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Embryotoxicity and molecular alterations of monoamine reuptake inhibitory drugs in a teleost fish

Pedro Miguel Carneiro Rodrigues

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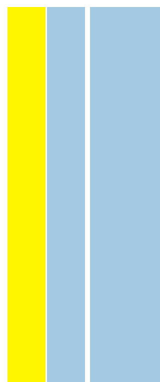

Pedro Miguel Carneiro Rodrigues

Embryotoxicity and molecular alterations of monoamine reuptake inhibitory drugs in a teleost fish

Dissertação de candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas Abel Salazar

Orientadora: Doutora Laura Guimarães
Categoria: Investigadora Auxiliar
Afiliação: Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Coorientadora: Doutora Marta Ferreira
Categoria: Investigadora Auxiliar / Senior Lecturer
Afiliação: Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) / School of Marine Studies, Faculty of Science, Techonology and Environment, The University of South Pacific,

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“A challenge only becomes an obstacle when you bow to it.”

Ray A. Davis

Resumo

A presença de produtos farmacêuticos em ecossistemas aquáticos é um problema crescente, em particular a classe de drogas psicoativas, cujo consumo tem vindo a aumentar devido à crise econômica e outros problemas sociais. Além disto, as concentrações destas substâncias no ambiente têm vindo a aumentar, sendo os seus efeitos em espécies não alvo ainda desconhecidos. A norflluoxetina é um inibidor seletivo da reabsorção de serotonina (SSRI) e é o metabolito ativo da fluoxetina, um dos antidepressivos mais consumidos no mundo. A venlafaxina é um inibidor seletivo da recaptação de serotonina e de noradrenalina (SNRI). É comercializado sob a designação comercial de Effexor e é também um dos antidepressivos mais consumidos mundialmente. Estes compostos são duas das drogas mais frequentemente detetadas em ambientes aquáticos e o conhecimento dos seus efeitos em organismos aquáticos é ainda escasso. Portanto, é necessário compreender seu impacto nos organismos não-alvo. Os embriões de teleósteos (peixes-zebra) foram utilizados como modelo neste estudo para investigar os efeitos dessas substâncias. Foram escolhidos estes embriões devido à sua transparência e facilidade de manuseamento que permite monitorizar o desenvolvimento do embrião. Este trabalho tem como principais objetivos, compreender a influência das exposições individuais, tanto para norfluoxetina e venlafaxina no desenvolvimento embrionário do peixe-zebra, e avaliar os efeitos dessas drogas sobre os genes envolvidos no modo de ação e metabolização de ambas as substâncias. A investigação dos efeitos de um cocktail de ambas as substâncias sobre os níveis encontrados em sistemas aquáticos naturais foi realizada.

Os embriões foram recolhidos uma hora após a fertilização (HPF) e expostos durante 80 horas a diferentes concentrações de norfluoxetina (0,64, 3.2, 16, 80 e 400ng/L), venlafaxina (16, 80, 400, 2000 e 10000ng/L) ou um cocktail de 3.2ng/L norfluoxetina e 2000ng/L venlafaxina. Nesta gama de concentrações foram incluídos valores previamente encontrados em amostras recolhidas em ambiente natural. Durante este período, foram feitas observações ás 8, 32 e 80 hpf para registrar o número de

embriões mortos e anormalidades do desenvolvimento. A transcrição de mRNA dos genes de interesse foi medida por meios de qRT-PCR. Os genes alvo eram recetores e transportadores neuro-hormonais (serotonina, dopamina e norepinefrina), monoamina oxidase, transportador vesicular de monoaminas, vários recetores nucleares, bem como genes envolvidos em processos de desintoxicação e antioxidantes. Os resultados mostram que tanto a norfluoxetina como a venlafaxina não tiveram influência sobre as taxas de mortalidade, enquanto que o cocktail causou mortalidade significativa em todos os pontos de observação ($p<0.05$), em relação ao controlo. Este aumento de mortalidade foi, ainda assim, mais proeminente entre as 0 e 8 horas pós fertilização. Além disso, a exposição a norfluoxetina, venlafaxina ou um cocktail, causou um aumento de anormalidades totais encontrados durante o desenvolvimento embrionário. Os embriões expostos ás concentrações mais elevadas nos testes foram os mais afetados. Anormalidades na pigmentação, coluna/cauda e saco vitelino foram as mais significativas. As anomalias encontradas ao nível da pigmentação em todas as condições testadas podem ser utilizadas como indicador de exposição. Quanto à expressão de genes, foram encontradas mais diferenças significativas em relação ao controle nas exposições a venlafaxina do que a norfluoxetina ($p <0{,}05$). Para a norfluoxetina um padrão geral de luz de indução da expressão génica era visível. A maioria dos genes afetados foram *rxrga* (3,2 ng / L), *5-ht2c* (80 ng/L), *adra2c* (0.64ng/L) e *Cu/Zn sod* (400ng/L). Na venlafaxina, um padrão geral de inibição de expressão génica foi detetado. Os genes que sofreram maior inibição foram transportadores *abc*, recetores nucleares *rxr* e *ppar*, *5-ht2c*, *drd1b* e *vmat2*. Estes genes possuem uma resposta em forma de U na gama de 80-2000ng/L, em que a concentração de 400ng/L foi a mais afetada (inibida), o que poderá significar que as abordagens tradicionais nos cálculos de risco podem não ser as ideais para essa substância. Esses genes exibiram fortes correlações entre si, devendo o seu uso como possíveis marcadores de exposição ser estudado em maior detalhe. Estes genes mostraram uma forte correlação entre si e têm sido previamente associados com alterações no metabolismo dos lípidos e da estimulação de dopamina. Em embriões expostos ao cocktail foi testado um conjunto de genes mais

pequeno. A seleção foi baseada nos resultados obtidos para os compostos individuais. Os resultados mostraram diferenças entre genes neuro-hormonais e de metabolização, com inibição e respostas intermediárias maioritárias especialmente em relação ás exposições individuais, respetivamente. O padrão de resposta ao cocktail foi mais parecido com o da norfluoxetina.

No geral, os resultados obtidos para esta janela de desenvolvimento parecem contradizer a ideia geral de que a norfluoxetina é mais potente do que o composto parental. Deve também ser ressalvado que concentrações de venlafaxina na gama de 80-2000ng/L estão dentro do intervalo encontrado em meio aquático. Além disso, *abcb4*, a primeira linha de defesa dos peixes foi inibida em concentrações ambientais relevantes, que significa que esta substância pode pôr em perigo a sobrevivência do organismo. Os resultados do cocktail parecem sugerir uma ação antagonista entre os dois fármacos. Contudo, foram também visíveis algumas relações sinergéticas em mais pequena escala.

Em conclusão, a norfluoxetina, venlafaxina e o cocktail tiveram efeitos sobre o desenvolvimento embrionário do modelo teleósteo empregado. Sistemas neuro-hormonais e vias de desintoxicação foram particularmente afetadas pela venlafaxina, mas os efeitos poderão ser suavizados ou potenciados pela coocorrência de ambas as drogas. Pesquisas futuras devem concentrar-se em esclarecer estas questões, de forma a entender melhor os seus efeitos negativos sobre o meio ambiente natural.

Abstract

The presence of pharmaceuticals in aquatic ecosystems is a growing problem, in particular the class of psychoactive drugs, which consumption has been increasing due to the economic downturn and other social problems. Moreover, the concentrations of pharmaceuticals is increasing in aquatic ecosystems and the effects of these substances for non-target organisms is still unknown. Norflluoxetine is a selective serotonin reuptake inhibitor (SSRI) and is the active metabolite of fluoxetine, one of the most consumed antidepressants worldwide. Venlafaxine is a serotonin and norepinephrine reuptake inhibitor (SNRI). It is marketed under the trade name Effexor and is also among the most consumed antidepressants. These compounds are two of the drugs most frequently detected in aquatic environments and the knowledge of their effects on aquatic organisms is still scarce. In this study a teleost embryo (zebrafish) was used as model to investigate effects of these substances. The choice was due to its transparency, and easiness to handle, which allows to monitor the development of the embryo. The present work had the main objectives of understanding the influence of single exposures to both norfluoxetine and venlafaxine in the embryonic development of zebrafish, as well as, to evaluate effects of these pharmaceuticals in genes implicated in the mode of action and metabolisation of both substances. Investigation of the effects of a cocktail of both substances at levels found in natural aquatic systems was also carried out.

Embryos were collected 1-hour post-fertilization (hpf) and exposed for 80 hours to different concentrations of norfluoxetine (0.64, 3.2, 16, 80 and 400ng/L), venlafaxine (16, 80, 400, 2000 and 10000ng/L) or a cocktail of 3.2ng/L of norfluoxetine and 2000ng/L of venlafaxine. Those concentrations included values previously found in environmental samples. During this period, observations were made at 8, 32 and 80hpf to register the number of dead embryos and developmental anomalies. mRNA transcription of genes of interest was measured by qRT-PCR. The target genes were

neurohormonal (serotonin, dopamine and norepinephrine) receptors and transporters, monoamine oxidase, vesicular monoamine transporter, several ligand-binding nuclear receptors, as well as genes involved in detoxification and antioxidant processes. Results show that both norfluoxetine and venlafaxine had no influence in mortality rates. For the cocktail was visible a significant induction of mortality in all analysis points ($p<0.05$), relative to the control. However, this increase was prominent between 0 and 8hpf. On the other hand, exposure to norfluoxetine, venlafaxine and cocktail, caused an increase in the total anomalies found during embryonic development, although in different stages of the embryonic development. Embryos exposed to higher test concentrations were the most affected ones. Anomalies in pigmentation, vertebral column/tail and vitelline sac were the most significant. Anomalies in pigmentation found in all test conditions can be used as an exposure indicator. Concerning gene expression, more significant differences relative to control were found for venlafaxine than norfluoxetine ($p<0.05$). For norfluoxetine a pattern of slight gene induction was visible. Most affected genes were *rxrga* (3.2 ng/L), *5-ht2c* (80 ng/L), *adra2c* (0.64ng/L) and *Cu/Zn sod* (400ng/L). For venlafaxine, a general pattern of gene inhibition was detected. Most inhibited genes were *abc* transporters, *rxr* and *ppar* nuclear receptors, 5-ht2c, *drd1b* and *vmat2*. These genes presented a u-shape response in the range 80-2000 ng/L, where 400ng/L concentration was the most affected (inhibited), meaning that traditional approaches in risk calculations may not be the ideal for this substance. These genes showed strong correlations among each other and the use of them as exposure indicators should be study in more detail. These genes, have been previously associated to disturbances in lipids and xenobiotics metabolism and efflux, as well as, dopamine stimulation related to drug dependence in the CNS. In cocktail-exposed embryos a smaller pool of genes was tested. The selection was based on the results obtained for the single compounds. Results showed differences between neurohormonal and metabolisation genes, with inhibition and mostly intermediate responses in relation to the single exposures, respectively. The cocktail pattern of response resembled more closely that of norfluoxetine.

Overall, the results obtained for this developmental window counteract the general idea that norfluoxetine is more potent than the parental compound. Venlafaxine concentrations 80-2000ng/L are within the range found in aquatic compartments. Moreover, *abcb4*, the first line of defence of fish, was significantly inhibited at environmentally relevant concentrations meaning that this substance can jeopardise organism survival. The cocktail results, suggest an antagonistic action for the two substances in some of the tested genes, mainly the ones involved in the substance metabolism. However, some synergetic effects also occurred on a smaller scale.

In conclusion, norfluoxetine, venlafaxine and the cocktail had effects on embryonic development of the teleost model employed. Neurohormonal systems and detoxification pathways were especially affected by venlafaxine, but may be potentiated or attenuated by co-occurrence of both drugs, depending on the endpoint evaluated. Future research should focus on clarifying this to better understand its negative effects on the natural environment.

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Abbreviations

5-HT - 5-hydroxytryptamine

5-ht2c - serotonin receptor 5-ht 2c

5-ht1a - serotonin receptor 5-ht 1a

ABC - ATP-binding cassette

abcb - P-glycoprotein

abcc - multidrug resistance associated protein

abcg2 - breast cancer resistance-associated protein

adra2a - noradrenaline receptor alpha-2a

adra2b - noradrenaline receptor alpha-2b

adra2c - noradrenaline receptor alpha-2c

Ahr - Aryl hydrocarbon receptor

AOP's – Adverse Outcome Pathways

cat - catalase

cyp - Cytochrome P450

Cu/Zn sod - superoxide dismutase

dat - dopamine transporter

drd1b - dopamine receptor D1B

drd2b - dopamine receptor D2B

dpf - days post fertilization

GST - glutathione S-transferase

hpf - hours post fertilization

mao - monoamine oxidase

MXR - multixenobiotic resistance

net - norepinephrine transporter

NRs - nuclear receptors

PCR - polymerase chain reaction

ppars - peroxisome proliferator activated receptor

pxr -pregnane X receptor

qRT-PCR - quantitative real time PCR

ROS - reactive oxygen species

rxr- retinoid X receptor

serta - serotonin transporter

SNRI – serotonin and norepinephrine reuptake inhibitor

SSRI - selective serotonin reuptake inhibitor

vmat2 - vesicular monoamine transporter

WWTP’s - Wastewater Treatment Plants

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Chapter 1: Introduction and objectives

1.Introduction

1.1. Pharmaceuticals as environmental pollutants

Contamination of aquatic ecosystems by pharmaceuticals is a problem that has been rising continuously in the last decades. Substances in this class of pharmaceuticals are nowadays recognized as emerging contaminants of public and scientific concern (Evgenidou *et al.*, 2015). Considering current social habits, increased life expectancy and population growth on the planet, consumption and generation of pharmaceuticals is globally increasing. Cconsequently, detection of these substances is becoming more usual and persistent in different sampling environments like natural water, drinking water and wastewater (Corcoran *et al.*, 2010; Ortiz de García *et al.*, 2014). Hence, there is an increased interest in the study of occurrence, fate and impacts of pharmaceutical products on the aquatic environment that led to a large number of published papers in recent years (Ortiz de García *et al.*, 2014; Evgenidou *et al.*, 2015).

The first studies in this area date back to the 1970s. However, only in the 1990s investigation and knowledge about contamination by pharmaceuticals in the aquatic environment increased, mainly due to advances reached in analytical methods (Fent *et al.*, 2006). Pharmaceuticals are very soluble and occur in small concentrations in the aquatic environment. Because of that methods traditionally employed to analyse aquatic pollutants were ineffective (Daughton and Ternes, 1999). Only in the 1990s the panorama changed and new methods, with higher separatory efficiencies and ability to find more polar compounds appeared, allowing scientists to detect concentrations in environmental compartments in the order of ng/L and μg/L (Daughton and Ternes,1999; Fent *et al.*, 2006; Santos *et al.*, 2010).

Pharmaceuticals are designed to have a specific mode of action, in order to exert their desired therapeutic effect. However, these are also characteristics that make them persistent in the environment and, ultimately, cause potential toxicity to fauna and flora (Fent *et al.*, 2006). This is even more visible in the aquatic environment. Pharmaceuticals are polar and non-volatile. Their distribution is made by aqueous

transport, which retains pharmaceuticals in water, making aquatic organisms susceptible to these pollutants (Santos *et al.*, 2010). Although risks to wildlife are known, existing knowledge in this field is still scarce in important aspects such as toxicity of metabolites, conjugates and degradation products of pharmaceuticals (Evgenidou *et al.*, 2015). Risks to wildlife exist not only at an acute toxicity level. There are also risks of long-term exposure, though these ones remain mostly unknown (Ortiz de García *et al.*, 2014; Corcoran *et al.*, 2010). Another problem is that pharmaceutical products can raise concern not only as sole compounds *per se*, but also in complex mixtures of different substances (Evgenidou *et al.*, 2015).

1.2 Sources of environmental contamination by pharmaceuticals

Pharmaceutical products are continuously released in the environment, although in small quantities (Santos *et al.*, 2010). After being consumed by humans, pharmaceuticals pass through liver phase I and II of drug metabolism, where they become more water soluble (phase I) and subsequently conjugated and/or excreted (phase II). Due to these reactions, pharmaceuticals are excreted by humans in two different forms: unchanged or in the form of metabolites (Brown *et al.*, 2015). Hospital effluents and direct elimination (e.g. through inadequate sanitary disposal) of unused pharmaceuticals in sewage are among the most important sources of contamination (Santos *et al.*, 2010) (Figure 1.).

Effluents are treated in WWTPs (Waste Water Treatment Plants) by three main processes of pollutants' removal, although only two are legally required. In the first treatment, the removal of suspended solids occurs. This treatment has a low degree of efficiency in the removal of micro pollutants like pharmaceuticals. In the second treatment several types of reactions occur, such as: dilution, partition, biotic and abiotic transformation (Luo *et al.*, 2014). In this treatment the level of efficiency is variable depending on the pharmaceutical in question (Luo *et al.*, 2014). The third treatment is optional and is related to health questions to humans or specific purposes of the treated water and consists in further removal of some compounds like nitrogen or phosphorus (Luo *et al.*, 2014; Guardabassi *et al.*, 2002). After this processing, in

some cases, effluent concentrations can be higher than influent ones (Luo *et al.*, 2014). This can be explained by the presence in influents of some substances, like metabolites, that can be transformed back into the parental compound during the biological treatment in WWTPs. Pharmaceuticals can also be imprisoned in faecal matter and released in the water during biological treatment increasing the overall concentration of the substance (Luo *et al.*, 2014). This shows that WWTPs are still not designed to treat pharmaceuticals and the existing treatments are not fully efficient in the removal of this kind of pollutant. This results in discharges of pharmaceuticals, together with treated effluents, in surface and ground water and their consequent occurrence in drinking water (Luo *et al.*, 2014; Santos *et al.*, 2010).

The use of pharmaceuticals is not exclusive of humans. This kind of substances is also used in agriculture and aquaculture to treat diseased animals. Such pharmaceutical products are excreted in the urine and faeces of animals, entering in the environment without any kind of treatment and contaminating the soil, and consequently ground water (Santos *et al.*, 2010). Other anthropological activities also act as sources of contamination. Industry discharges, sometimes illegally, or the use of WWTPs sludge contaminated with pharmaceuticals as fertilizer, are examples of these (Santos *et al.*, 2010).

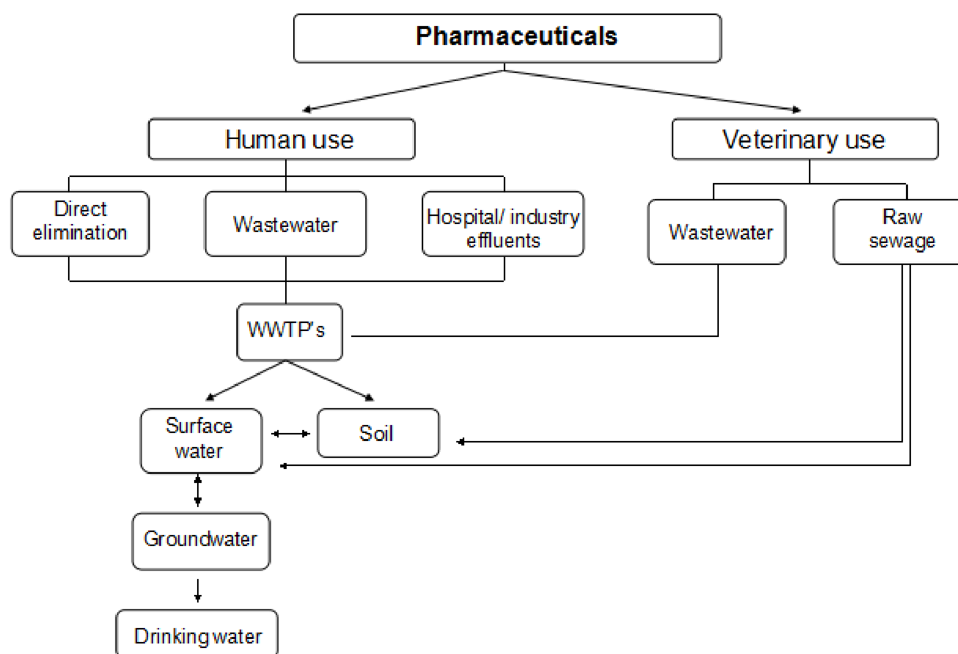


Figure 1. Schematic representation of the sources and fate of pharmaceuticals in the environment (adapted from Kummerer, 2001)

Nowadays, all classes of pharmaceuticals are detected in the aquatic environment. Concentrations found vary according to the class of pharmaceuticals and the zone of the globe where sampling is conducted (Fent *et al.*, 2006; Santos *et al.*, 2010). Some classes of pharmaceuticals are more consumed than others and that can contribute to a higher detection of some classes in the environment. This relationship between the use and detection of pharmaceuticals, however, is not always correct (Fent *et al.*, 2006). The presence of a pharmaceutical in the environment is also connected to the physico-chemical properties of the pharmaceutical that influence the persistence and durability of the substance in the water. Also the percentage of removal in WWTPs is linked to different levels of occurrence of pharmaceuticals in the water given that, like previously mentioned, the quantities of removal differ greatly from substance to substance; this can explain in part differences in the quantities detected in the aquatic environment (Santos *et al.*, 2010; Luo *et al.*, 2014)

1.3 The case of antidepressants

With the human population exposed to increased stress as a result of the current economic and social context, antidepressants' consumption has risen on a large scale around the world. Indeed, this class of drugs is one of the most detected in the environment (Santos *et al.*, 2010; OECD, 2014). In Europe, recent data show that consumption of antidepressants has risen considerably since 2000 (OECD, 2014). Portugal, in particular, is one of the European Union countries where antidepressant consumption was higher in 2012 (Figure 2.) (OECD, 2014). This increasing consumption is also linked with the massive appearance of the selective serotonin reuptake inhibitors (SSRI) type of antidepressants in the market and most recently with the appearance of serotonin–norepinephrine reuptake inhibitors (SNRI) (Gusmão *et al.*, 2013; Melnyk-Lamont *et al.*, 2014).

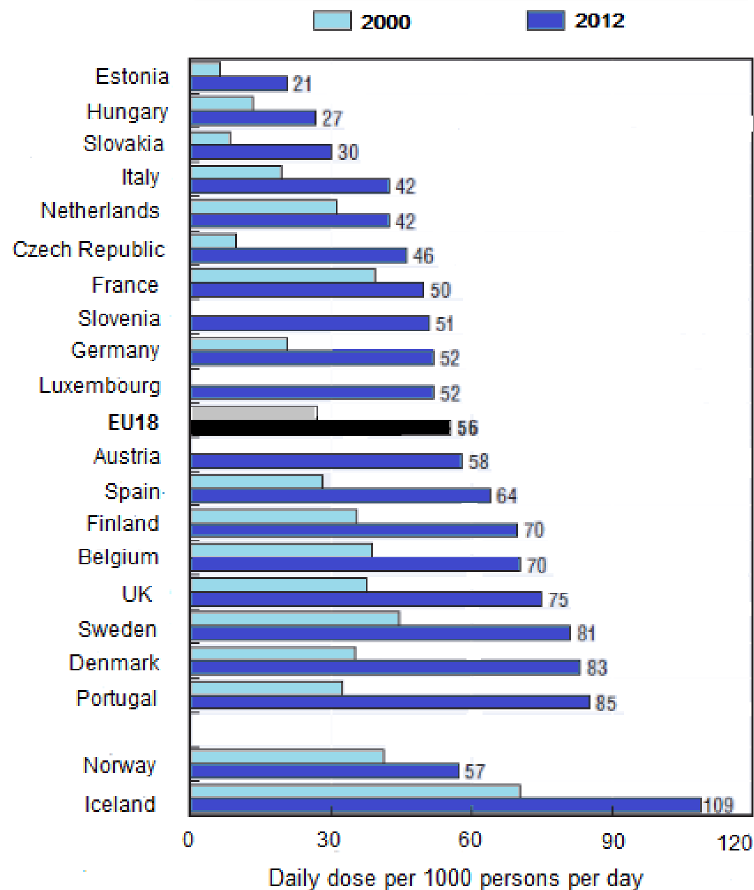


Figure 2. Evolution of consumption of antidepressants between 2000 and 2012 in Europe (adapted from *Health at a Glance: Europe 2014, OECD*)

1.3.1 Selective Serotonine Reuptake Inhibitors (SSRI): fluoxetine and norfluoxetine

SSRI are a class of antidepressants that comprise six different main compounds: fluoxetine, sertraline, escitalopram, paroxetine, fluvoxamine and citalopram (Patel *et al.*, 2015). In this work we are going to focus in fluoxetine metabolite, norfluoxetine.

