes, since differences with mammals *mao* exists (Kreke and Dietrich, 2008).

6. Conclusions

In conclusion, the present study showed that antidepressant FX can decrease the levels of anxiety in European sea bass juveniles, nonetheless FX haven't increase the preference for upper regions of the aquarium. Our results showed no influence of FX on shoaling. Also, anorectic effects of FX were not observed in any group during the assay, however food intake increased during the assay in all groups. The present study was not able to investigate the effects of FX on sea bass juveniles swimming velocity, since the number of fishes that swam through the raceway was reduced not allowing to have meaningful results, highlighting the need to perform further studies. No apparent effects on HSI levels were observed after the exposure to FX.

The expression of *sert* has increased from day 21 to 28 after exposure to 50 µg/ L of FX, which could be related to anxiolytic results observed in scototaxis tests. The expression of mRNA of *5-ht*$_{3B}$ and *mao* suffered few alterations, indicating that no important alterations could occur after the exposure to FX.

Chapter 3. Final remarks

The on growing use of antidepressants and the lack of appropriated methods to eliminate them from wastewaters, lead to the problem of ecosystems contamination. However, the investigation of the effects of antidepressants on aquatic organisms or the ecosystems is recent and therefore still need a more thorough collection of information. This study is part of a major study whose objectives were to evaluate the effects of antidepressants in European sea bass at several levels. There is no information in what regards the effects of the antidepressant FX in this species, however due to the conservation of target sites in vertebrates it was expected that similar effects to those already observed in other animals could also occur in our study.

FX has already been described as capable of inducing alterations at several levels of the fish, such as behavioral and physiological changes. Indeed, our study was able to show some of these changes also in juvenile sea bass. For example, the decrease of anxiety that we observed was an example of the anxiolytic effects of FX. This decrease of anxiety can make juveniles less cautious to danger and can affect their search for shelter, especially in juveniles, where the mortality rate is high. Little is known about the effects of FX on gene expression, and our results showed that high concentrations of FX could alter the expression of gene *sert*, which is a pre synaptic serotonin transporter. *sert* protein has already been described as important to modulation of several behaviors (as anxiety and feeding) especially in mammals, so it is important to assess the regulation of the gene in fishes in order to assess possible changes after exposure to FX.

It was not possible to evaluate if FX can alter the swimming velocity of European sea bass juveniles. Swimming capacity is very important for a fish, namely a predator species, to be able capture prey or avoid predators and migrations. If this capacity is compromised, this could bring serious problems to the population dynamics. Even if this important point of the study was not able to be study, it is important that more tests should be made to assess if this behavior could be compromised or not by the exposition to antidepressant FX.

Further studies about the effects of FX and other antidepressants on fishes should be made in the future, not only at behavior level but also at physiological. Also, more tests about target FX genes, namely in fishes should be made in order to increase knowledge about this area and to assess effects that could occur after exposure to antidepressants.

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Annex

Tables

Table I: Sheet used to register the behavior parameters.

	Analisys					
Data	**Morning (Hour)**			**Afternoon (Hour)**		
Parameters						
Solitary						
Top						
Bottom						
Notes						
Feeding						

Table II: Sheet with results of swimming tests. Legend: * time (s) that took to swim the 3 m raceway; ** time (s) resisting water flow; - Individuals that didn't swam. Frequency of individuals that swam (f(S)) and individuals that exhibit flow resistance (f(R)) was also calculated.

Aquarium 5 (Ct)						
Fish/ Day	1	3	7	15	21	28
1	-	-	60**	-	-	-
2	-	-	-	-	-	-
3	-	40**	22**	-	-	-
4	-	-	-	-	-	6*
5	-	-	-	60**		
6	-	1.27*	-	60**		
7	-	30**	-	-		
8	-	60**	-	-		
f (S)	0%	13%	0%	0%	0%	25%
f (R)	0%	38%	25%	25%	0%	0%

Aquarium 8 (CtS)						
Fish/ Day	1	3	7	15	21	28
1	60**	-	-	-	20*	43**
2	-	-	-	60**	-	-
3	60**	60**	-	60**	-	11*
4	60**	60**	60**	24*	-	-
5	-	60**	60**	-		
6	-	-	-	60**		
7	60**	60**	-	-		
8	-	60**	60**	-		
f (S)	0%	0%	0%	13%	25%	25%
f (R)	50%	63%	38%	38%	0%	25%

Aquarium 14 (FX0.5)						
Fish/ Day	1	3	7	15	21	28
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	60**	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-		
6	-	-	-	-		
7	-	-	-	-		
8	-	-	-	-		
f (S)	0%	0%	0%	0%	0%	0%
f (R)	13%	0%	0%	0%	0%	0%

Aquarium 13 (FX5)						
Fish/ Day	1	3	7	15	21	28
1	-	-	-	-	-	-
2	-	-	40*	-	-	60**
3	-	-	20**	-	-	60**
4	60**	60**	60**	20*	-	-
5	-	30**	-	17*		
6	20*	-	60**	-		
7	-	-	60**	6*		
8	60**	-	-	24*		
f (S)	13%	0%	13%	50%	0%	0%
f (R)	25%	25%	50%	0%	0%	50%

Aquarium 2 (FX50)						
Fish/ Day	1	3	7	15	21	28
1	-	-	-	-	-	-
2	-	-	-	-	-	19*
3	-	-	-	-	-	-
4	-	-	60**	22*	-	-
5	60**	-	60**	-		
6	-	-	-	6*		
7	60**	-	-	-		
8	-	-	60**	-		
f (S)	0%	0%	0%	25%	0%	25%
f (R)	25%	0%	38%	0%	0%	0%

Figures

Figure I: Raceway device used for the swimming tests.

Figure II: Aquarium (with black and white compartment) used for anxiety tests.

Protocols

Protocol I

Note: Always start the process from the control group to the highest concentration of FX.

1. Collect the animal and put him in a small aquarium for a short time, for cleaning the animal;

2. Take the animal from the aquarium and put him carefully in the raceway device;

3. Start to count when the animal reach the starting point (0 meters) ;

4. Stop the count when the animal reach the finish point (3 meters), and register time he toke to reach it;

5. If the animal doesn't swim but resist to the water flow, time he spend resisting was also registed

6. Take the animal from the raceway device and put him again in the test aquarium;

7. Repeat the procedure to the other fishes in each aquarium.

Protocol II

Note: Always start the process from the control group to the highest concentration of FX.

1. Put the sliding doors in the scototaxis aquarium;

2. Collect the animal and put him in a small aquarium for a short time, for cleaning the animal;

3. Take the animal from the aquarium and put him carefully in the central zone of the aquarium;

4. After a short period of acclimatization, remove the sliding doors at the same time;

5. Wait until the fish choose a zone of the aquarium and stabilize, and register his preference;

6. Take the animal from the aquarium and put him again in the test aquarium;

7. Repeat the procedure to the other fishes in each aquarium.