Like previously mentioned fluoxetine is an antidepressant of the SSRI class. Its main indications are for use in moderate to severe depression, obsessive-compulsive disorder, food and panic disturbances and anxiety (Dulawa *et al.*, 2004). It is used as fluoxetine hydrochloride, commonly under the trade name Prozac, in two different forms: oral solution and a capsule formulation. It is one of the most used and prescribed antidepressants worldwide (Mennigen *et al.*, 2011; Winder *et al.*, 2012). The marketed drug, is a racemic mixture of two different enantiomers, r-fluoxetine and s-fluoxetine. The two enantiomers have similar potency (Baumann *et al.*, 2002). The bioavailability of the two different marketed forms is almost similar (De Vane., 1999). The overall bioavaibility of fluoxetine, however, tends to be low due to the presystemic metabolism of vertebrates (DeVane, 1999). Fluoxetine is also stable in water and has frequently been detected in aquatic environments, in concentrations in the order of ng/L to ug/L (Barry, 2013).

The mode of action of SSRI antidepressants involves the inhibition by presynaptic receptors of serotonin (5-hidroxitroptamine, 5-HT) reuptake. This increases the overall concentration of active serotonin in the synaptic cleft, potentiating its effect (Mennigen *et al.*, 2011) (Figure 3.). Serotonin is a neurotransmitter involved in neural and hormonal mechanisms participating in the regulation of endocrine regulatory functions. Thus, an alteration in serotonin levels can cause changes in various physiological processes and the behaviour of an organism (Fent *et al.*, 2006). In this SSRI mechanism, in addition to the 5-HT receptors, there are other role players like monoamine oxidase (MAO) or vesicular monoamine transporter (VMAT). MAO is a monoamine responsible for oxidative deamination of a great number of amines, where serotonin is included, independently of its endogenous or exogenous origin

(Sallinen *et al.*, 2009). In mammalians two different MAO isoforms are known: MAO A and MAO B, while in zebrafish there is only one MAO form. In this fish species, MAO presents high affinity to 5-HT and studies suggest that MAO has a major role in 5-HT metabolism in adult individuals (Anichtchick *et al.*, 2006). VMAT is a transport protein whose function is the uptake of different monoamines, serotonin included, into storage vesicles and subsequent release in the central nervous system (CNS). In humans, two different kinds of VMATs are known, with different distribution in the tissues: VMAT1 and VMAT2. VMAT 1 is mostly present in neuroendocrine cells, while VMAT2 is mainly expressed in the CNS (Wimalasena, 2011). Although different, VMAT1 and VMAT2 have similar binding affinity to serotonin (Wimalasena, 2011). Different studies showed that VMAT2 is related with several neurological disorders and stress response, like Parkinson's disease where VMAT2 is used as a diagnostic tool (Tillinger *et al.*, 2010; Wimalasena, 2011)

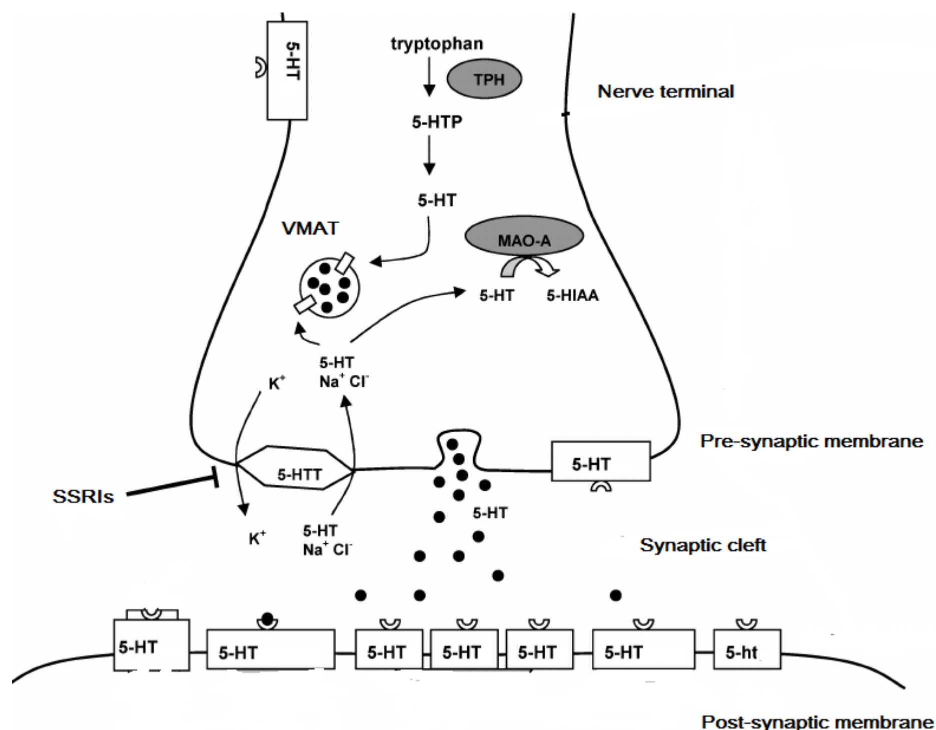


Figure 3. SSRI mechanism of action (adapted from: Kreke and Dietrich, 2008)

5-HT receptors have been found in vertebrates and invertebrates. They are considered as very conserved from an evolutionary point of view (Kreke and Dietrich.,

2008). However, their specific role at the physiological level and their mode of action is still unknown in many species, making difficult to evaluate the impact of environmental exposure to SSRI, particularly in aquatic ecosystems (Kreke and Dietrich, 2008; Connors *et al.*, 2014). As SSRI, fluoxetine acts on neural receptors leading to alterations in the level of serotonin in the synaptic cleft. Fluoxetine may also interact with dopaminergic and adrenergic systems (Kreke and Dietrich, 2008). Previous studies on aquatic organisms, particularly in zebrafish, demonstrated that serotonin (Schultz *et al.*, 2011) and dopamine (Fontaine *et al.*, 2013) are involved in embryonic development, while adrenergic receptors are also responsible for the pigmentation in embryos and adults (Xu and Xie, 2011). 5-HT receptors in aquatic organisms were found in different parts of the brain and at very early stages of embryo development (Kreke and Dietrich, 2008). In fact, in zebrafish, the development of the serotonergic system occurs between 1 and 4dpf (days post fertilization). This system has two different pathways: the first one occurs between 1 and 2dpf with separate populations of serotonergic neurons with growth cones in the spinal cord; the second one occurs between 3 and 4dpf with the development of the raphespinal projection (Airhart *et al.*, 2012). Furthermore, there is evidence that serotonin and 5-HT receptors are also involved in reproduction, immune system and behaviour (Kreke and Dietrich, 2008). That said, it is likely that exposure of aquatic vertebrates to SSRIs such as fluoxetine, induces changes in the development and behaviour of these animals. This is confirmed by several studies that have linked exposure to fluoxetine in fish with the disruption of endocrine functions involved in various physiological processes at the level of reproduction, growth, stress, immune system and behaviour (Mennigen *et al.*, 2011).

Beyond the parental compound, fluoxetine, its main metabolite norfluoxetine (Figure 4.) can also be a possible source of problems to aquatic organisms, since it has been discovered in fish tissues and different environmental water samples in the order of ng/g and ng/L, respectively (Brooks *et al.*, 2005; Santos *et al.*, 2010). Norfluoxetine is considered an SSRI and its use as antidepressant was investigated, but the development of the drug was never completed and consequently it did not

come to the global market (PubChem, 2015). Like the main compound, norfluoxetine presents two different enantiomers, r-norfluoxetine and s-norfluoxetine, but contrary to fluoxetine, norfluoxetine enantiomers have different potency, being s-norfluoxetine 20-fold more potent than r-norfluoxetine (Stanley *et al.*, 2007). In this study was used norfluoxetine in solid crystals containing a mixture of both enantiomers. After the consumption of fluoxetine by humans, the compound is metabolised in the liver, giving rise to norfluoxetine by demethylation reactions (*Fong et al.*, 2008). The n-demethylation of r- and s-fluoxetine into its metabolites r- and s-norfluoxetine is catalysed by different types of genes from the Cyp (Cytochrome P450) family, in humans (Ring *et al.*, 2001). Different Cyp genes are involved in the n-demethylation of the two different enantiomers of fluoxetine. Genes involved in the formation of r-norfluoxetine are the following: Cyp2D6, Cyp2C9, Cyp2C19, Cyp3A4 and Cyp3A5. Among these, Cyp2D6 and Cyp2C9 are the ones with major role in this process. In the case of the formation of s-norfluoxetine, Cyps involved are the following ones: Cyp2D6, Cyp2C19, Cyp3A4 and Cyp3A5, with Cyp2D6 playing a major role in the process (Ring *et al.*, 2001; Margolis *et al.*, 2000).

Although r- and s-fluoxetine are similar in terms of potency, s-fluoxetine presents a higher potential to inhibit Cyp2D6. Studies showed that s-fluoxetine is six times more potent at inhibiting Cyp2D6 than r-fluoxetine (Stevens and Wrighton, 1993). Data obtained from patients, also showed that s-norfluoxetine concentrations in plasma were the double of the r-norfluoxetine ones, but the ratio between them could vary from 1.5 to 3 (Potts and Parli, 1992; Torok-Both *et al.,* 1992). Moreover, only a small percentage (20 to 30%) of fluoxetine remains unchanged in the urine; the remaining percentage is a mixture of different metabolites where norfluoxetine is the most active one (Fong *et al.*, 2008). Another difference between fluoxetine and norfluoxetine, lies in the half-life time, which is higher for norfluoxetine (Brooks, 2014). Studies have also shown that norfluoxetine is more potent than fluoxetine (Hiemke and Hartter., 2000). However, only a sparse quantity of studies is available regarding the effects of norfluoxetine in aquatic organisms. The first published study in this area dates back to 2008 and showed that norfluoxetine induces spawning and parturition in bivalves

(Fong *et al.*, 2008).

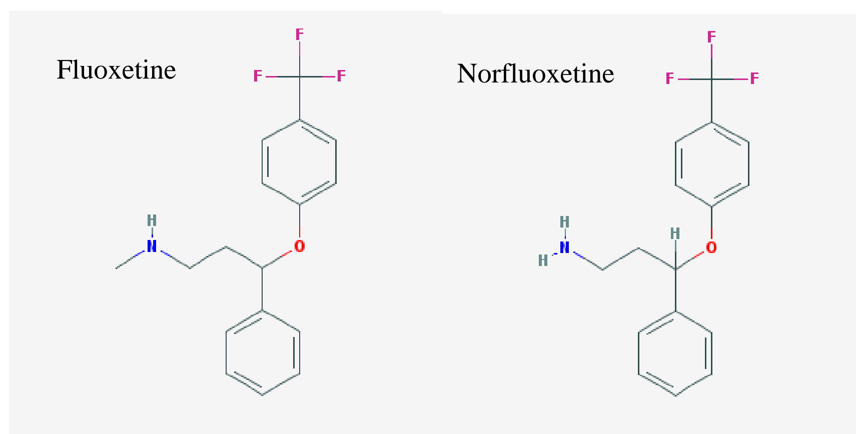


Figure 4. Comparison between fluoxetine and norfluoxetine 2D structures (source: PubChem)

1.3.2 Serotonin–norepinephrine reuptake inhibitors (SNRI): venlafaxine

SNRI are a class of antidepressants that comprises six different main compounds: venlafaxine, desvenlafaxine, duloxetine, milnacipran, levomilnacipran and sibutramine (FDA, 2016; Sansone and Sansone., 2014). In this work we are going to focus in venlafaxine (Figure 5.).

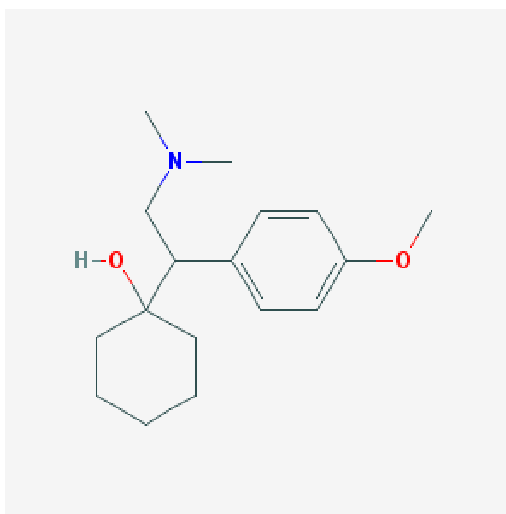


Figure 5. Venlafaxine 2D structure (source: PubChem)

Like previously mentioned venlafaxine is an antidepressant of the SNRI class. Its main indications are for use in the treatment of major depressive disorders, generalised anxiety disorders, chronic pain syndromes and social phobia (Gutierrez *et*

al., 2003; Sansone and Sansone, 2014). It is used as venlafaxine hydrochloride under the trade name Effexor®, in two different forms: immediate release and extended release. It is one of the most prescribed antidepressants in the last years (Sansone and Sansone, 2014; Melnyk-Lamont *et al.*, 2014). Those two forms are very similar. Their main differences are the half-life time (14 hours for extended release and 5 hours to immediate release), dose per day (twice a day for immediate release and once a day to extended release) (Olver *et al.,* 2004; Sansone and Sansone, 2014). The extended release form was found to cause less nausea and dizziness. Venlafaxine has been detected in the aquatic environment with concentrations ranging from few ng/L to 2ug/L in wastewater effluents (Schultz *et al.*, 2010; Gonzalez Alonso *et al.*, 2010).

Both serotonin and norepinephrine are neurotransmitters involved in neural and hormonal mechanisms participating in the regulation of endocrine regulatory functions. Thus, an alteration in serotonin and norepinephrine levels can cause changes in the behaviour of an organism (Fent *et al.*, 2006; Melnyk-Lamont *et al.*, 2014).

The mechanism of action of SNRIs is similar to that of SSRIs. The main difference is that SNRIs, besides the inhibition of presynaptic receptors of serotonin reuptake, also inhibit the presynaptic receptors of norepinephrine reuptake (Figure 6.). This leads to an increase in the overall concentration of serotonin and norepinephrine in the synaptic cleft, potentiating their action (Gutierrez *et al.*, 2003; Mennigen *et al.*, 2011).

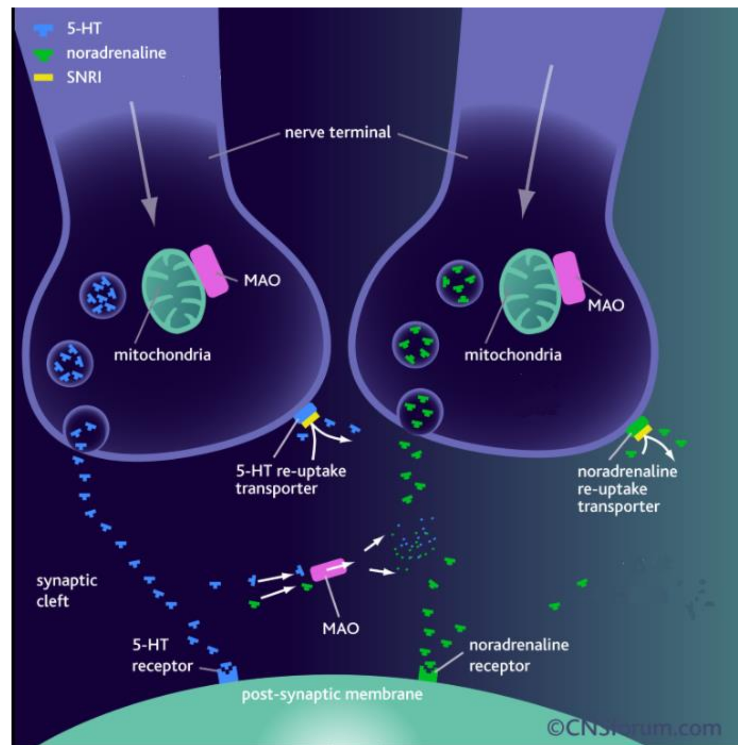


Figure 6. SNRIs mechanism of action (from CNS Forum)

Norepinephrine pathways are also very conserved in fish (Kreke and Dietrich, 2008), however their specific role on the physiology of non-target organisms, like fish, are still unknown (Kreke and Dietrich, 2008; Melnyk-Lamont *et al.*, 2014). However, these monoaminergic pathways are linked to different kinds of behaviours like feeding, locomotion and aggression (Kreke and Dietrich, 2008). In mammals' venlafaxine shows different grades of inhibition depending on the concentration. At lower doses, venlafaxine acts as an SSRI, inhibiting only the serotonin reuptake, while at higher doses it inhibits both serotonin and norepinephrine (Redrobe *et al.*, 1998). This can be explained by the higher affinity of venlafaxine to serotonin receptors than to norepinephrine receptors (Montgomery, 2008). Although low, venlafaxine has also affinity to dopamine and at high doses may have effects in the reuptake of dopamine (Sansone and Sansone, 2014). Like the 5-HT receptors, noradrenergic ones are also present in different parts of the brain and at very early stages of embryonic development (Kastenhuber *et al.*, 2010). In fact, the first zebrafish adrenergic neurons appear after 24hpf, and the overall adrenergic system is complete at 5dpf exhibiting high similarity to the system described in adults (Kastenhuber *et al.*, 2010). Since

venlafaxine is a SNRI acting in neural receptors, which lead to alterations in the levels of serotonin, norepinephrine and even dopamine in the synaptic cleft, it can lead to significant alterations in several physiological functions of the organisms (Redrobe *et al.*, 1998). In fact, several studies regarding the possible effects of venlafaxine in fish, showed that this pharmaceutical may cause alterations in reproduction and behaviour (Galus *et al.*, 2013; Schultz *et al.*, 2011). Moreover, alterations in the adrenergic system, one of this SNRI targets, can alter pigmentation of zebrafish embryos and adults (Xu and Xie, 2011; Ruuskanen *et al.*, 2005); gene expression of neural development and regulation (Thomas *et al.*, 2012) and may also block epinephrine-induced glucose production affecting the stress response of fish exposed to venlafaxine (Ings *et al.*, 2012).

1.4 Biotransformation of xenobiotics (pharmaceuticals) by the organism

The body has several detoxification mechanisms to deal with the presence of toxic substances, which eliminates them substances either in their original state or in the form of metabolites. Among these mechanisms are biotransformation enzymes of phase I and phase II, transport proteins from phase 0 and phase III, and antioxidant enzymes. Cytochrome P4501A (CYP1A) and cytochrome P4503A (CYP3A) are main phase I enzymes. Glutathione S-transferase (GST) is a major phase II enzyme. Membrane proteins of phase 0 and III are ABC transporters (ATP-binding cassette), while some main antioxidant enzymes are catalase (CAT) and superoxide dismutase (SOD).

To realize the regulation of genes encoding enzymes of phase I and II, and its carriers, it is essential to understand the signalling mechanisms that involve these genes and the nuclear receptors (NRs) involved (Wang and Lecluyse, 2003). Receptors involved in detoxification mechanisms are already known in mammals. Among these receptors are: pregnane X receptor (PXR), peroxisome proliferator activated receptors (PPARs) and retinoid X receptors (RXR) (Xu *et al.*, 2005). PXR among other functions is known to regulate the expression of CYP3A gene, a main

player in phase I biotransformation in humans and rodents (Lehmann *et al.*, 1998). PPARs include 3 subtypes (PPARα, PPARβ and PPAR gamma), which have been described in several vertebrates, including fish (Ibabe *et al.*, 2005). These receptors can induce at least one type of CYP's (Zhao *et al.*, 2006). RXR comprise a family of three elements: RXRα, RXRβ and RXRλ. These receptors can form heterodimers with other nuclear receptors facilitating the activation and specific binding of all nuclear receptors known to date (Wang and Lecluyse, 2003). They can also form heterodimers with retinoic acid receptors (RARs) acting at the level of gene promoters, modulating its transcription rate (Dollé, 2009). Another important receptor is the Aryl Hydrocarbon Receptor (Ahr) that recognizes various structures such as aromatic compounds (Hankinson, 1995). Correlations between Ahr and expression of genes involved in detoxification (CYP1 and phase II enzymes) in mammals (Nebert *et al.*, 2004; Rushmore and Kong, 2002) were previously described. In fish, in particular zebrafish, the possibility of such interactions, especially in terms of CYP's was recently described (Kubota *et al.*, 2015).

Members of the superfamily ABC (ATP-binding cassette) in aquatic species are part of the mechanism denominated MXR (multixenobiotic resistance) (Kurelec, 1992) and are considered the first line of defence against toxic substances and its metabolites at the cellular level (Bard, 2000). In mammals, ABC proteins are divided into different families which include ABCB (P-glycoprotein), multidrug resistance associated protein (MRP's) ABCC and breast cancer resistance-associated protein (BCRP) ABCG2. All these have shown xenobiotic transport capacity and, consequently, have ecotoxicological relevance (Epel *et al.*, 2008). In zebrafish the functional P-glycoprotein is ABCB4, performing the same functions as the ABCB1 in mammals (Fisher *et al.*, 2013), which is the efflux of unmodified compounds from the cells (phase 0) (Figure 7.). However, this first mechanism of defence is sometimes overpast by high doses of xenobiotics, that accumulate in the cell (Bard, 2000). In those cases, the organism has to apply to other mechanisms of defence. In a second defence line, metabolisation or biotransformation of compounds occurs. This can be defined as a catalytic/enzymatic conversion of a xenobiotic into a more hydrophilic

compound that can be more easily excreted than the parent compound (Van der Oost *et al.*, 2003). Biotransformation occurs over two stages; stage I leads to several non-synthetic modification reactions including oxidation, reduction or hydrolysis (via Cytochrome P450) converting the parent compound into a metabolite. In phase II, the metabolite resulting from stage I, can be combined with an endogenous compound such as glutathione (Xu *et al.*, 2005). In a third phase (phase III), transport proteins, such as ABCC and ABCG, efflux metabolites resulting from phase I and II biotransformation (Bard, 2000). Recent works show correlations between the expression of ABC transporters and phase I and II enzymes, in fish exposed to xenobiotics (Costa *et al.*, 2012).

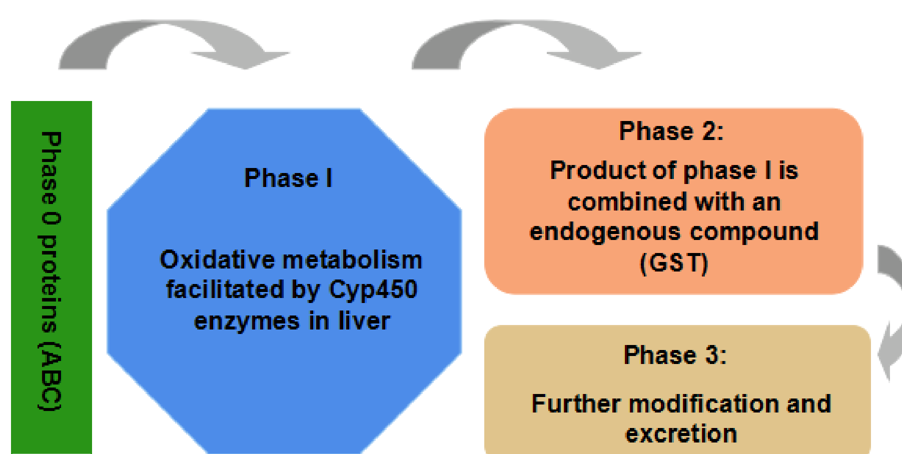


Figure 7. Schematic representation of the different xenobiotic metabolisation phases

Many compounds can cause oxidative stress (Van der Oost *et al.*, 2003). Oxidative stress is defined as damage caused to cellular macromolecules by reactive oxygen species (ROS) which can lead to cell death (Van der Oost et al, 2003). However, there are mechanisms to combat oxidative stress; namely antioxidant enzymes such as catalase (CAT), or superoxide dismutase (SOD) which transform ROS into non-reactive molecules (Van der Oost *et al.*, 2003).

In toxicology and ecotoxicology, in vivo assays are the main tools used to obtain crucial data about the effects of a wide range of toxicants in living organisms. This information is crucial for ecological risk assessment, allowing decision-makers to apply different regulatory strategies for those toxicants (Ankley *et al.*, 2010). However,

this method has low efficiency, extensive animal use, high costs and is time consuming; it is thus against the current demands for higher efficiency and less animal use (Ankley *et al.*, 2010; Volz *et al.*, 2011). To respond to such demands, in recent years, new developments in bioinformatics, measurement technologies and toxicological knowledge at the molecular level were reached and new types of assays start to be proposed like *in silico* and *in vitro* screening (Ankley *et al.*, 2010; Volz *et al.*, 2011). In order to conciliate the most recent tools available in predictive testing with knowledge at the molecular level and the necessity of create new strategies to ecological risk assessment, Ankley *et al.*, (2010) proposed the Adverse Outcome Pathways (AOP's) as model.

An AOP is a conceptual framework that links an initial molecular event of interaction between a toxicant and a biomolecule to an adverse outcome at a higher organisation level that is relevant to ecological risk assessment (Ankley *et al.*, 2010; Kramer *et al.*, 2011). This means that AOP's take into account a myriad of events that occur through several levels of biological organisation (Figure 8.) (Ankley *et al.*, 2010). The different types of relationships between the diverse levels of biological organisation, and the information supporting those relationships, can be obtained from *in vivo, in vitro* or i*n silico* assays (Ankley *et al.*, 2010). This framework and the different linkages that it addresses makes it a very important tool to predictive tactics in toxicology and environmental risk assessment (Ankley *et al.*, 2010; Kramer *et al.*, 2011). Nowadays, AOP's are widely used in different study subjects, since human health, interactions between chemicals and algae, early development in fish and obviously risk assessment evaluations (Bal-Price *et al.*, 2016; Vogs and Altenburger, 2016; Volz *et al.*, 2011; Kramer *et al.*, 2011).

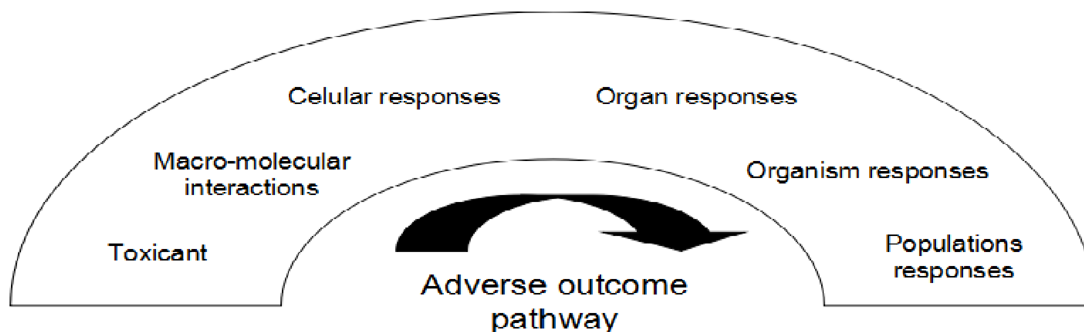


Figure 8. AOP sequence of biological organization levels

1.5 Test model: zebrafish (*Danio rerio*)

In this study, the used test model was zebrafish (*Danio rerio*) (Figure 9). Zebrafish is a small freshwater fish that belongs to the family Cyprinidae and has origin in the North of India, mainly in the Ganges and Brahmaputra river basins (Spence *et al.*, 2008; Avdesh *et al.*, 2012; Parichy, 2015). In the lastest years, it has been widely used as a vertebrate model in scientific studies in biomedical and environmental sciences (Parichy, 2015). Males and females are easily differentiable, mainly before spawning, given that females present a more curled belly shape, due to the eggs carried, while males have straight shape (Lammer *et al.*, 2009). Another difference between males and females is the anal fin that tends to be bigger and yellow in males (Spence *et al.*, 2008).

Figure 9. Zebrafish (*Danio rerio*) specimen

Zebrafish has a range of characteristics that makes this species a widely used vertebrate model. It is easy to obtain, maintain and reproduce in laboratory environment with low costs (Avdesh *et al.*, 2012); it has high fecundity allowing to obtain a large quantity of eggs per spawn (Spencer *et al.*, 2008); eggs have a considerable size (0.7mm diameter), are non-adhesive and transparent, which allows the monitoring of embryonic development (Kimmel *et al.*, 1995; Spence *et al.*, 2008; Soares *et al.*, 2009); embryonic development is fast, approximately 72 hours, enabling faster researcher and diagnosis results (Kimmel *et al.*, 1995). Adding to this, the zebrafish genome is completely sequenced and publicly available at the National Centre for Biotechnology Information (NCBI) Zebrafish Genome Page. These characteristics make zebrafish a first choice in different fields such as genetics, pharmacology and behaviour (Avdesh *et al.*, 2012). This species is also considered a good model for toxicological and ecotoxicological studies since they show clear dose responses to different toxics and allow the evaluation of different sets of endpoints. Guidelines from OECD are already available for toxicological tests in different phases of zebrafish development (Zhang *et al.*, 2003). The embryonic and larval stages are the most sensitive and exposure to xenobiotics in these phases may have adverse effects on animal development, with consequences at the population level. The formation of zebrafish brain starts at 9 hpf (Kimmel *et al.*, 1995). Knowing that norfluoxetine and venlafaxine are active in neuronal receptors that start to be put in place during very early developmental stages (first two days after fertilization), makes this species a good model for studying their mode of action and potential toxic effects towards young teleost fish.

1.6 Objectives

As previously mentioned, generation and consumption of antidepressants is increasing all over the world and this tendency should continue in the near future. Consequently, increased discharge of these pharmaceutical products into the environment is expected. Consequences for non-target organisms, resulting from

those discharges are still little known and should therefore continue to receive attention from the scientific community. Research should focus particularly in evaluating effects of exposure to single substances and their by-products, as well as mixtures of pharmaceutical products, fields where existing gaps in knowledge are bigger (Corcoran *et al.*, 2010; Evgenidou *et al.*, 2015).

The present work, therefore, had the main objectives of understanding the influence of single exposures to both norfluoxetine and venlafaxine in the embryonic development of zebrafish, as well as, to evaluate effects of these pharmaceuticals in genes implicated in the mode of action and metabolisation of both substances. Investigation of the effects of a cocktail of both substances at levels found in natural aquatic systems was also carried out. The work developed was based on the zebrafish embryotoxicity assay with exposures to the selected substances and their mixture. Survival and hallmark developmental endpoints, including gross anomalies, were recorded throughout the exposures. At the end of assays, embryos were collected and used to evaluate the expression of 38 genes involved in neurohormonal and detoxification processes selected according to available knowledge on mode of action of these substances in other vertebrates.

This research adds to the knowledge base about the responses of teleost embryos to SSRI or/and SNRI exposure. Moreover, it provides information and cues to develop early diagnosis of exposure to these environmental contaminants, as well as empirical data useful for risk calculations.

After this general introduction, chapter 2 presents the materials and methods employed in the research presented herein. Chapter 3 is dedicated to the presentation of the results obtained for norfluoxetine and venlafaxine evaluations, respectively, as well as the responses elicited to exposure to the cocktail tested. The general discussion of the results obtained is presented in Chapter 4. Finally, Chapter 5 regards the conclusions and future perspectives of the research developed, and Chapter 6 presents the list of references supporting the work.

Chapter 2: Material and Methods

2. Material and methods

2.1 Used pharmaceuticals

In this study, two pharmaceuticals were used: norfluoxetine (15900) from Cayman Chemical Company® and venlafaxine from the European Pharmacopeia Reference Standard®.

2.2 Zebrafish maintenance and reproduction

Zebrafish (*Danio rerio*) specimens used in this study, were born and maintained in CIIMAR, Porto, Portugal, certified facilities for aquatic organisms. Breeders were maintained in 70L tanks with continuous aerification and water circulation at 27 ± 1ºC, photoperiod was 12 hours' light and another 12 hours' obscurity, and the animals were fed twice a day with proper fish food (Tetramin®) and with artemia once a day.

Males and females were placed in a maternity in a 1:2 ratio (female:male) for reproduction. The maternity was placed inside a 30L tank for 12 hours, for acclimatisation, and had a net bottom covered with glass marbles to mimic natural conditions.

2.3 Zebrafish embryo toxicity test

After reproduction, in the beginning of the light period (at dawn), embryos (0-1 hours post fertilisation, hpf) were collected, washed and counted. The assays were carried out in 24 well plates. Ten embryos were placed in each well in a final volume of 2ml. Embryos were exposed to different norfluoxetine (0.64, 3.2, 16, 80 and 400 ng/L) and venlafaxine (16, 80, 400, 2000 and 10000 ng/L) concentrations during 80 hours. In cocktail assays a mixture of 2000ng/L of venlafaxine and 3.2ng/L of norfluoxetine was tested. Concentrations ≤3.2ng/L for norfluoxetine, ≤2000ng/L for venlafaxine and the ones used in the cocktail are environmentally relevant, which means that these concentrations are found in the ecosystems. These assays also included single treatments of norfluoxetine and venlafaxine at the concentrations in the mixture for comparative purposes and better data interpretation. Solutions were prepared from a

stock solution of each test substance diluted in ultrapure water, followed by a dilution series. A control group only containing ultrapure water was also included in each assay. In the day before the each assay the microplate wells were filled with the correspondent treatment volume, in order to avoid adsorption by the testing plates. The culture medium was renewed every day during the assay to avoid the growing of microscopical organisms that could affect the embryos and/or cause degradation of the test substances. Three assays, with two replicates each, were conducted for each test substance and the cocktail, in a total of 18 plates (6 per substance/mixture), to obtain enough biological material for gene expression determinations. At the end of the exposures (80hpf) embryos were collected and preserved in RNA*later* for further processing and gene expression analysis.

In this study, three specific time points were selected to evaluate normal progression of embryological development, monitoring main embryonic developmental stages. At 8hpf one can observe the 75% epiboly stage where the embryo general plan is evident, and abnormal tissue masses or developmental delay can be checked. At 32hpf embryo segmentation is visible, as well as heartbeats, spontaneous movements and some pigmentation; embryo anomalies can be easily detected by following these hallmarks. At 72hpf embryos typically hatch. However, in some cases, hatching can be delayed taking more time until occurrence (Kimmel et al, 1995). Because of this, embryos or ecloded larvae were observed at 80hpf to detect possible hatching delays and other morphological anomalies. Table I presents the list of endpoints recorded in each developmental stage.

Table 1. Parameters analysed at different observation points.

Parameter	8hpf	32hpf	80hpf
Cumulative mortality	x	x	x
Delay/pause in the development	x	x	x
Abnormal masses	x	x	x
75% Epiboly	x		
Anomalies in the eyes		x	x
Anomalies in the head		x	x
Anomalies in the tail		x	x
Anomalies in the vitelline sac		x	x
Pericardium edema		x	x
Unhatched embryos rate			x

2.4 RNA extraction and cDNA synthesis

RNA was extracted from organisms exposed after 80 hours of exposure to norfluoxetine and venlafaxine, or their cocktail, using Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer protocol. RNA quality was verified by electrophoresis on an agarose gel with analysis of the 18s and 28s bands and by measuring the optical density ratio at λ260/280nm. RNA was quantified using a micro-volume (2 μL) quantification method, using Take3 micro-volume plates in a BioTek microplate spectrophotometer. Then, 1μg of total RNA was subjected to digestion of genomic DNA using deoxyribonuclease I Amplification Grade (Invitrogen) and cDNA synthesis was subsequently performed using iScript cDNA Synthesis Kit (Biorad) following the manufacturer's protocol.

2.5 Primers design, PCR (polymerase chain reaction) and cloning

Table II indicates the genes which activity was assessed in the exposed zebrafish larvae. These were dopamine, serotonin and noradrenaline receptors, the vesicular monoamine transporter and the monoamine oxidase gene, serotonin, dopamine and norepinephrine transporters, several nuclear receptors, ABC transporters, biotransformation and antioxidant enzymes. Reference genes assessed are also

indicated in Table II and were an elongation factor, the widely employed actin β1 and a ribosomal protein. Pairs of primers (forward and reverse) for the genes of interest (Appendix A1) are based on gene sequences available in GenBank and were designed in Primer 3 Plus program.

Table 2. Assessed genes and their function in the organism

Assessed gene	Function in the organism
serta	Serotonin transporter
5-ht2c	Serotonin receptor
5-htt1aa	Serotonin receptor
dat	Dopamine transporter
drd1b	Dopamine receptor
drd2b	Dopamine receptor
net	Norepinephrine transporter
adra2a	Norepinephrine receptor
adra2b	Norepinephrine receptor
adra2c	Norepinephrine receptor
vmat2	Vesicular monoamine transporter
mao	Monoamine oxidase
pxr	Nuclear receptor
ahr2	Nuclear receptor
raraa	Nuclear receptor
rarab	Nuclear receptor
rarga	Nuclear receptor
rxraa	Nuclear receptor
rxrab	Nuclear receptor

Table 2. Assessed genes and their function in the organism

Assessed gene	Function in the organism
rxrbb	Nuclear receptor
rxrga	Nuclear receptor
rxrgb	Nuclear receptor
pparα	Nuclear receptor
pparβ	Nuclear receptor
pparγ	Nuclear receptor
abcb4	ABC transporter
abcc2	ABC transporter
abcc1	ABC transporter
abcg2a	ABC transporter
Cyp1a1	Phase I biotransformation enzyme
Cyp3a65	Phase I biotransformation enzyme
gstπ	Phase I biotransformation enzyme
Cu/Zn sod	Antioxidant enzyme
cat	Antioxidant enzyme
ef1	Reference gene
actb1	Reference gene
rpl8	Reference gene

To confirm the identity of the sequences, PCR (polymerase chain reaction) was performed in a Biometra thermocycler with a mixture of 2µL cDNA (cDNA obtained from pools of about 40 zebrafish larvae with 80hpf). PCR reaction was performed with the following components, volumes and final concentrations, in a final volume of 20µL per reaction: 4µL of 5x buffer, 2µL MgCl2 (2.5mM), 1µL of forward primer (1uM) 1µL of

reverse primer (1μM), 0.4 μL of DNTP's (0.2 mM), 9.5μL water, 0.1μL of TaqPolimerase (Promega) (2.5U) and 2μL of cDNA template. In the thermocycler, the reaction was carried out under the following conditions: 2 minutes of denaturation at 94ºC; 40 cycles of denaturation for 30 seconds, 30 seconds of annealing or hybridization at 51ºC, 54ºC, 55 ºC (51ºC for VMAT, 55ºC for receptor of serotonin 5-HT2c and dopamine drd1b; 54ºC for the remaining genes), 30 seconds of polymerisation at 72 ºC and 10 minutes at 72 ºC for a final elongation. The size of the bands was evaluated on a 2% agarose gel buffered with 1μL of TAE 1x Gel Red and visualized under UV light. Bands of the expected size were excised from the gel and purified using the illustra GFX PCR DNA and Tm Gel Band Purification Kit (GE Healthcare). Cloning and identification of sequence identity was made according to the protocol described by Costa et al. 2013. The fragments were inserted into the vector pGEM (pGEM(R) - T Easy Vector Systems - Promega) and introduced into *E. coli* using New Blue Competent Cells (Novagen). Colonies of interest were selected (white ones) and developed on solid medium for 10 hours (35 g/L of LB Broth, ampicillin 0.1 mg/ml, IPTG 0.1mM and X-gal 100mM) at 37ºC. Plasmids were isolated from 5mL of culture medium and incubated overnight with 5μL ampicillin at 37ºC, with constant stirring. For subsequent DNA extraction the Wizard Kit Plus SV Minipreps DNA Purification System (Promega) was used, according to the manufacturer instructions. PCR products were sequenced by Stabvida (Portugal) and the identity of the sequences was verified with the Alignment Basic Local Search Tool (Blast) at the National Centre for Biotechnology Information (NCBI) (http: //www.ncbi.nlm.nih gov).

2.6 Gene expression

Evaluation of gene expression was performed by quantitative real time PCR (qRT-PCR). Optimal concentrations of primers to use in qRT-PCR were determined after evaluating the highest fluorescence signal in the shortest Cycle threshold (Ct). To determine the efficiency of the PCR reactions, standard linear curves were made for all pairs of primers, using 8 dilutions of 0.05 to 50 ng/μL from an initial mixture of

cDNA. The slope of the regression lines was used to evaluate PCR efficiency. qPCR was performed in an Eppendorf Mastercycler realplex 4 qPCR system (Eppendorf, Hamburg, Germany). The following components and volumes were placed in each well: 10µL of SybrGreen (Biorad), 4µL of water, 2µL of forward primer, 2µl of reverse primer and 2µL of cDNA in a total volume of 20µL. All the reactions were performed in duplicate using the following protocol: 1 cycle at 95ºC for 3 min followed by 40 cycles at 95ºC for 10 sec; 51ºC, 54ºC or 55ºC (51ºC to VMAT, 55ºC to receptor of serotonin 5-HT-2C and dopamine drd1b; 54ºC for the remaining genes) for 30 sec; and 72ºC for 30 sec. A blank sample was performed for each gene studied as well as a melting curve in order to check for the formation of nonspecific products. Quantitation of gene expression was performed by normalizing to reference genes actb1 and rpl8 for norfluoxetine and the cocktail and ef1 and rpl8 for venlafaxine as determined using the Normfinder algorithm (Urbatzka *et al.*, 2013). Relative expression was calculated using the efficiencies of the real-time qPCR using the mathematical template of Pfaffl (Pfaffl., 2001).

2.7 Data analysis

Differences between treatments in the embryo toxicity assay were evaluated by means of crosstable χ^2 test at 5% significance level, avoiding repeated measures designs to fulfil test assumptions. Data is presented as mean ± standard error of the mean (SE) of malformation frequencies obtained in the three independent replicates.
Differences in mRNA expression were evaluated by means of a one-way analysis of variance (ANOVA), followed by a Dunnett's test at a 5% significance level. Data were log-transformed in order to fit ANOVA assumptions. When ANOVA assumptions could not be met after transformation, the non-parametric Kruskal-Wallis test was used to investigate differences in expression among treatments.

A Principal Component Analysis (PCA) to search for specific responses patterns among genes evaluated and associate them with exposure conditions. Factor maps were generated for individuals (with calculation of confidence ellipses) and variables

to assist results interpretation, respectively. PCA was performed using FactoMineR package developed by Lê and colleagues (Lê *et al.*, 2008).

Hierarchical cluster analysis (HCA) was also applied to further depict relationships among genes assayed. HCA was based on the Pearson correlation coefficient with single linkage, using GENE-E software available at the Broad Institute (http://www.broadinstitute.org). Gene expression was plotted into a heatmap according to HCA results, using GENE-E.

Chapter 3: Results

3. Results

3.1. Embryonic exposure to norfluoxetine

3.1.1. Mortality and gross malformations

Results of the toxicity tests carried out showed that mortality was generally low in both the control and the exposed groups (Figure 10). Average cumulative mortality recorded was always below 15%. Though, a statistically significant reduction of average cumulative mortality was observed at 8hpf in zebrafish embryos exposed to 16 ng/L norfluoxetine in relation to controls. At 32 and 80 hpf this difference was no longer detected (Figure 10).

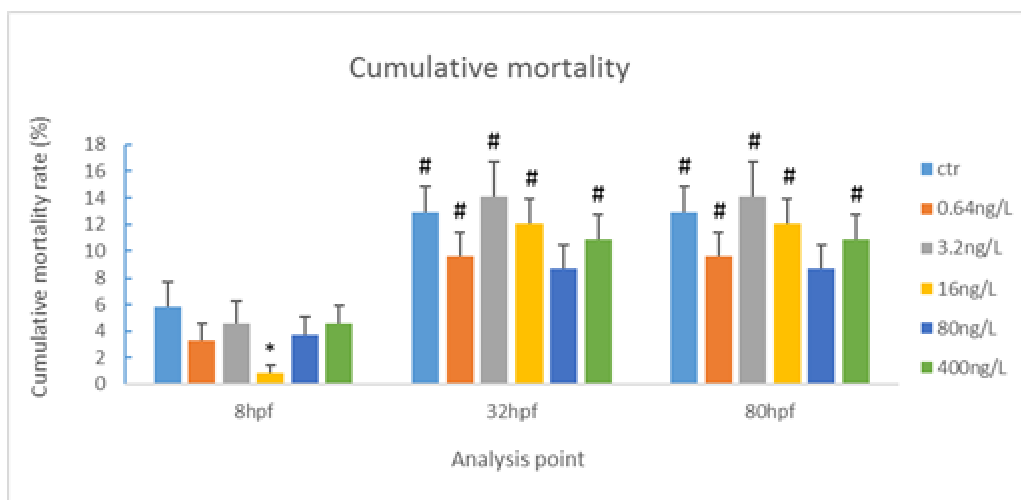


Figure 10. Cumulative mortality of embryos exposed to different concentrations of norfluoxetine at different developmental stages. Values represent the mean ± SE of three independent replicates. Significant differences ($p<0.05$) in relation to the control are marked with an asterisk. Significant differences ($p<0.5$) at 32 and 80hpf, relative to the respective treatment at 8hpf, are marked with #.

At 32 and 80hpf recorded average cumulative mortality was significantly higher than at 8hpf in all treatments tested, except in the 80ng/L concentration. No additional mortality was detected between 32 and 80 hpf (Figure 10).

In contrast to the results observed for mortality, the total number of anomalies found increased over time in zebrafish embryos exposed to norfluoxetine (Figure 11).

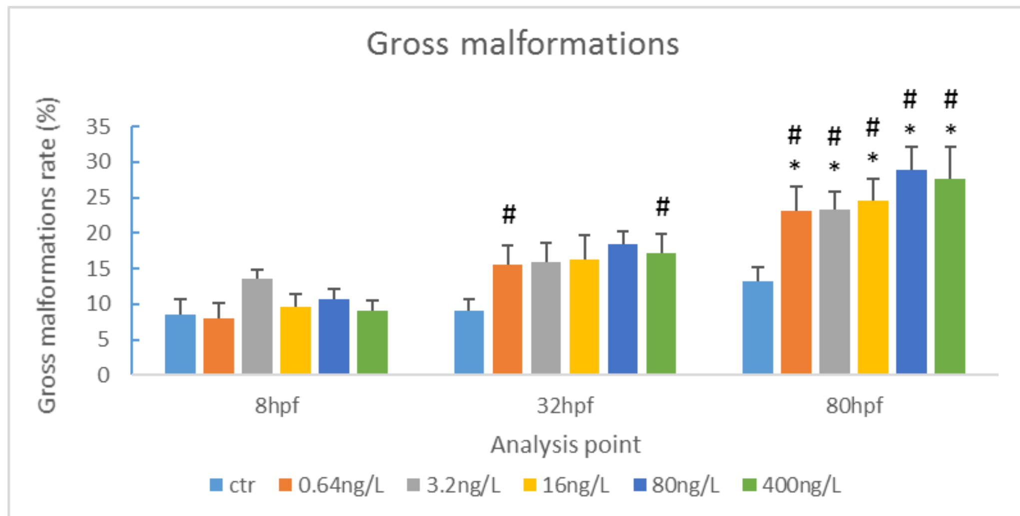


Figure 11. Rate of gross malformations in embryos exposed to different concentrations of norfluoxetine at different developmental stages. Significant differences (p<0.5) in relation to control are marked with an asterisk. Significant differences (p<0.5) at 32 and 80hpf, relative to the respective treatment at 8hpf, are marked with #.

At 32hpf, significant increases in the frequency of malformations were found for 0.64 and 400ng/L norfluoxetine treatments, compared to the frequency observed in the respective treatments at 8hpf. At the end of the test, the frequency of embryo malformations was significantly higher in all norfluoxetine concentrations, compared to the control group (Figure 11). The 80ng/L norfluoxetine treatment was the one showing higher number of anomalies at 80hpf. The significant differences in relation to the control, resulted from an increase in occurrence of different anomalies, although statistically significant differences could only be found for delay in pigmentation (Figure 12).

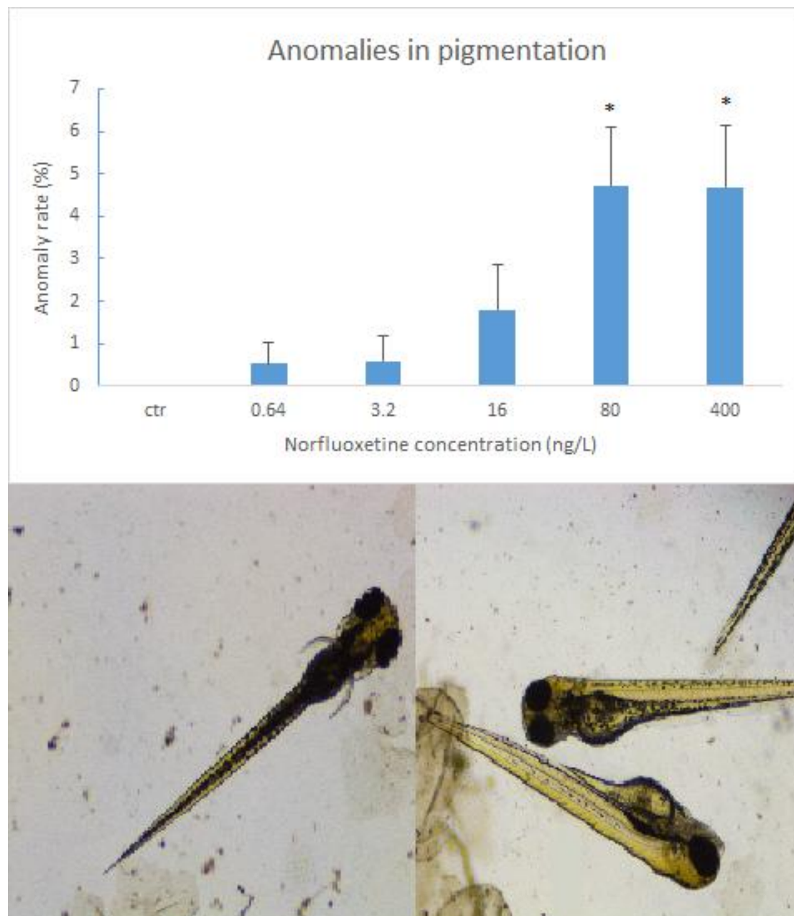


Figure 12. Total of pigmentation anomalies found at 80hpf and illustrative example of the anomaly. Significant differences in relation to the control are marked with an asterisk. In bottom left is indicated a control and at bottom right is indicated a pigmentation anomaly observed in treated larvae.

3.1.2. Molecular responses

Reference genes

A multiple reference gene approach was employed in this work. The selection of reference genes for this approach was based on the software NormFinder (model-based approach). Higher stability in mRNA expression among groups is indicated by values closer to zero. NormFinder evaluated *rpl8* as the most stable gene (stability value: 0.005) if used as single reference gene, and actb1 + rpl8 (stability value: 0.005) as the most stable combination for multiple reference genes, since combination of genes with intergroup variations orientated in opposite directions is recommended. Hence, for norfluoxetine experimental data, mRNA expression of the genes tested was corrected by the normalization factor of rpl8+actb1.

Expression of receptor, transporter, biotransformation and antioxidant genes

Figure 13 presents gene expression levels obtained for the serotonin transporter *serta* and serotonin receptors *5-ht2c* and *5-ht1a*. Globally, expression levels determined for these genes were similar among treatments. The only exception was *5-ht2c* for which induction of expression was found in larvae exposed to 80ng/L norfluoxetine (p<0.05).

Patterns of transcription of dopaminergic genes after exposure to norfluoxetine are shown in Figure 14. For *dat*, there was an apparent tendency for induction of gene transcription, although no significant differences between norfluoxetine and control groups could be found. For *drd1b* and *drd2b*, expression levels of larvae exposed to norfluoxetine were comparable to those of control organisms (Figure 14).

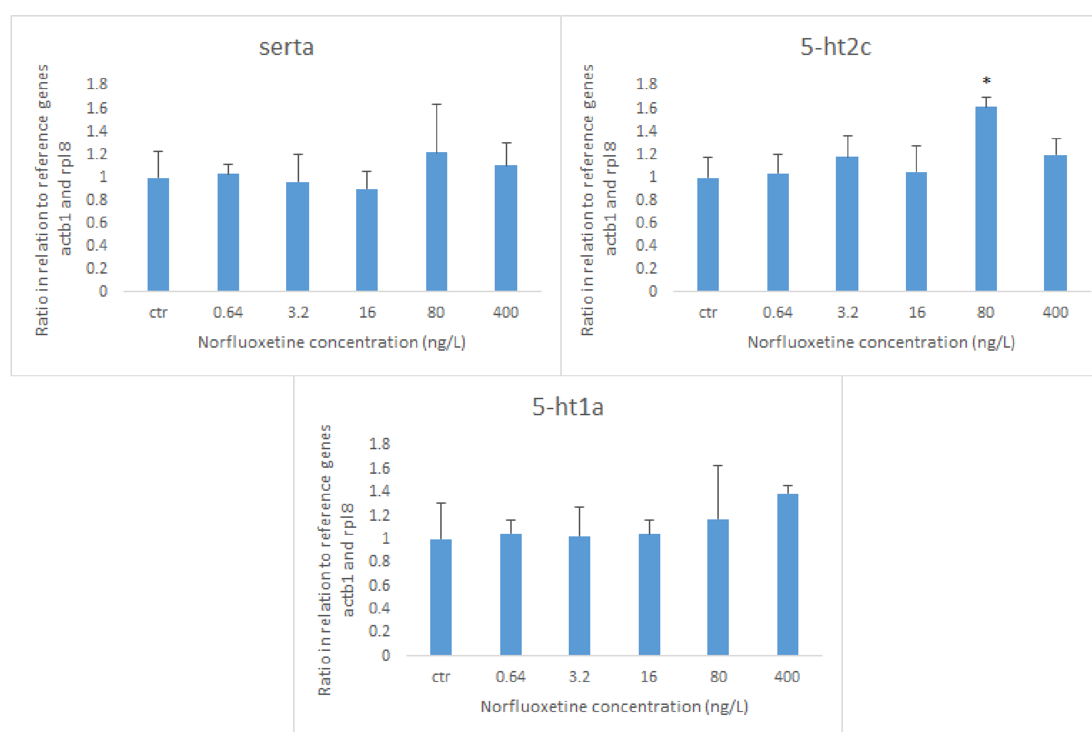


Figure 13. Expression of serotonergic genes after exposure to different concentrations of norfluoxetine for 80hpf. Significant differences (p<0.05) in relation to the control are marked with an asterisk.

Figure 15 presents gene expression data for norepinephrine transporter *net* and

norepinephrine receptors *adra2a*, *adra2b* and *adra2c*. In contrast to the remaining genes, *Adra2a* and *adra2c* show tendency for inhibition of transcription. Significantly lower ($p<0.05$) expression was found for *adra2c* at the lowest concentration of norfluoxetine tested (0.64ng/L), compared to the control group (Figure 15).

Expression of genes *vmat2* and *mao* is plotted in Figure 16. These genes appeared to exhibit a trend for opposite variation; inhibition for *vmat2* and induction for *mao*. Nevertheless, no significant differences in expression in relation to the control could be found for either gene.

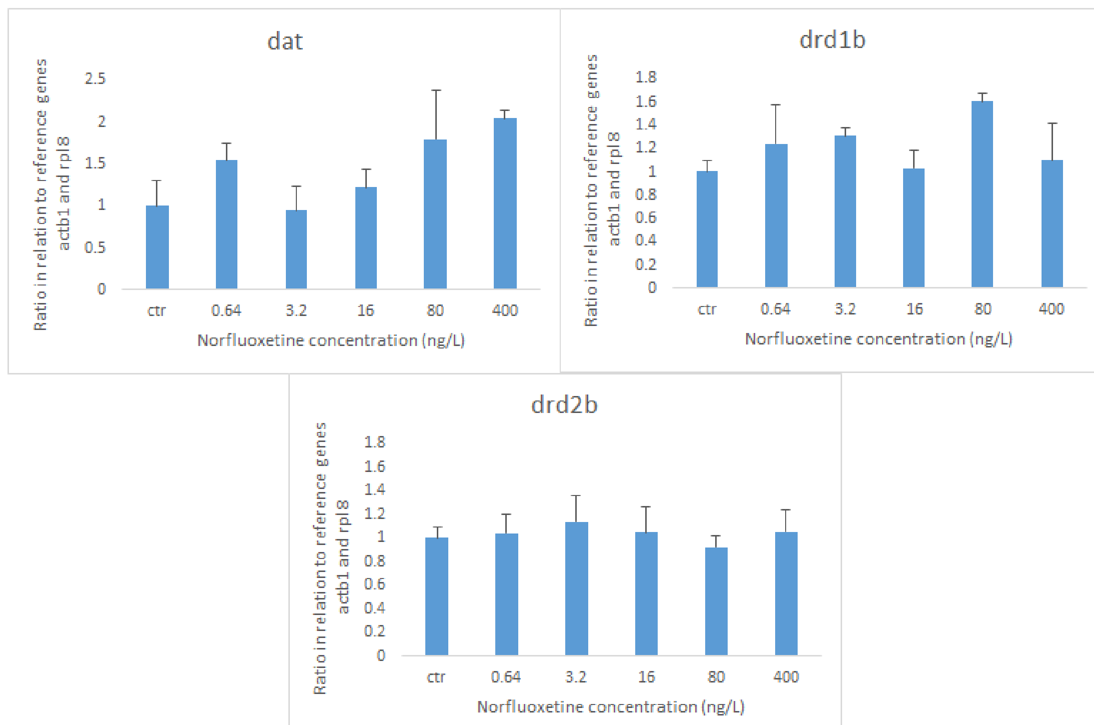


Figure 14. Expression of dopaminergic genes after exposure to different concentrations of norfluoxetine for 80 hpf.

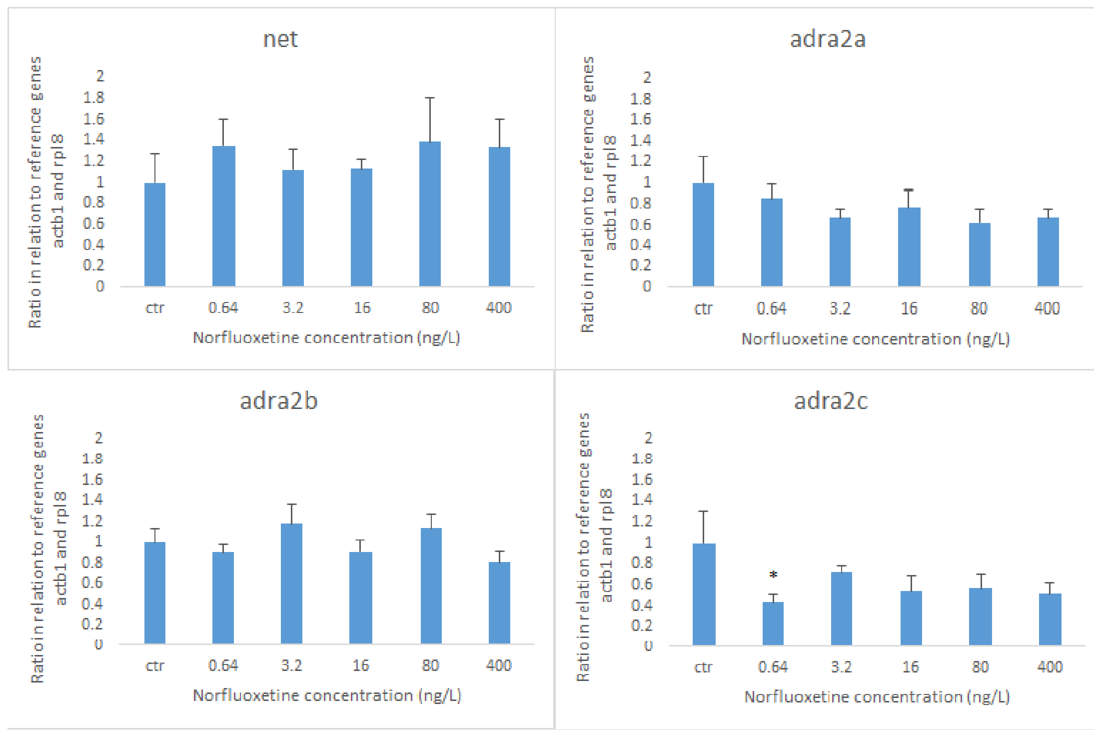


Figure 15. Expression of noradrenergic genes after exposure to different concentrations of norfluoxetine for 80 hpf. Significant differences (p<0.05) in relation to the control are marked with an asterisk.

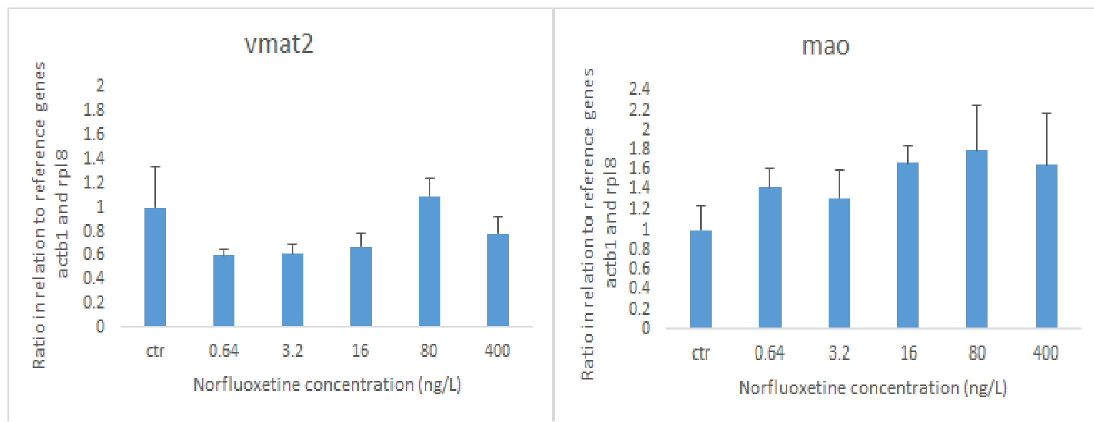


Figure 16. Gene expression of *vmat2* and *mao* after exposure to different concentrations of norfluoxetine for 80 hpf.

The pattern of transcription of nuclear receptors *pxr* and *ahr2* obtained after exposure to norfluoxetine is shown in Figure 17. Though some variation among treatments was observed for *pxr*, sometimes suggesting inhibition and other times suggesting induction, no significant differences in expression could be depicted. For *ahr2*, levels of expression in norfluoxetine treatments were similar to those of the control.

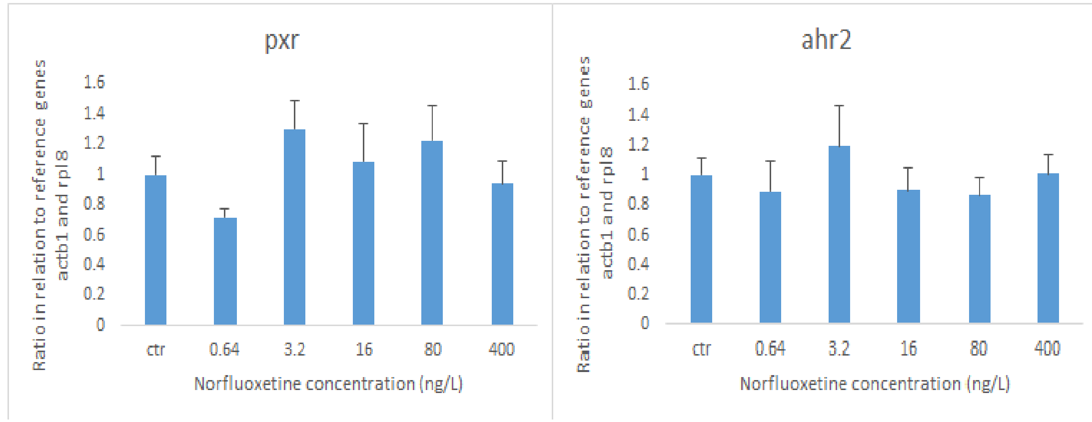


Figure 17. Gene expression of nuclear receptors *pxr* and *ahr2* after exposure to different concentrations of norfluoxetine for 80 hpf

Expression of *raraa* and *rarab* nuclear receptors of larvae exposed to norfluoxetine was similar to control levels (Figure 18). On the other hand, *rarga* presented a different expression response to norfluoxetine exposure. *rarga* expression level increased at low exposure concentrations, peaking at 3.2 ng/L norfluoxetine for which statistically significant differences relative to the control group were found (p<0.05). Thereafter, expression levels decreased to near control values.

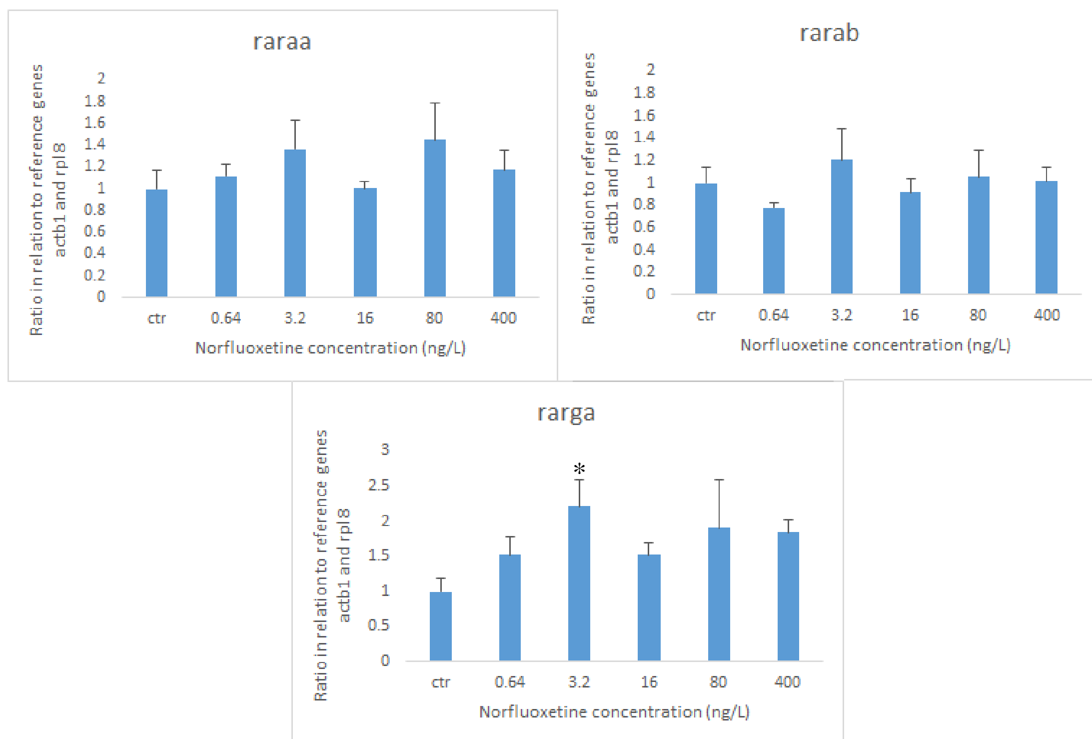


Figure 18. Gene expression of *rar* group of nuclear receptors after exposure to different concentrations of norfluoxetine for 80 hpf. Significant differences (p<0.05) in relation to the control are marked with an asterisk.

Figure 19. presents gene expression data of *rxr* group of nuclear receptors. Expression of genes of this group in larvae exposed to norfluoxetine was similar to that of controls, as indicated by ratios of relative mRNA expression around the unit.

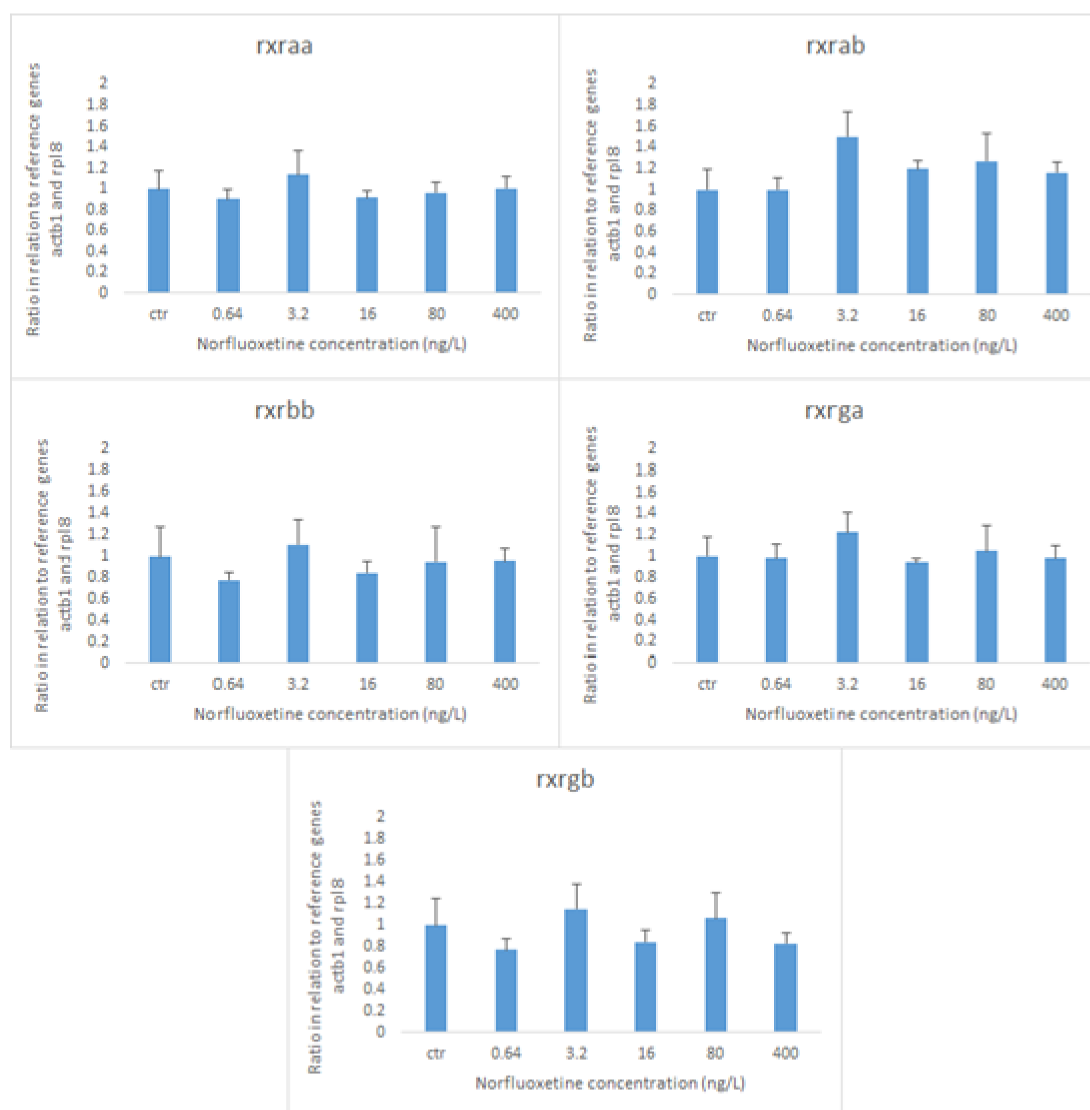


Figure 19. Gene expression of *rxr* group of nuclear receptors after exposure to different concentrations of norfluoxetine for 80 hpf

The pattern of transcription of *ppar* group of nuclear receptors obtained after exposure to norfluoxetine is shown in Figure 20. Interestingly, also for this group no significant differences in gene expression were found among treatments, suggesting these were not affected by norfluoxetine during embryonic development.

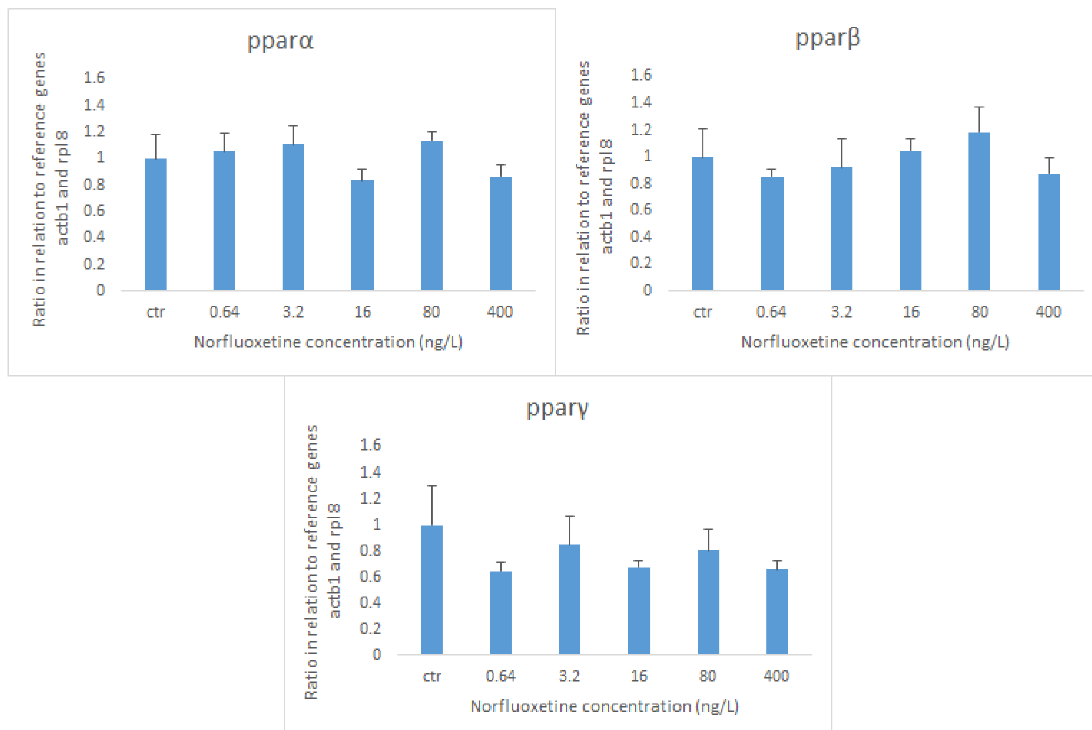


Figure 20. Gene expression of *ppar* group of nuclear receptors after exposure to different concentrations of norfluoxetine for 80 hpf

Similarly, gene expression of *abcc2* and *abcg2a* transporters, showed no obvious pattern of induction or inhibition (figure 21). For *abcb4*, low exposure (0.64 ng/L) caused inhibition of expression ($p<0.05$) that was no longer observed at concentrations ≥3.2 ng/L. Conversely, larvae exposed to 3.2 ng/L showed induction ($p<0.05$) of *abcc1* expression. For the remaining test concentrations, expression levels were within control values.

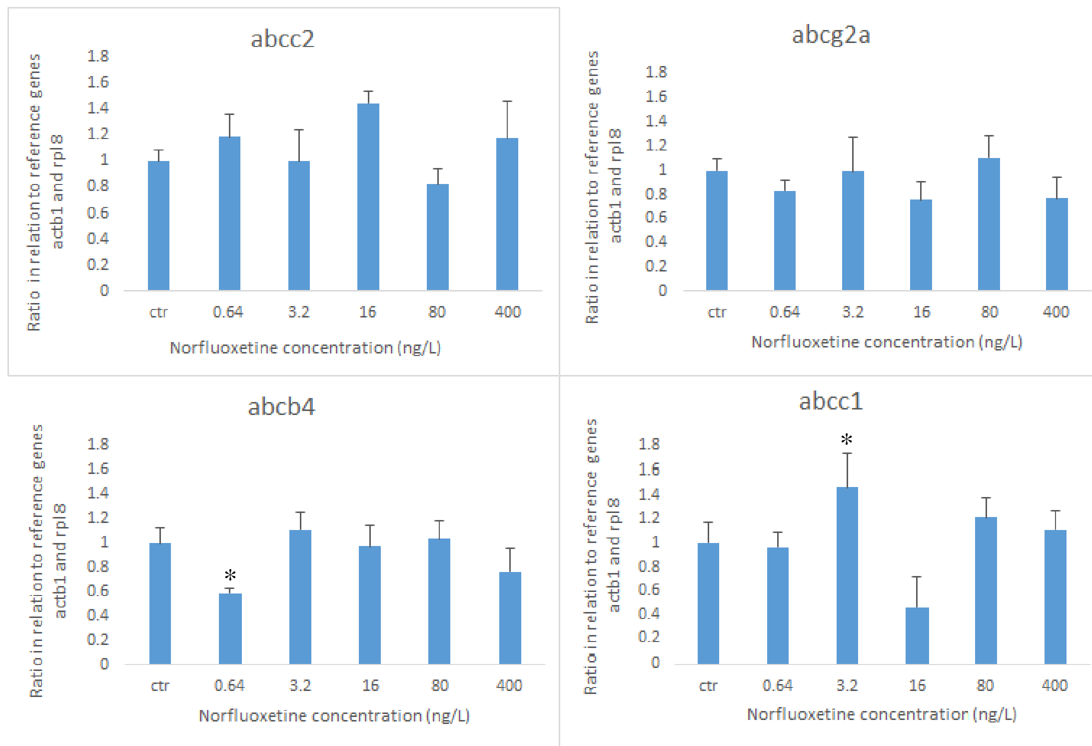


Figure 21. Gene expression of ABC transporters (Phase 0 / III) after exposure to different norfluoxetine concentrations for 80 hpf. Significant differences (p<0.05) in relation to the control are marked with an asterisk.

Like observed for other genes, no clear pattern of alteration of gene expression by norfluoxetine was visible for most biotransformation and antioxidative enzymes evaluated (Figures 22 and 23). Apart from *sod*, no differences in expression levels were detected among treatments for *cyp1a1*, *cyp3a65*, *gstπ* and *cat*. For Cu/Zn *sod* significant transcriptional inhibition was found in organisms exposed to high (400 ng/L) norfluoxetine concentration (p<0.05).

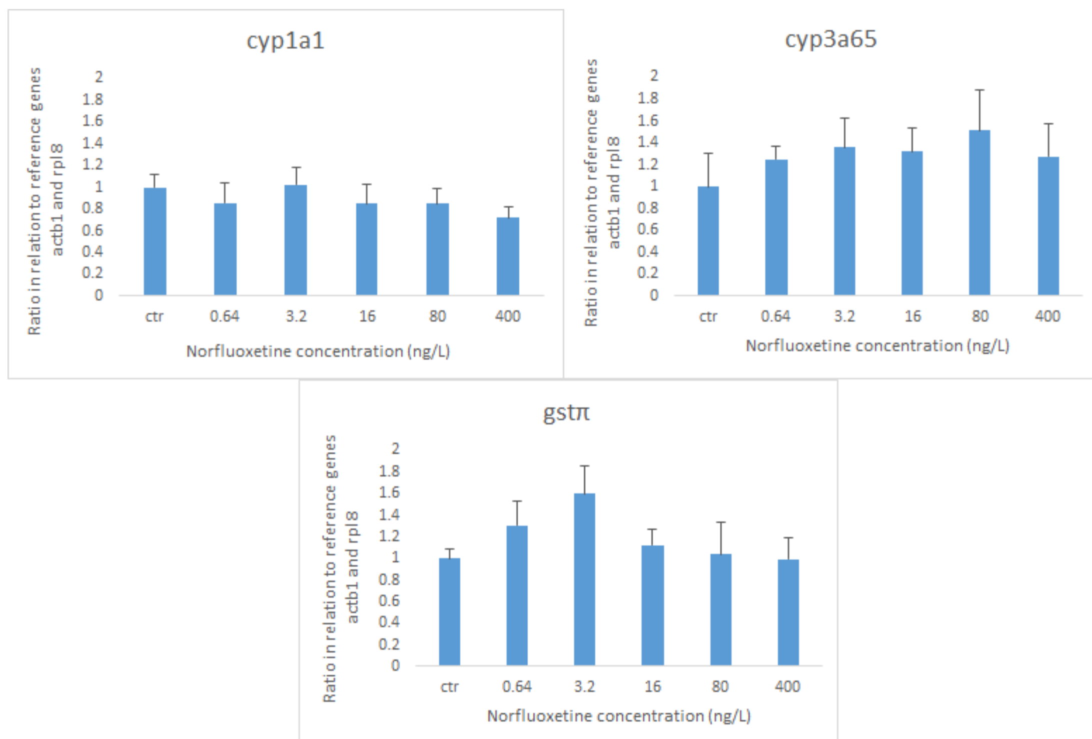


Figure 22. Gene expression of biotransformation enzymes (phase I and II) after exposure to different norfluoxetine concentrations for 80 hpf.

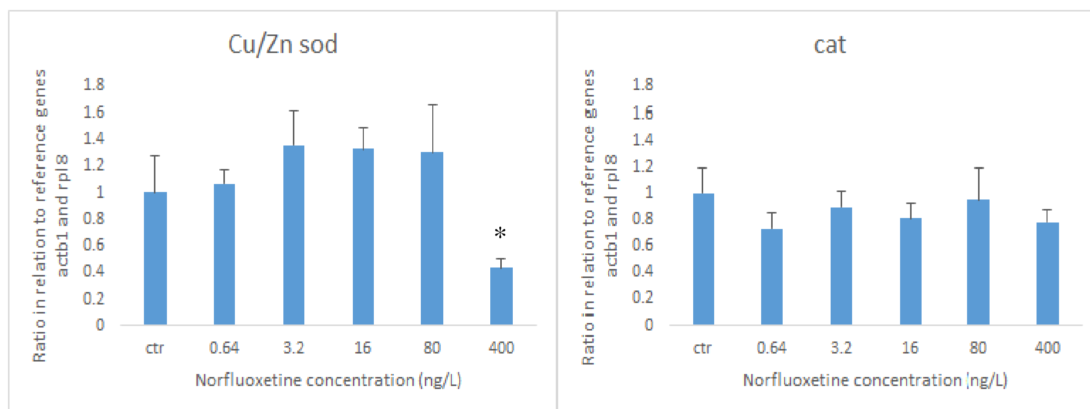


Figure 23. Gene expression of oxidative stress enzymes after exposure to different norfluoxetine concentrations for 80 hpf. Significant differences ($p<0.05$) in relation to the control are marked with an asterisk.

Main patterns of variation in gene expression are summarized in Figure 24 in the form of a heatmap. The heatmap is colour-coded according to the percentage of variation relative to the control group. The levels of most genes were found to be fairly stable across exposure concentrations. Nevertheless, hierarchical cluster analysis identified a group of concentrations sharing some similarity in gene expression. This group comprised 16, 0.64, 400 and 80 ng/L norfluoxetine. The group showed a

tendency for slight inhibition several genes, such as *abcg2a, rxrbb, rxrxgb* and *adra2c*, and slight induction of *dat, serta* and *net*. The concentration of 3.2ng/L was associated with an inverse expression response of these genes.

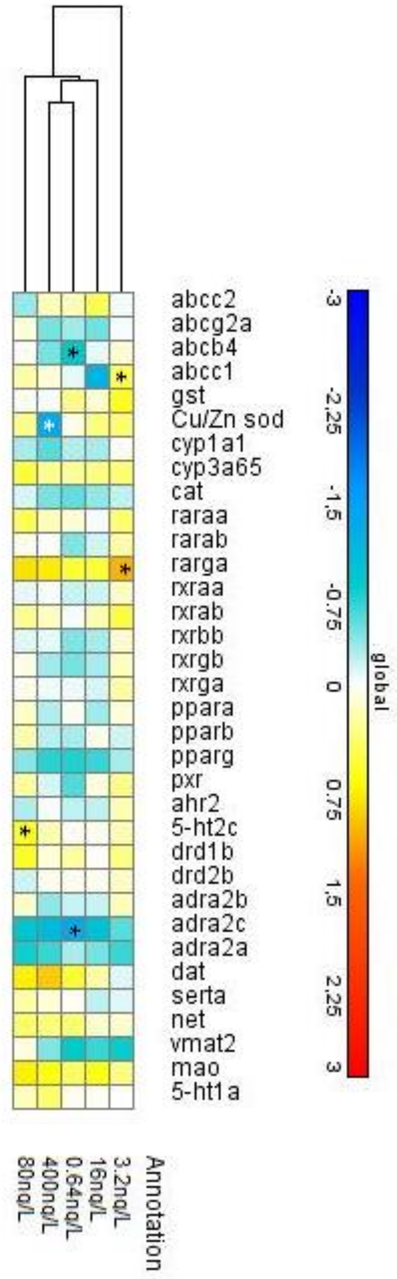


Figure 24. Heatmap of gene expression variation in organisms exposed to different concentrations of norfluoxetine for 80hpf. The colour scale reflects the direction and magnitude of variation in relation to the control group. The asterisk indicates significant differences relative to the control at $p < 0.05$.

For more detailed investigation of the patterns of response of the genes evaluated, and their possible association to exposure concentrations, PCA analysis was then

carried out. According to results obtained, seven components were extracted, expressing 87% of the total variability observed in the data. Of these, the first two principal components (PCs) retained 60% of the total inertia (Figure 25). Interpretation of PCA results was, therefore, based on the first two components extracted. The first component (horizontal) was mainly associated with the expression of genes involved in detoxification and to a lesser extent with monoamine transporter and receptors: *rxrga* and *rxrgb*, showing positive correlations (*r*) with the axis above 0.90; with *rxrab, raraa, serta, cyp3a65, rarab, pparg, rxrbb, rxraa* all with *r*>0.8; with *net, mao, cyp1a1, 5-ht1a* and *pparβ* all with *r*>0.7; and with *pxr, gstπ, sod, pparα, rarga, adra2b, dat* and *ahr2* (*r*>0.6). This pattern is visible in the variables factor map of Figure 25. The second axis (vertical) was mainly associated with the expression of dopamine and serotonin receptors and two ABC transporters: *drd1b, 5-ht2c, abcg2a* and *abcc1* all positively correlated with the axis (*r*>0.6). Analysis of the individuals factor map (Figure 25) shows no remarkable differences between the control group and the concentrations of norfluoxetine tested. Centroids for 3.2 and 80 ng/L concentrations were the most distant from the control, however the confidence ellipses of these three groups are intersected.

Individuals factor map (PCA)

Figure 25. Results of the PCA defined by the expression of genes evaluated in zebrafish larvae exposed to norfluoxetine for 80hpf. The cloud of genes is represented in the variables factor map; sample are represented in the individuals factor map (bottom). Centroids of each treatment group, and respective confidence ellipses, are also shown.

3.2. Embryonic exposure to venlafaxine

3.2.1. Mortality and gross malformations

Mortality recorded during the toxicity tests performed was very low, particularly in the control group (Figure 26). Cumulative mortality was below 5% in all test treatments at 8 hpf.

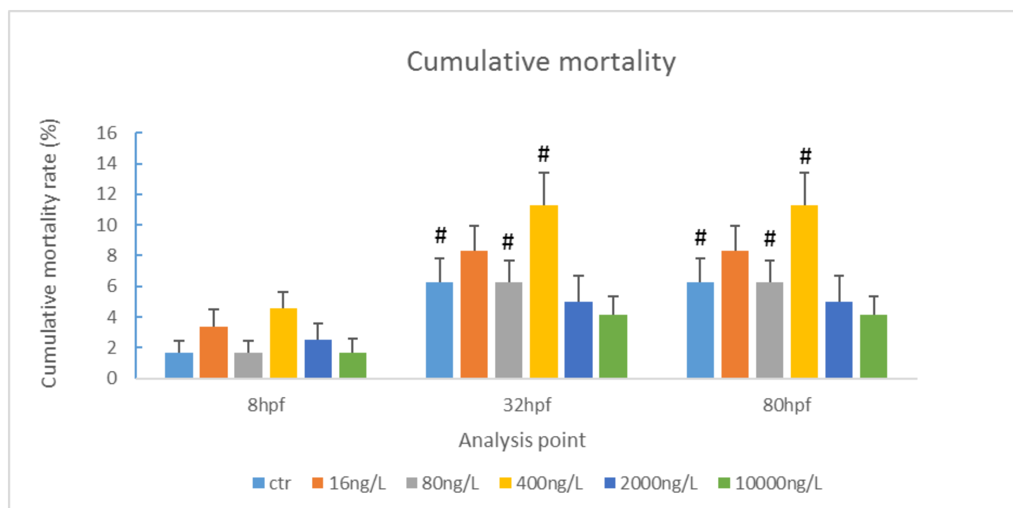


Figure 26. Cumulative mortality of embryos exposed to different concentrations of venlafaxine at different developmental stages. Values represent the mean ± SE of three independent replicates. Significant differences (p<0.05) relative to 8 hpf are marked with #.

A slight increase in cumulative mortality, to about 10% on average, was recorded at 32hpf in the control and in embryos exposed to 80 and 400ng/L venlafaxine, compared to the respective groups at 8hpf (p<0.05) (Figure 26). After this point, no additional dead embryos were found. The highest cumulative mortality was always observed in the group exposed to a venlafaxine concentration of 400ng/L, independently of the time window assessed.

In contrast to the results observed for mortality, a high rate of malformations was detected. At 8hpf the frequency of malformations was very low (≤5%) (Figure 27). Controls continued to exhibit low frequency of malformations (<10%) over time. However, in exposed embryos, a clear tendency for increase in malformations with the increase in venlafaxine concentration was evident at 32pf.

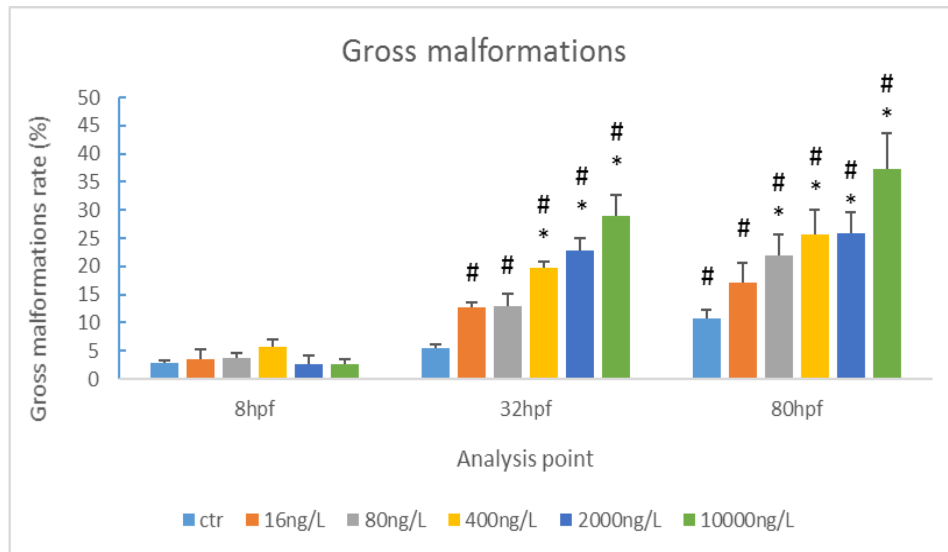


Figure 27. Gross malformations in embryos exposed to different concentrations of venlafaxine at different analysis points. Significant differences in relation to the control are marked with (*) and relative to 8 hpf with (#), with p <0.05

At 32 hpf significant increases (p<0.05) in the number of anomalies were found in all venlafaxine concentrations in relation to the previous developmental window analysed (8 hpf). Significant differences relative to the control group were found for the highest concentrations tested (400, 2000 and 10000ng/L). Malformations contributing to this increase were a delay in the pigmentation and anomalies in the vitelline sac (Figure 28)

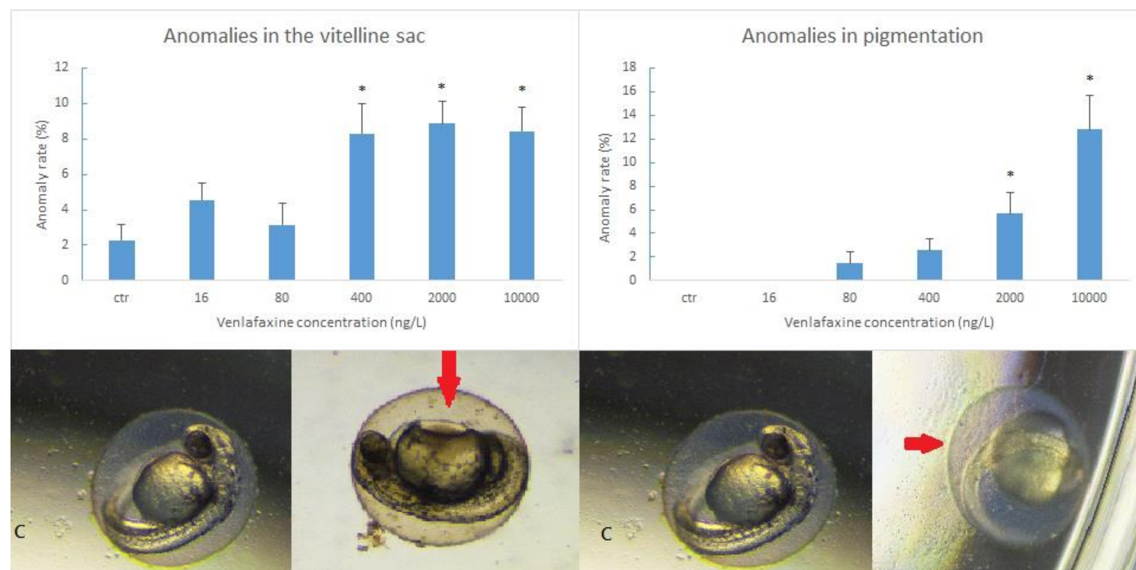


Figure 28. Frequency of total and pigmentation and vitelline sac anomalies found at 32 hpf. Normal embryo is indicated by C (left) and an example of anomalies observed (right) indicative by the red arrow. Significant differences (p<0.05) in relation to the control are marked with an asterisk.

At the end of the test (80 hpf), with individuals already in larval stage, statistically relevant increases ($p<0.05$) in the rate of malformations, in relation to the control group, were found for exposure concentration ≥ 80ng/L venlafaxine. In these treatments the rate of total anomalies ranged on average from 20% (80ng/L group) to 30% (10000 ng/L). Delayed pigmentation and spinal anomalies were the malformations contributing mostly to the pattern observed (Figure 29).

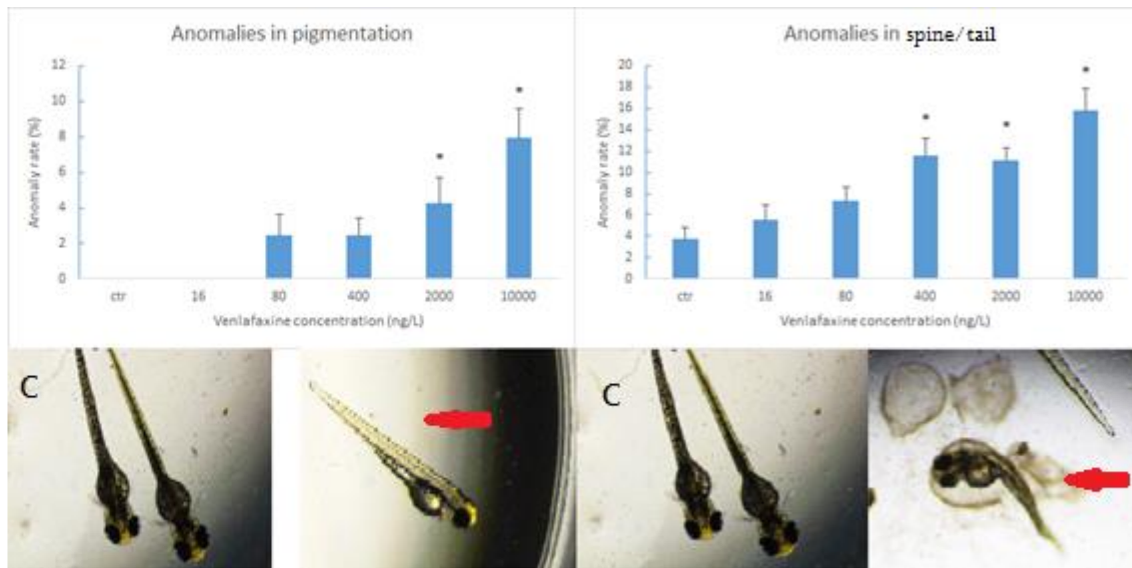


Figure 29. Frequency of total pigmentation and spine/tail anomalies found at 80 hpf. Normal embryo is indicated by C (left) and an example of anomalies observed (right) indicative by the red arrow. Significant differences ($p<0.05$) in relation to the control are marked with an asterisk.

3.2.2. Molecular responses

Reference genes

NormFinder evaluated *rpl8* as the most stable gene (stability value: 0.005) if used as single reference gene. The combination of *ef1 and rpl8* (stability value: 0.006) was found to be the most for multiple reference genes. mRNA expression of the genes evaluated after venlafaxine exposure was therefore corrected by the normalization factor of *rpl8* + *ef1*.

Expression of receptor, transporter, biotransformation and antioxidant genes

Figure 30. presents the gene expression of serotonin transporter *serta* and serotonin receptors *5-ht2c* and *5-ht1a*. Globally, there was a clear tendency for inhibition of transcription of all these genes, though with different trends. For the serotonin transporter *serta*, the group exposed to 10000ng/L exhibited the highest inhibition ($p<0.05$), of more than half the control level. Conversely, expression of *5-ht2c* receptor increased in the lowest test concentration (16ng/L) relative to the control, decreased thereafter to levels well below control at 80 ($p<0.05$) and 400ng/L ($p<0.05$), then recovering to controls levels. For serotonin receptor *5-ht1a*, significant inhibition was found at 10000ng/L venlafaxine ($p<0.05$) (Figure 30).

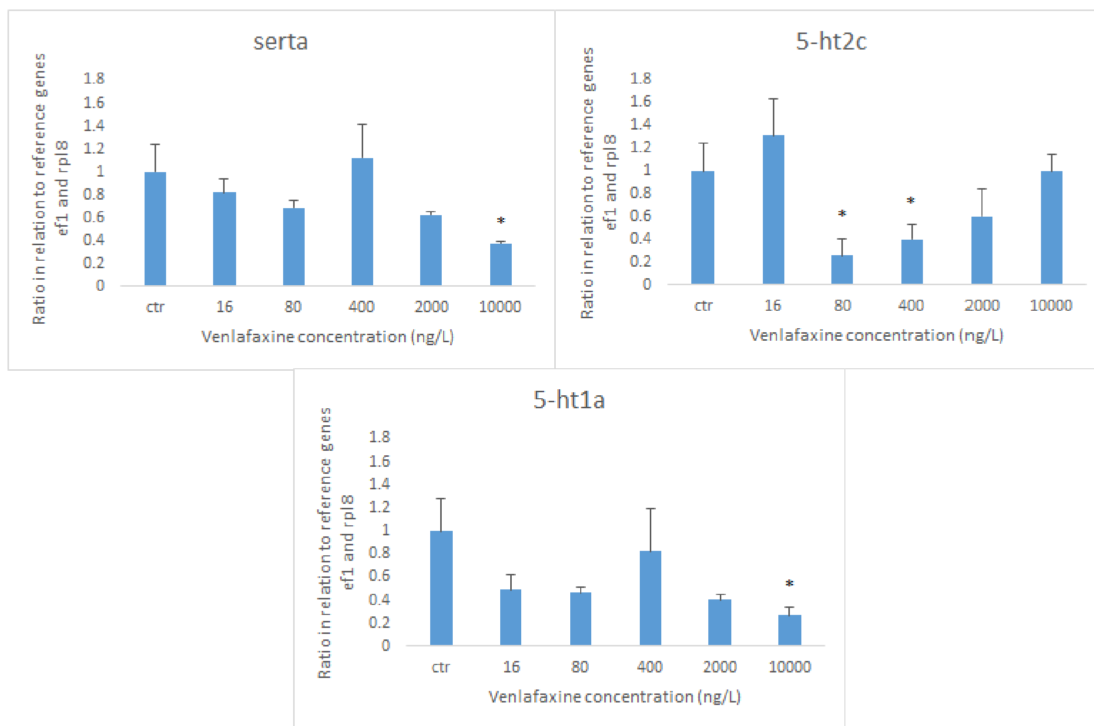


Figure 30. Gene expression of serotonergic genes after exposure to different concentrations of venlafaxine for 80 hpf. Significant differences in relation to the control are marked with (*)

Patterns of transcription of dopaminergic genes after exposure to venlafaxine are shown in Figure 31. Dopamine transporter *dat* was induced at lower concentrations, peaking in larvae exposed to 400ng/L ($p<0.05$). In larvae exposed to high venlafaxine concentrations it was near control expression levels. Receptor *drd1b* showed an

opposite trend; expression was decreased in organisms exposed to the lower venlafaxine concentrations, with significant differences relative to the control at 80 and 400ng/L ($p<0.05$). In the highest test concentrations there was a recovery to control levels, in a U-shape response curve (Figure 31). For *drd2b*, no significant alterations were found in venlafaxine treatments, in relation to control expression levels; the values of exposed organisms were similar to those of the control group.

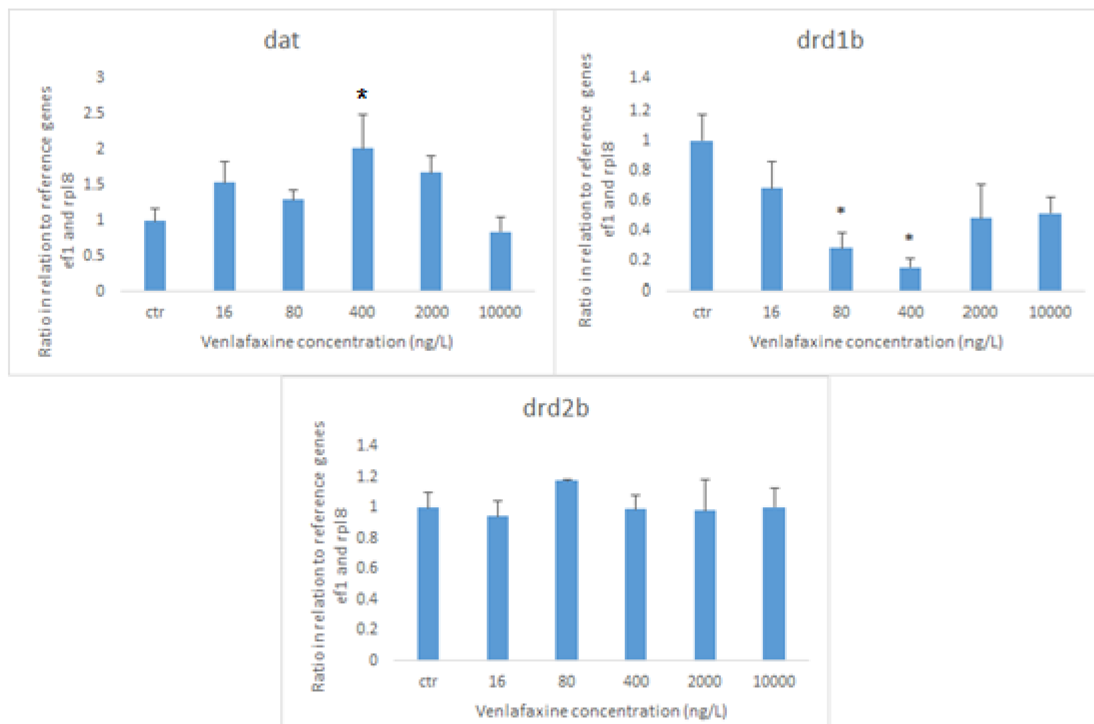


Figure 31. Gene expression of dopaminergic genes after exposure to different concentrations of venlafaxine for 80 hpf. Significant differences in relation to the control are marked with (*)

Figure 32. presents the gene expression of norepinephrine transporter *net* and norepinephrine receptors *adra2a*, *adra2b* and *adra2c*. For *net*, trend of inhibition of transcription was visible in organisms exposed to venlafaxine. Statistically significant differences relative to the control were found for the highest concentration tested (10000ng/L) ($p<0.05$). For *adra2b*, there was a clear induction of gene expression, with organisms exposed to ≥400ng/L showing significant 2-3 fold increases in expression ($p<0.05$). No significant differences among treatments were detected for the expression levels of either *adra2a* or *adra2c*.

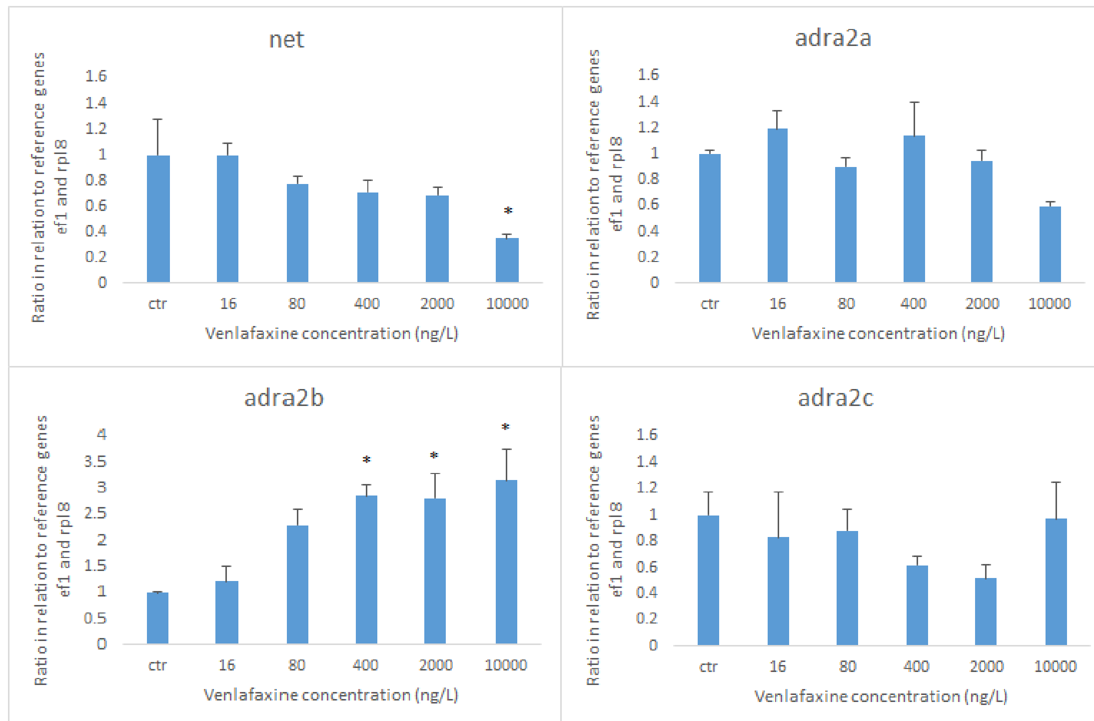


Figure 32. Gene expression of noradrenergic genes after exposure to different concentrations of venlafaxine for 80 hpf. Significant differences in relation to the control are marked with (*)

Gene expression of *vmat2* and *mao* genes is shown in Figure 33. *vmat2* expression appeared to increase in larvae exposed to the lowest concentration (16ng/L), followed by significant inhibition ($p<0.05$) of the gene transcription at 80 and 400 ng/L. At 2000ng/L and 10000 ng/L expression levels in exposed larvae were in the same range of those of control larvae. For *mao* inhibition of gene expression ($p<0.05$) was also observed in larvae exposed to the highest concentration of venlafaxine. No differences in *mao* expression were found between the controls and the remaining treatments.

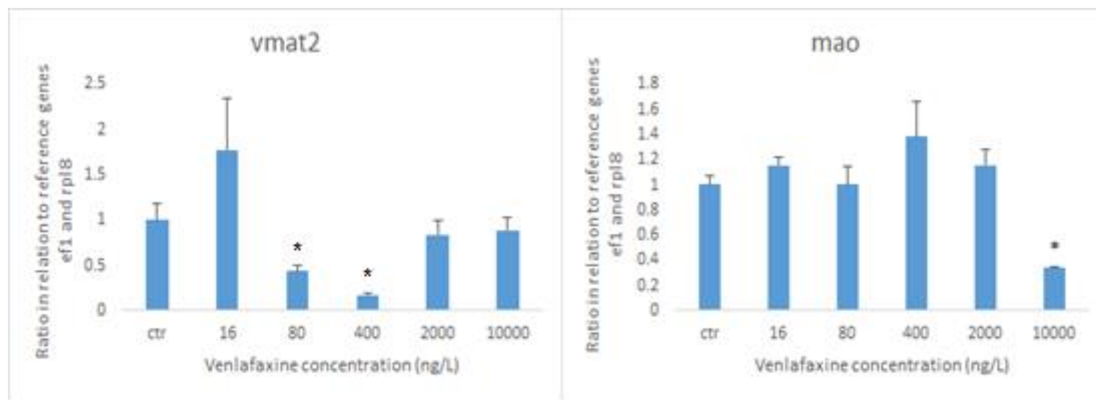


Figure 33. Gene expression of *vmat2* and *mao* after exposure to different concentrations of venlafaxine for 80 hpf. Significant differences in relation to the control are marked with (*)

The patterns of transcription of nuclear receptors *pxr* and *ahr2* after exposure to venlafaxine are shown in Figure 34. No significant differences among treatments were observed for either gene.

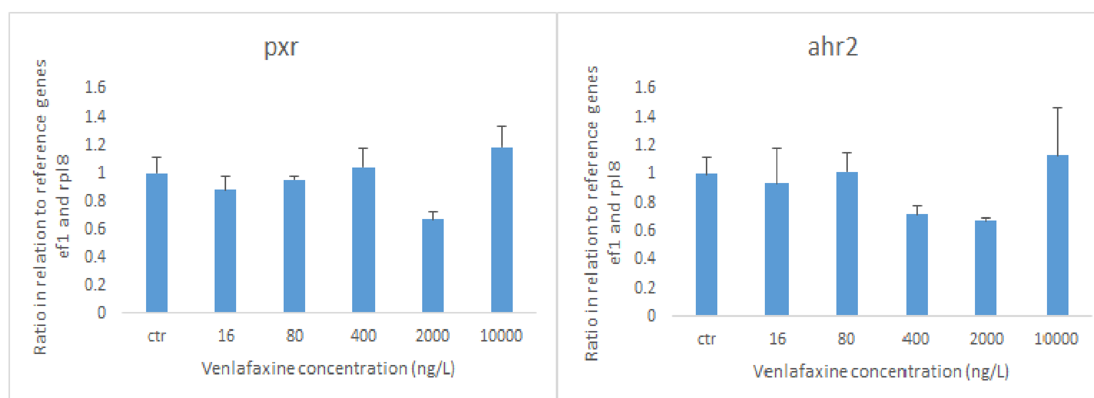


Figure 34. Gene expression of nuclear receptors *pxr* and *ahr2* after exposure to different concentrations of venlafaxine for 80 hpf

Likewise, no effects of venlafaxine exposure on retinoic acid receptors could be depicted (Figure 35.).

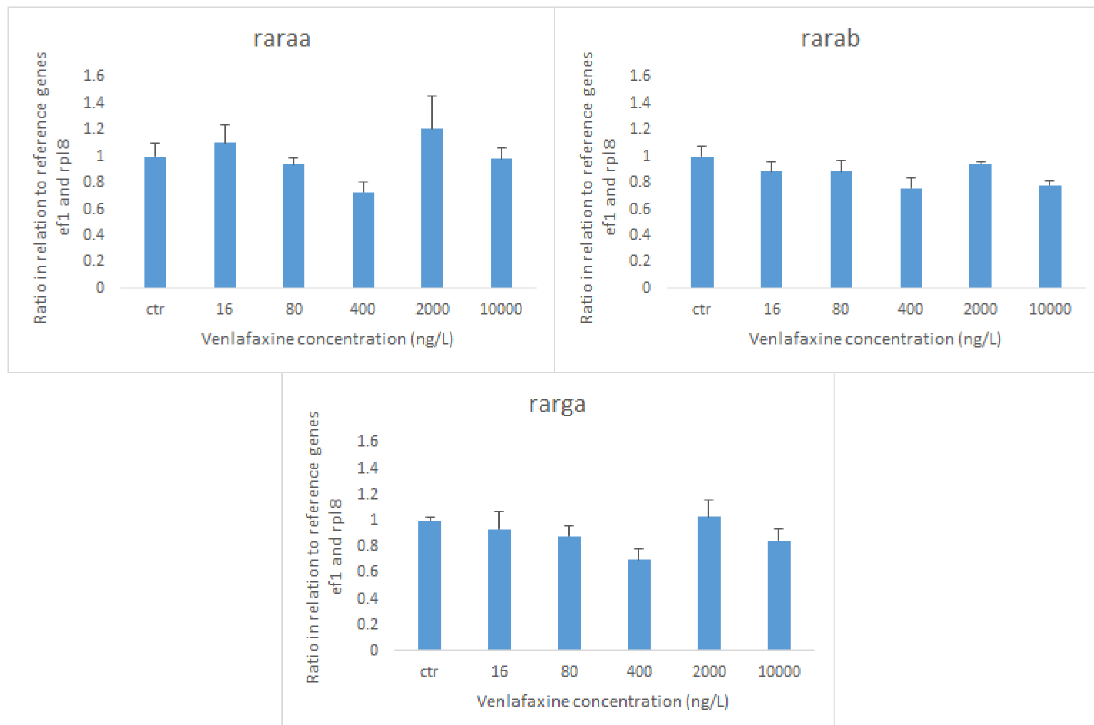


Figure 35. Gene expression of *rar* group of nuclear receptors after exposure to different concentrations of venlafaxine for 80 hpf

In contrast, exposure to venlafaxine caused inhibition of *rxrab* ($p<0.05$) and *rxrga* ($p<0.05$) expression in organisms exposed to 400 ng/L, compared to control organisms. Relative to the control group, significant inhibition of gene expression was also found for *rxrgb* in organisms exposed to 400ng/L ($p<0.05$) and 10000ng/L ($p<0.05$). No differences in exposed larvae were found for the expression of the other two retinoic x receptors evaluated (*rxraa* and *rxrbb*) when compared to controls.

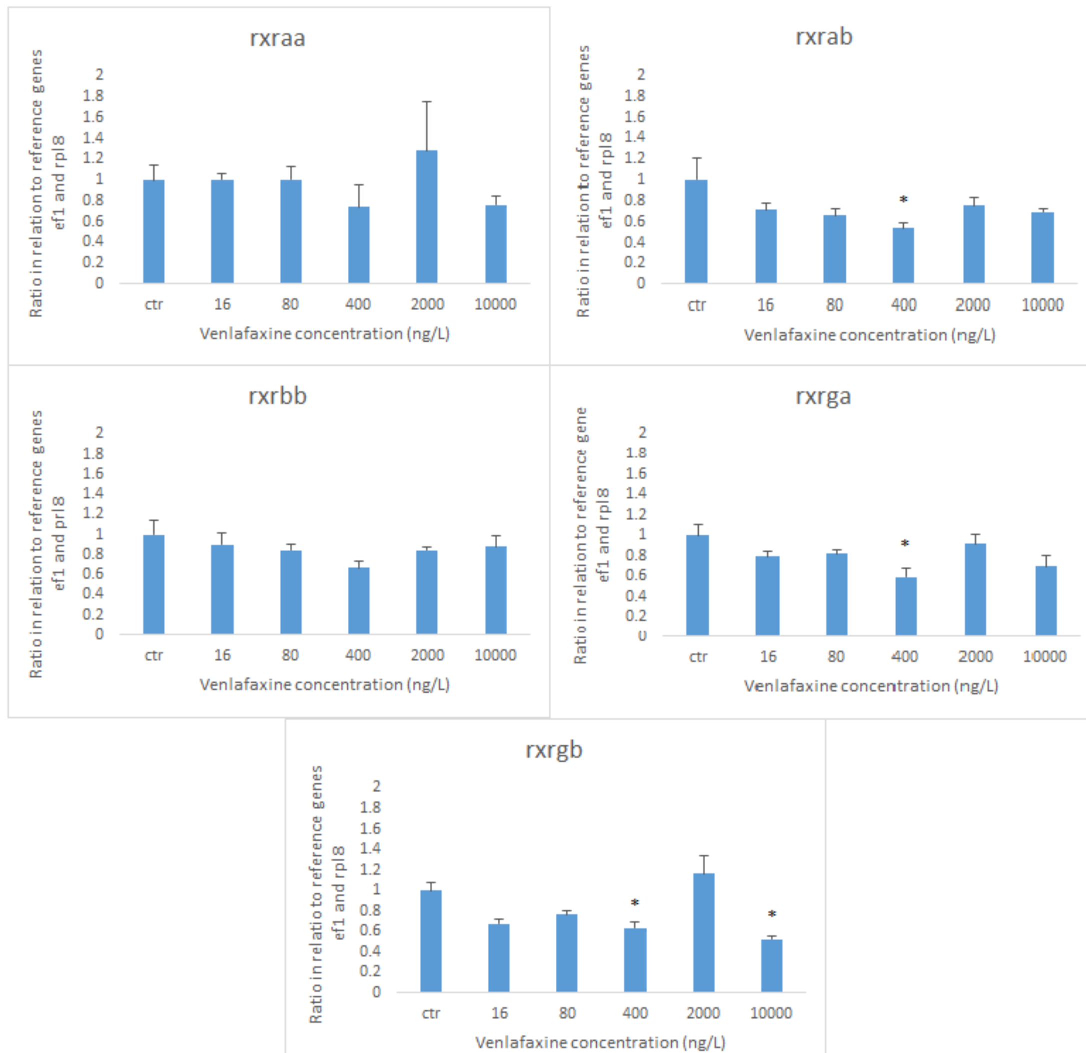


Figure 36. Gene expression of *rxr* group of nuclear receptors after exposure of zebrafish to different concentrations of venlafaxine for 80 hpf. Significant differences in relation to the control are marked with (*)

Patterns of transcription of *ppar* nuclear receptors after exposure to venlafaxine are shown in Figure 37. For this group of genes a U-shape response to venlafaxine exposure was visible. Significant inhibition of gene expression, relative to control, was found for *pparα* at 400 ng/L ($p<0.05$), for *pparβ* at 80 ng/L ($p<0.05$) and 400ng/L ($p<0.05$), and for *pparγ* at 80 ng/L ($p<0.05$), 400 ng/L ($p<0.05$) and 2000 ng/L ($p<0.05$) (Figure 37). For all genes the 400ng/L concentration was the one where the inhibition was more accentuated.

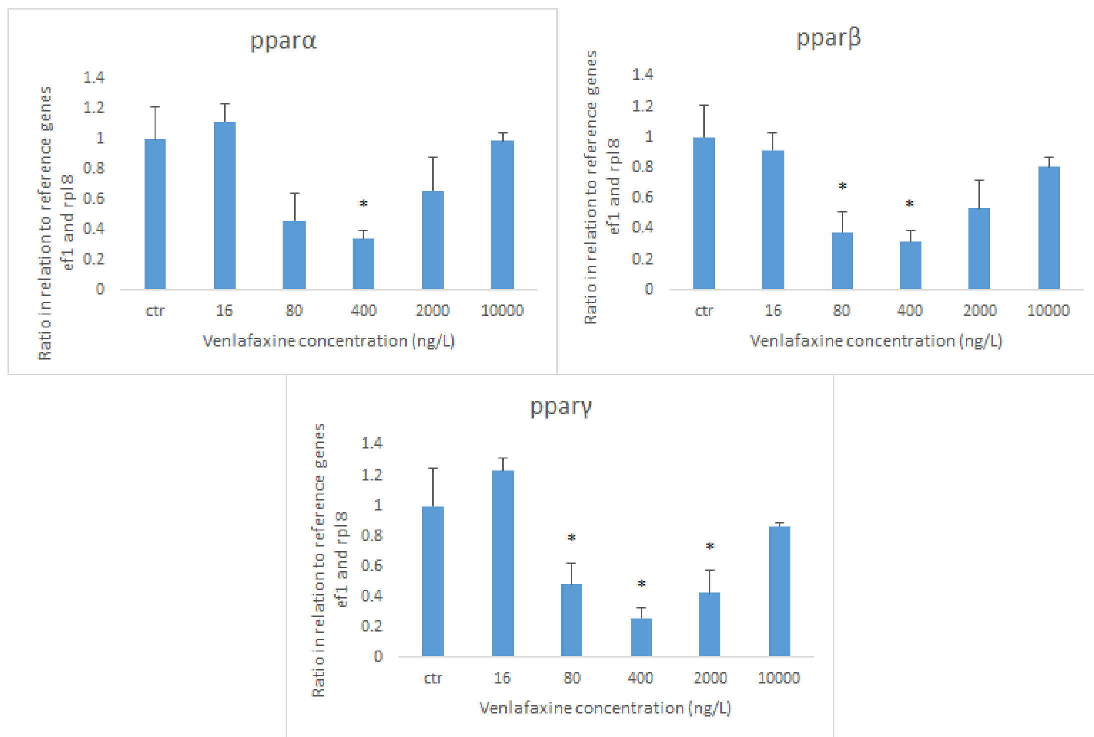


Figure 37. Gene expression of *ppar* group of nuclear receptors after exposure to different concentrations of venlafaxine for 80 hpf. Significant differences in relation to the control are marked with (*)

Patterns of transcription of ABC transporters after exposure to venlafaxine are shown in Figure 38. For *abcg2a* and *abcc1* a clear U-shape response pattern was observed. Compared to the control group, inhibition of gene expression was observed at 80 ($p<0.05$) and 400ng/L ($p<0.05$) venlafaxine concentrations for *abcg2a*, and at 400ng/L ($p<0.05$) for *abcc1*. For *abcb4*, larvae exposed to 2000ng/L concentration also exhibit inhibition of gene expression when compared to control larvae ($p<0.05$) (Figure 38.). No relevant differences among treatments were found for *abcc2* expression.

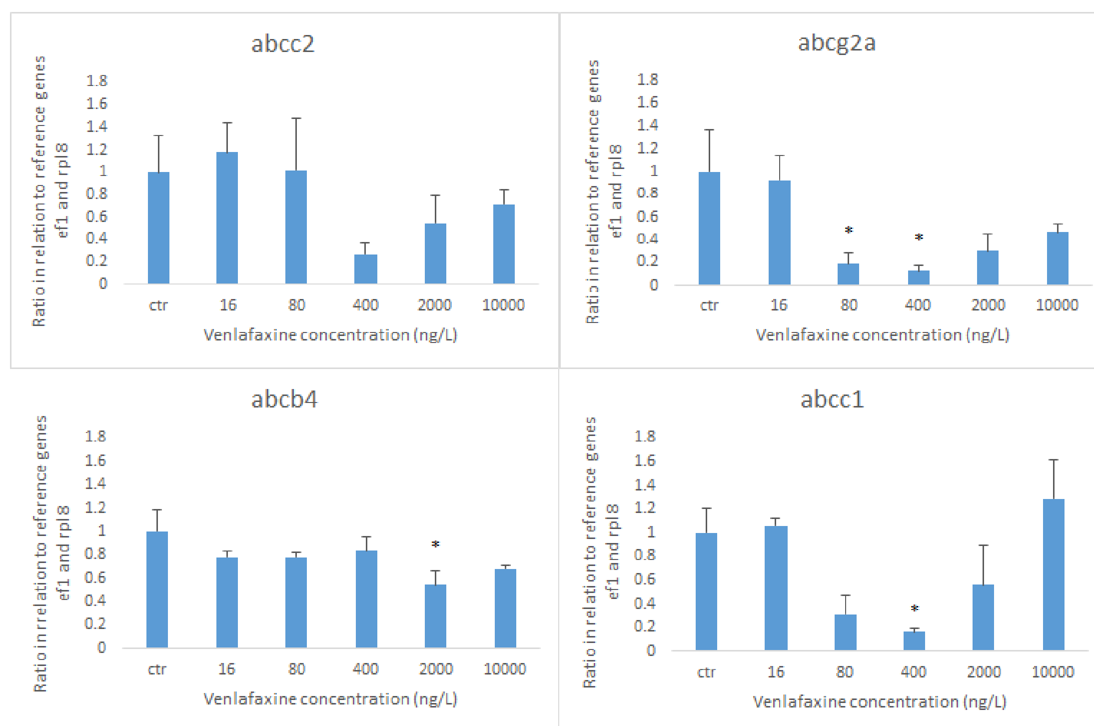


Figure 38. Gene expression of ABC transporters (Phase 0 / III) after exposure to different venlafaxine concentrations at 80 hpf. Significant differences in relation to the control are marked with (*)

Figure 39. presents the gene expression for biotransformation enzymes determined after exposure to venlafaxine for 80hpf. For phase I enzymes, *cyp3a65* expression was significantly decreased (p<0.05) in organisms exposed to 10000 ng/L venlafaxine, compared to the control group. No other alterations in gene expression were found, including for *cyp1a1* and phase II biotransformation enzyme *gstπ.* Gene expression of antioxidant enzyme Cu/Zn *sod* also showed a trend for decreased expression, which was significantly inhibited in the 10000ng/L group (p<0.05), compared to the control (Figure 40.). Levels of *cat* expression in exposed organisms were fairly stable, compared to those of control larvae.

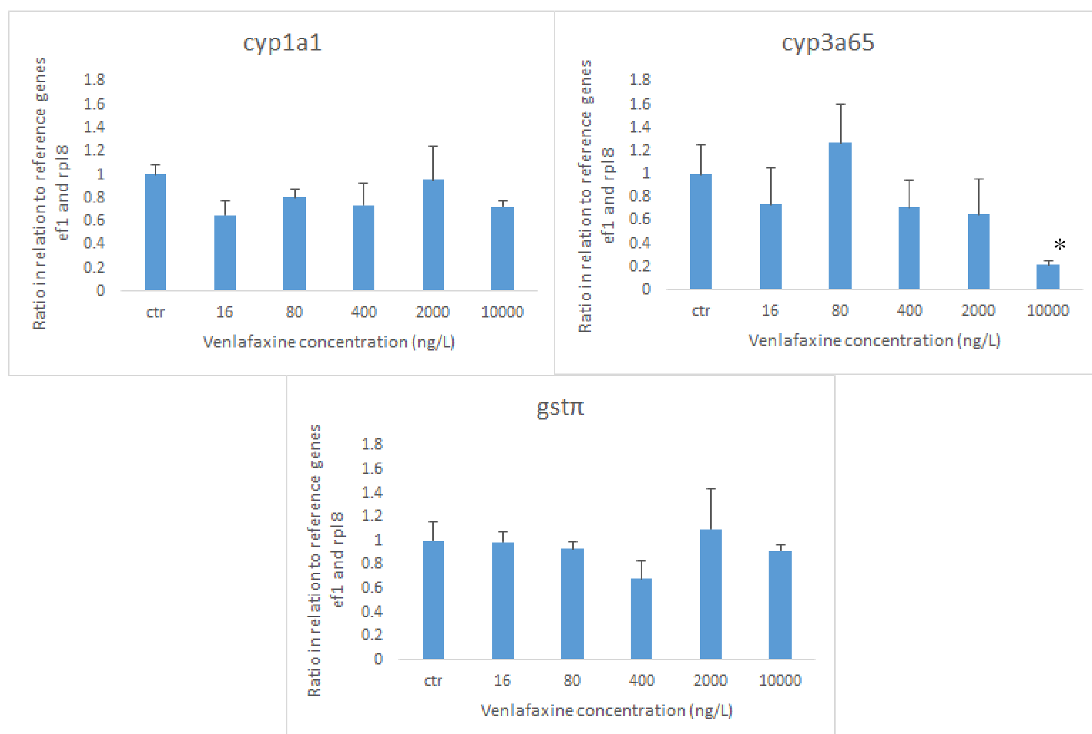


Figure 39. Gene expression of biotransformation enzymes (phase I and II) after exposure to different venlafaxine concentrations at 80 hpf. Significant differences in relation to the control are marked with (*)

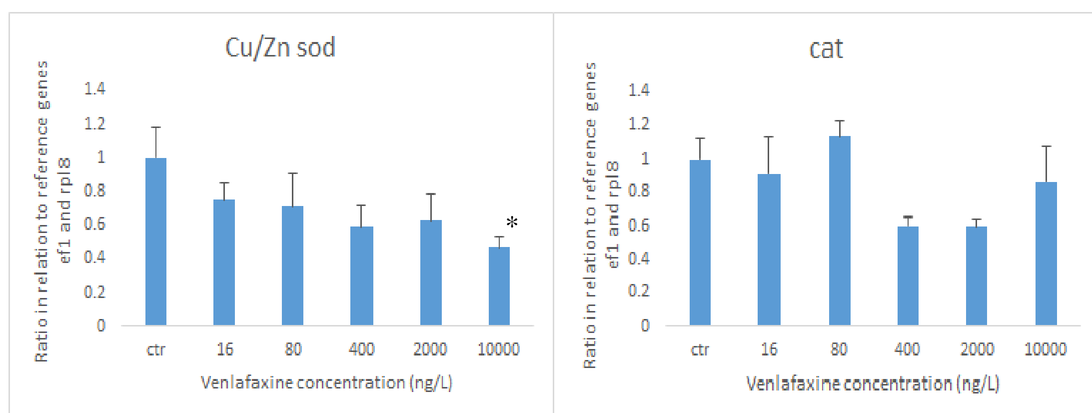


Figure 40. Gene expression of oxidative stress enzymes after exposure to different venlafaxine concentrations for 80 hpf. Significant differences in relation to the control are marked with (*)

A summary of the pattern of variation of gene expression obtained in the venlafaxine assays is shown in the heatmap of Figure 41. In contrast to norfluoxetine, where slight tendency for upregulation was observed, the heatmap for venlafaxine shows the clear tendency for downregulation of gene expression identified previously, as indicated by the prevalence of colder colours. Genes for which higher downregulation was observed were: *abcg2a*, *abcc1*, *drd1b, ppar* receptors, *5-ht2c*, *vmat2*, *sert*, *net* and *5-ht1a*. On the other hand, *adra2b* was the gene showing the

highest level of induction. Apart from this, upregulation was only observed for *dat*, though in a very low level. Hierarchical cluster analysis identified two groups of concentrations sharing some similarity in gene expression (Figure 41.). The first group aligned together concentrations of 80, 400 and 2000 ng/L venlafaxine. Here *abcg2a* and *abcc1* transporters, *ppar* nuclear receptors, *drd1b* and *5-ht2c* showed resembling patterns of expression, characterized particularly by downregulation. The second group aligned together the most extreme concentrations (16 and 10000 ng/L), confirming the U-shape response described above. Most genes in this group showed no alterations relative to control or very slight downregulation, in contrast to the former group.

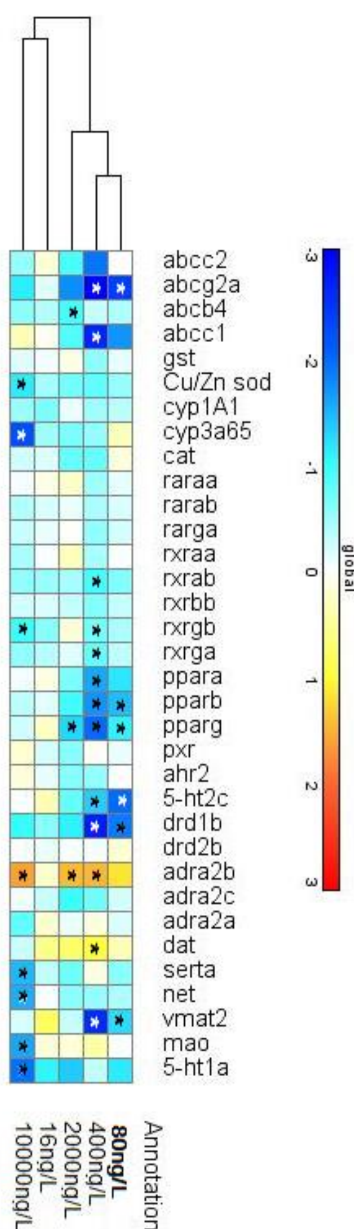
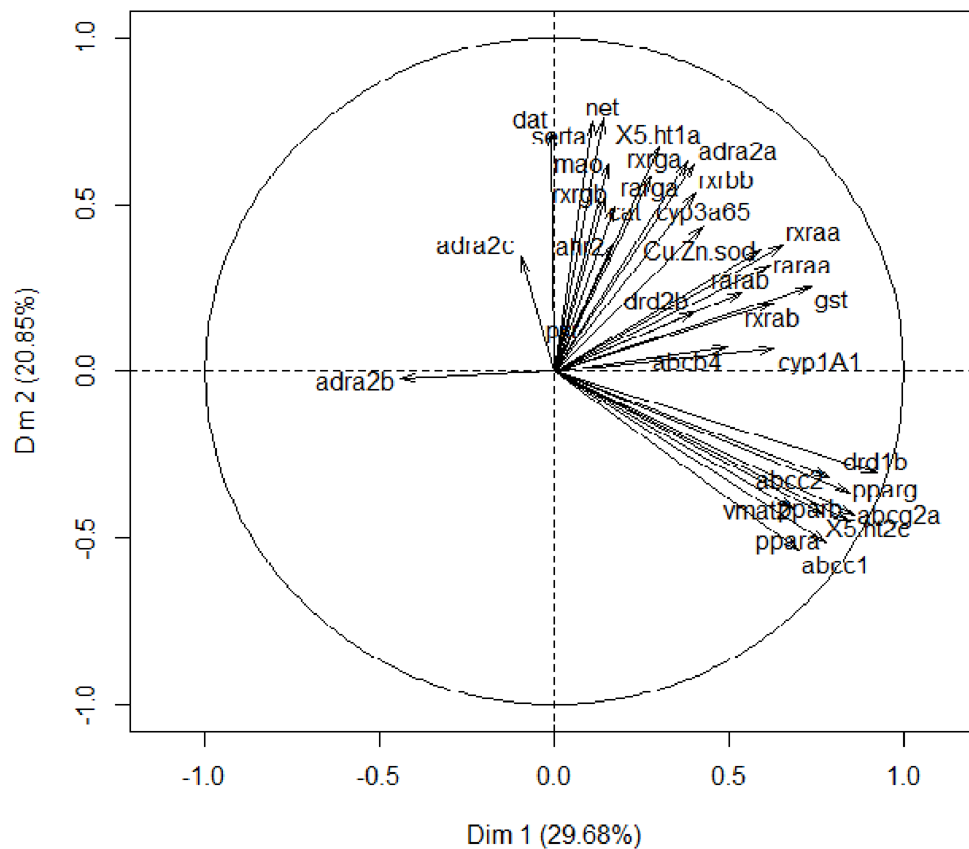


Figure 41. Heatmap of gene expression variation in zebrafish early larvae after exposure to different concentrations of venlafaxine for 80hpf. The colour scale reflects the direction and magnitude of variation in relation to the control group. The asterisk indicates significant differences relative to the control at $p < 0.05$.

In the PCA performed for this dataset seven components were extracted, expressing 88% of the total variability in the data. Of these, the first two principal components (PCs) retained 51% of the total inertia (Figure 42). The first component was mainly associated with the expression of ABC transporters, dopamine and serotonin receptors, and several nuclear receptors related to the detoxification process: drd1b (r>0.9); *abcg2a, pparγ, pparβ* showing correlation with the axis above 0.8;

abcc2, pparα, 5-ht2c and *gstπ* with r>0.7; *abcc1, vmat2, rxraa, cyp1a1, rxrab* and *raraa* with *r*>0.6. This axis clearly separated the control group from the 400 ng/L treatment, which tend to show upregulation of *adra2b* and downregulation of *abcc2, abcg2a, abcc1, ppar* nuclear receptors*, drd1b* and *vmat2* (Figure 42). In the venlafaxine treatment, the second axis was mainly associated with the expression of monoamine transporters and receptors: *net, serta* and *dat*, showing correlation with the axis above 0.7; and with *5-ht1a, rxrga, adra2a* and *mao* (*r*>0.6). This axis further discriminated the highest test treatment (10000ng/L) from the remaining groups.



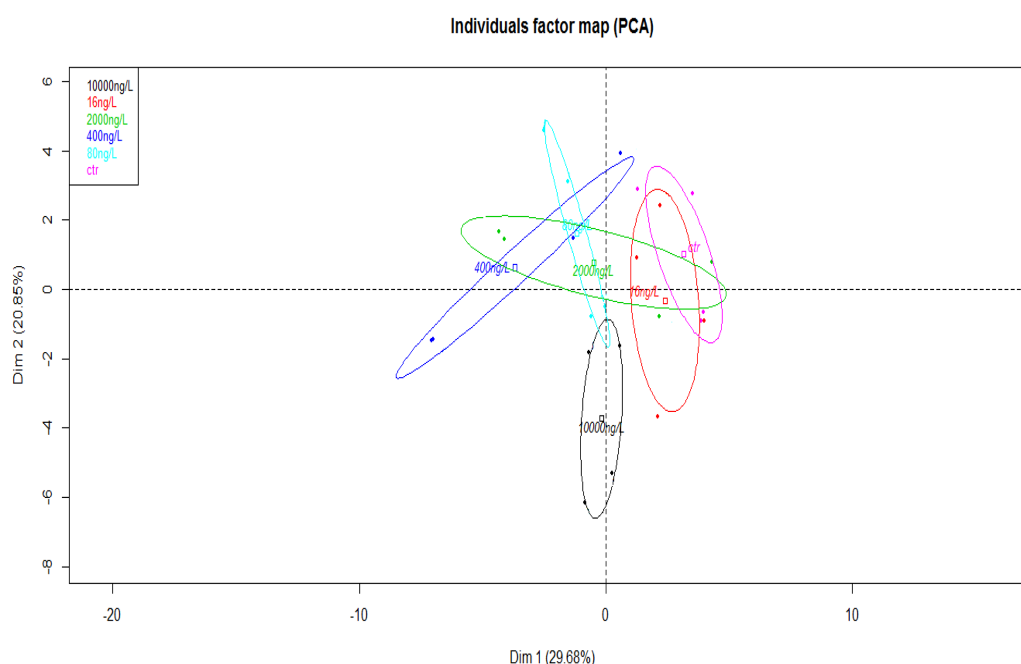


Figure 42. Results of the PCA defined by the expression of genes evaluated in zebrafish larvae exposed to venlafaxine for 80hpf. The cloud of genes is represented in the variables factor map; sample are represented in the individuals factor map (bottom). Centroids of each treatment group, and respective confidence ellipses, are also shown.

3.3 Exposure to a cocktail of psychotropic drugs

3.3.1. Mortality and gross malformations

Results of the embryo toxicity assays performed with the cocktail of norfluoxetine and venlafaxine showed this combination elicited a higher rate of mortality in relation to control in all time windows of embryonic development of zebrafish analysed (Figure 43.). In comparison to organisms exposed to the single compound, the mixture also showed an increase in the mortality rate. However, no differences were found between mortality at 8 hpf and the following time windows evaluated. Observation of Figure 43. also shows that mortality in the cocktail occurred just until 32hpf, because no dead embryos were found between 32 and 80hpf in any of the test conditions. Results obtained for the single compounds are in the same range of those presented in the previous sections.

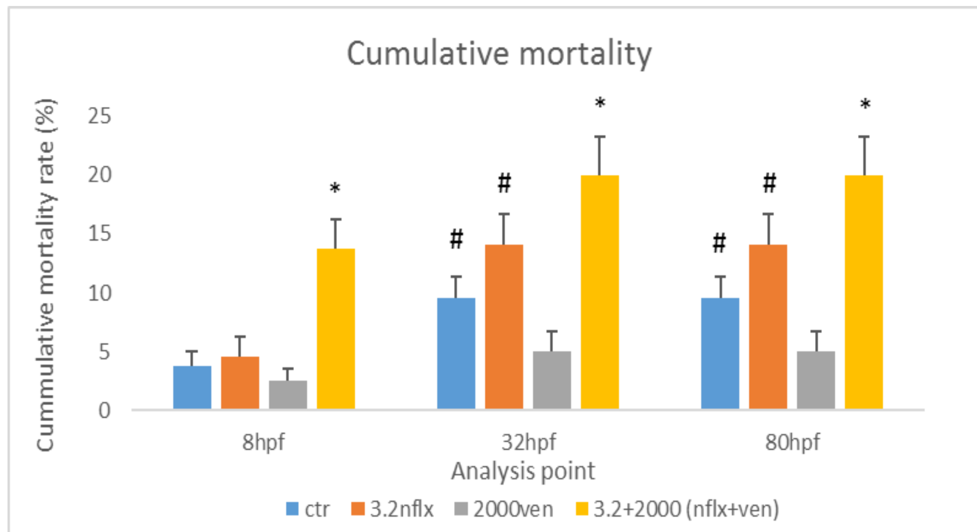


Figure 43. Cumulative mortality of embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mixture of both. Significant differences ($p<0.05$) in relation to the control are marked with *; differences relative to 8 hpf are indicated with #.

Regarding the gross anomalies found, the cocktail showed different results, according to the time window analysed (Figure 44.). At 8 hpf embryos anomalies were seldom observed in any test group. At 32 hpf the paradigm changes and embryos exposed to the single compounds or the mixture had a significant increase in the total of anomalies found in relation to the respective control and also in relation to the respective test group at 8 hpf. Embryos exposed to the mixture were the ones presenting more anomalies, mainly due to anomalies in pigmentation (Figure 45.), pointing out a possible synergetic relationship between both pharmaceuticals.

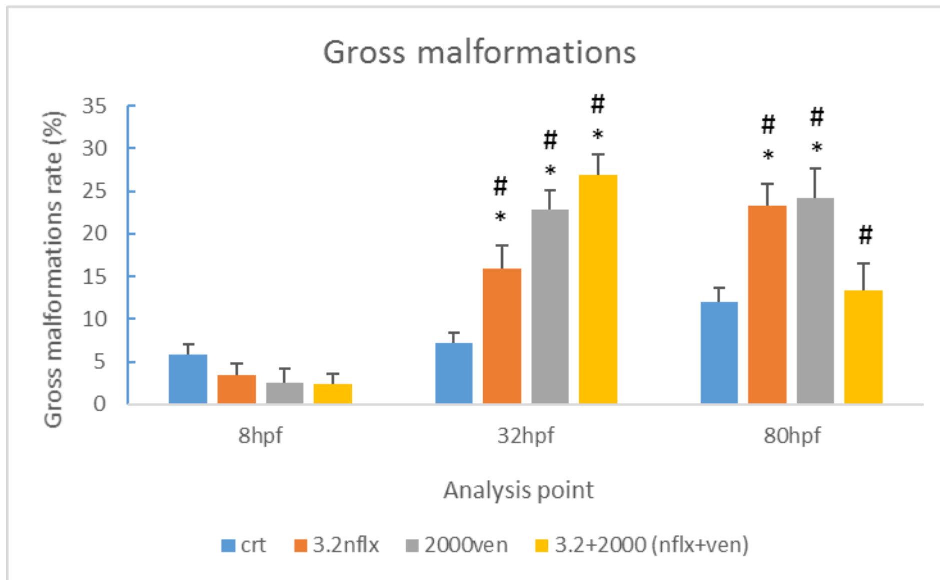


Figure 44. Gross malformations in embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mixture of both psychotropic drugs. Significant differences ($p<0.05$) in relation to the control are marked with *; significant differences relative to 8 hpf are marked with #.

At 80 hpf, the frequency of malformations exhibited by embryos exposed to single compounds was still higher than that of controls. The frequency of malformations detected in embryos exposed to the cocktail was similar to that of the control group (Figure 44.), pointing out a possible antagonist effect between both pharmaceuticals in this development period. Globally, in relation to embryos exposed to single compounds, the results are similar to those obtained in the previous sections of this work. Though, in this case, for the norfluoxetine treatment a significant increase in the anomalies was visible at 32hpf, in relation to control and 8hpf.

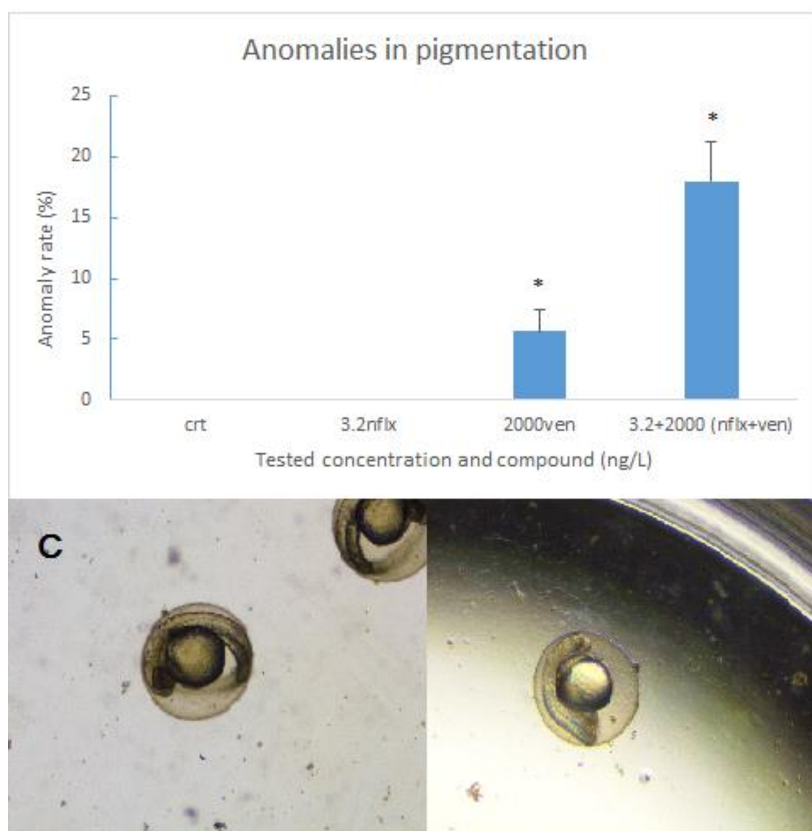


Figure 45. Total of pigmentation anomalies found at 32hpf and illustrative example of the anomaly. Significant differences in relation to the control are marked with an asterisk. (C) indicates a control at bottom right is indicated a pigmentation anomaly observed in treated embryo.

3.3.2. Molecular responses

Genes evaluated in the cocktail experiments were selected from those showing relevant alterations in the previous experiments. Reference genes were evaluated simultaneously with the genes of interest and their stability was investigated with NormFinder as described previously. The results obtained are described in the following subsections.

Reference genes

NormFinder evaluated *actb1* as the most stable gene (stability value: 0.008) if used as single reference gene. The combination of *actb1* and *rpl8* (stability value: 0.007) was found to be the most for multiple reference genes, due to opposite

intergroup variation. mRNA expression of the genes evaluated after venlafaxine exposure was therefore corrected by the normalization factor of *actb1* + *rpl8*.

Expression of monoamine receptors, nuclear receptors and ABC transporters

Figure 46. presents the gene expression of neurohormonal receptors *5-ht2c, drd1b* and *adra2b* involved in norfluoxetine and venlafaxine mode of action. The results show that embryos exposed to the mixture of norfluoxetine and venlafaxine suffered an inhibition of expression of *5-ht2c* ($p<0.05$) and *drd1b* ($p<0.05$) genes.

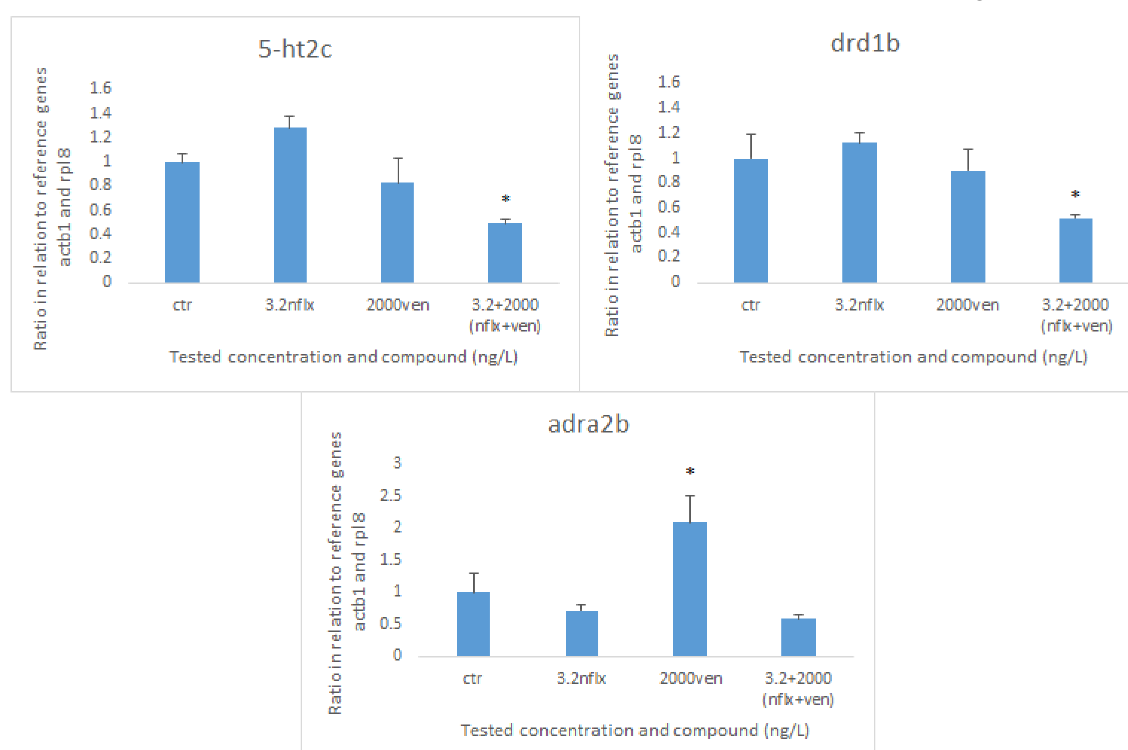


Figure 46. Gene expression of neurohormonal receptors in embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mix of both previous mentioned concentrations and compounds. Significant differences in relation to the control are marked with an asterisk (*).

In comparison to exposure to single compounds, embryos exposed to the mixture exhibited inhibition of gene expression previously observed for venlafaxine. Focusing only in the embryos exposed to single compounds, the results are within the response range obtained in previous sections of the work. Interestingly, for *adra2b*, despite a significant increase observed for venlafaxine, exposure to the cocktail resulted in inhibition of expression ($p<0.05$). These results suggest an antagonistic effect of these

two psychotropic drugs.

Figure 47. presents the gene expression of *vmat2*. The results show that embryos exposed to the mixture of 3.2ng/L of norfluoxetine and 2000ng/L of venlafaxine suffer a statistically significant inhibition of expression ($p<0.05$). Like for the neurohormonal receptors, in comparison to exposure to the compounds alone, embryos exposed to the mixture suffered inhibition of gene transcription, showing effects even when exposure to single compounds elicited no alterations, relative to the control.

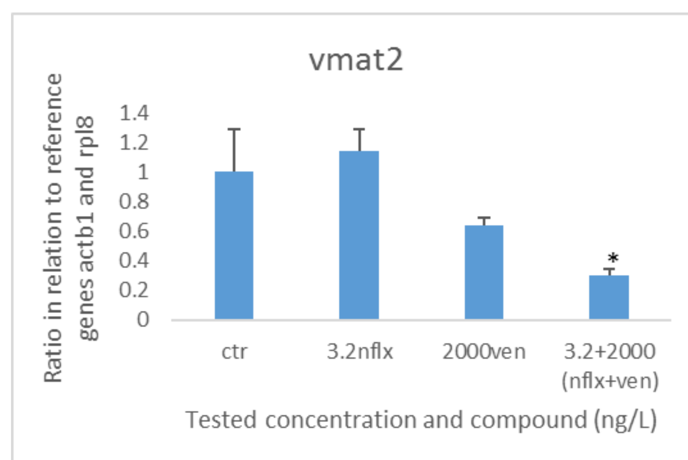


Figure 47. Gene expression of vmat2 in embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mix of both previous mentioned concentrations and compounds. Significant differences in relation to the control are marked with (*)

The pattern of transcription of *ppar* group of nuclear receptors after exposure to 3.2 ng/L of norfluoxetine, 2000ng/L of venlafaxine and a mixture of both concentrations is shown in Figure 48. The results showed that embryos exposed to the mixture of 3.2ng/L of norfluoxetine and 2000ng/L of venlafaxine suffer different responses in *ppar* genes ($p<0.05$).

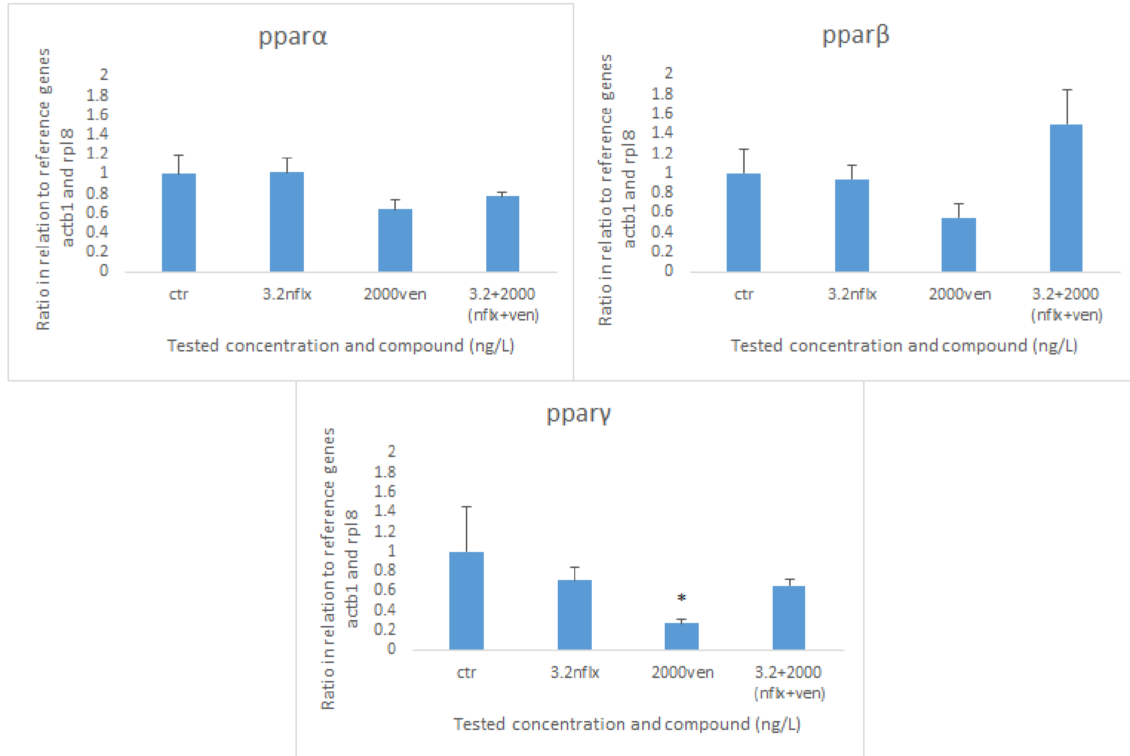


Figure 48. Gene expression of *ppar* group of nuclear receptors in embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mix of both previous mentioned concentrations and compounds.

For *pparγ* inhibition of gene expression was visible for the venlafaxine treatment, while for the mixture no differences relative to the control could be found (Figure 48), again suggesting an antagonistic effect of the two test substances. For the remaining *ppar* nuclear receptors, no differences among treatments were found. Focusing only in embryos exposed to the single compounds, the results for both substances are in the response range obtained in previous sections of the work.

Patterns of transcription of ABC transporters after exposure to 3.2 ng/L of norfluoxetine, 2000ng/L of venlafaxine and a mixture of both concentrations and compounds are shown in Figure 49. The results showed that embryos exposed to the mixture of 3.2ng/L of norfluoxetine and 2000ng/L of venlafaxine had different responses of ABC transporters. For *abcc2* there was an induction of gene expression following exposure to 3.2ng/L norfluoxetine ($p<0.05$), but no effect of the mixture, compared to the control group. For *abcc1* there was a clear induction of transcription

after exposure to norfluoxetine alone ($p<0.05$) and inhibition of transcription after exposure to venlafaxine alone ($p<0.05$). However, no significant differences relative to the control were found after expression to the cocktail evaluated.

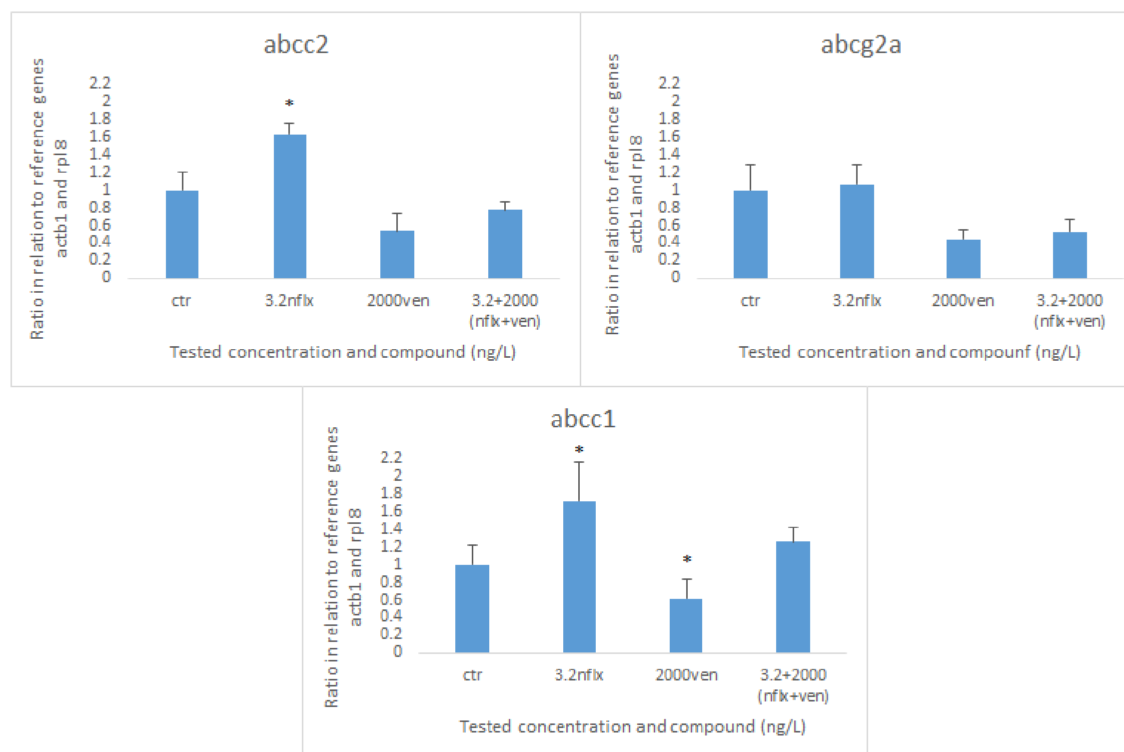


Figure 49. Gene expression of ABC transporters (Phase 0 / III) in embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mix of both previous mentioned concentrations and compounds. Significant differences in relation to the control are marked with (*)

Comparing the results obtained in the mixture those of exposures to single compounds, embryos exposed to 2000ng/L of venlafaxine suffered an inhibition but a recuperation to values close to the control was visible in the mixture which had an intermediate gene expression value between norfluoxetine and venlafaxine. These results again suggest an opposite effect of the two substances when provided together.

The hierarchical cluster analysis (HCA) for embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mix of both show some differences among tested genes. A summary of the variation in gene expression observed is shown in the heatmap of Figure 50. The clusters identified show differences between genes involved in the detoxification mechanisms and neurohormonal receptors. Also,

expression of *adra2b* is different from all the other tested genes. The heat map shows a general tendency for inhibition in the 2000ng/L venlafaxine treatment, relative to control. The norfluoxetine (3.2ng/L) and the mixture treatments were grouped together by the HCA. These show opposite responses to the venlafaxine treatment. For norfluoxetine, no alterations or slight induction of gene expression were prevalent. For the mixture, neurohormone genes were the most affected, showing inhibition of expression (Figure 50). The higher levels of inhibition of neurohormonal receptors in the mixture in relation to single compounds is clear in the heatmap. In addition to this, for ABC transporters and *ppar* genes it is also visible the tendency for the mixture to have intermediate values of expression, between those obtained for single exposure to venlafaxine or norfluoxetine.

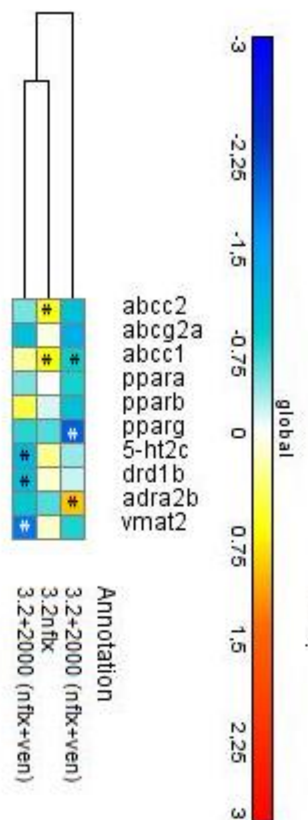


Figure 50. HCA and heatmap for embryos exposed exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mix of both previous mentioned concentrations and compounds for 80h. The asterisk indicates significant differences relative to the control at $p < 0.05$.

The analysis of the PCA individuals factor map showed differences between the control group and the some of the concentrations tested. Individuals exposed to 3.2ng/L of norfluoxetine only, didn't show differences in relation to control since the ellipses of both group intersect with each other (Figure 51.). The same didn't happen in individual exposed to 2000ng/L of venlafaxine and to the mixture of 3.2ng/L of norfluoxetine and 2000ng/L of venlafaxine, where ellipses didn't intersect with the control group. The mixture seems to be more different from control than the venlafaxine concentration alone, however, this can be explained by the greater cohesion of the values obtained in the mixture.

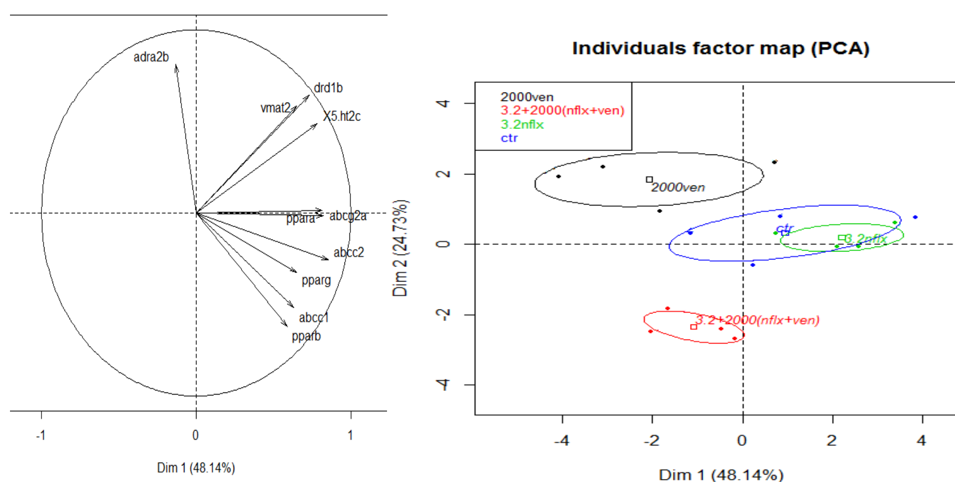


Figure 51. Results of the PCA defined by the expression of genes evaluated in zebrafish larvae exposed to norfluoxetine (3.2ng/L), venlafaxine (2000ng/L) and a mixture of both for 80hpf. The cloud of genes is represented in the variables factor map; samples are represented in the individuals factor map (bottom). Centroids of each treatment group, and respective confidence ellipses, are shown.

In the PCA performed for cocktail dataset three components were extracted, expressing 84% of the total variability in the data. The first two components explained high a proportion (73%) of the total inertia (Figure 51.). The analysis to the variables factor map show interesting patterns of expression of tested genes in embryos exposed to 3.2ng/L of norfluoxetine, 2000ng/L of venlafaxine and to a mixture of both. The first component was associated with the expression of ABC transporters, *ppar* nuclear receptors and serotonin and dopamine receptors as well. The component significantly differentiated norfluoxetine and venlafaxine treatments (Figure 51). In the

map there are visible differences between genes involved in the mode of action of the studied drugs (*drd1b*, *vmat2* and *5-ht2c*) and genes involved in detoxification mechanisms (*ppar* receptors and abc transporters genes). The second component opposed the adrenergic and dopaminergic receptors to *pparβ*. This axis significantly discriminated the venlafaxine treatment from the cocktail (Figure 51). Moreover, *adra2b* is clearly separated from all the others assessed. Looking to the map, one can also observe a strong positive correlation between some specific genes like *vmat2* and *drd1b* or *pparα* and *abcg2a*, that have almost equal representation.

Chapter 4: Discussion

4.Discussion

The use of embryos of aquatic organisms in bioassays, is useful and effective since the early stages of development are usually more sensitive than other life cycle phases. They also allow to monitor parameters that otherwise would be impossible to analyse later in life. In this study two different kinds of antidepressants were evaluated, a SSRI (norfluoxetine) and a SNRI (venlafaxine), single and in mixture. Study of pharmaceutical metabolites is very pertinent since they are often active, exhibiting pharmacological properties. However, investigation of these compounds has been neglected in scientific studies (Evgenidou *et al.*, 2015). In this work, the choice felt on norfluoxetine because this is an active metabolite of a very well-known and studied parental compound (fluoxetine). Moreover, in recent studies it is the most representative metabolite found in Portugal (Santos *et al.*, 2016). Venlafaxine was chosen because it is widely prescribed all over the world and is found in aquatic systems in relatively high concentrations, in relation to other antidepressants like fluoxetine (Schultz *et al.*, 2010; Gonzalez Alonso *et al.*, 2010).

The study of the action of multiple substances has also great environmental relevance given that in the environment the most common situation is the presence of several compounds that interact to each other (Evgenidou *et al.*, 2015). Despite its importance, knowledge about the action of mixtures of substances is still scarce and, thus, the reason why testing a mixture of these drugs was considered very relevant in the scope of this work.

The results obtained in this work, showed that norfluoxetine, venlafaxine and the combination of both compounds, have significant effects in the embryonic development and transcription patterns of genes related with mechanisms of detoxification and neurohormonal systems in zebrafish, although each compound alone had different response magnitudes.

Regarding embryo toxicity assays, exposure to different concentrations of venlafaxine or norfluoxetine caused low mortality of the embryos, similar to that of the control group. The mortality rate increased over time, mainly from 8hpf to 32hpf,

raising the question of whether this could be related to increased sensitivity to the test drugs due to development of serotonergic and adrenergic systems, which start to form at 24hpf in zebrafish embryos (Airhart *et al.,* 2012; Kastenhuber *et al.,* 2010). However, increased mortality in this developmental window was also found in the control group, within the same range of exposed groups, ruling out this possibility. In contrast, exposure to a mixture of norfluoxetine and venlafaxine, caused increased mortality in relation to the control, indicating the combined exposure should have more severe embryotoxic effects than the single compounds. This mortality increase was particularly proeminent at 8hpf, suggesting that the mixture could be acting in pathways other then the serotonergic and adrenergic routes, since these are not yet developed at this stage. Further investigation is needed in order to clarify this possibility. Mortality elicited by the combination of the two substances stabilised between 32 and 80hpf. This could be linked to the developmental processes taking place in the embryo. Organogenesis occurs during this period (Kimmel *et al.*, 1995), with the establishment of systems that could make the embryo more apt to deal with harmful compounds to the body.

The psychotropic drugs investigated caused the appearance of abnormalities in exposed embryos, at 80hpf for norfluoxetine and at 32 and 80hpf for venlafaxine and 32 hpf for the cocktail. The anomalies described were caused by exposure levels in the range found in aquatic systems (≥0.64ng/L for norfluoxetine, ≥400ng/L at 32hpf and ≥80ng/L at 80hpf for venlafaxine, 3.2+2000ng/L at 32hpf for the cocktail). Up-to-date no report is available in the literature describing the presence of embryonic anomalies elicited by either norfluoxetine or venlafaxine in fish embryos. In fact, data from a previous study suggests that exposure of zebrafish embryos to venlafaxine would result in very low frequency of appearance of anomalies (Galus *et al.*, 2013). These authors exposed zebrafish embryos to 0, 0.5 and 10μg/L of venlafaxine for 96h. The rate of anomalies was approximately 1% in exposed embryos, meaning that was a decrease of 3- to 7-fold decrease of anomalies, in 0.5 and 10μg/L concentrations respectively. However, results presented herein show significant increase in the appearance of anomalies in relation to the control, at 32 and 80hpf, for norfluoxetine

(~20%) and venlafaxine (~25%). The differences observed may be due to the use of different exposure conditions and/or differences in sensitivity of the test organisms, as well as prevalence of anomalies in pigmentation found in this work weren't analysed by the work of Galus and colleagues. Furthermore, in this study, exposure to the mixture of 3.2ng/L norfluoxetine and 2000 ng/L venlafaxine, suggests the combined substances may initially have stronger effects than exposure to each of the single compounds, causing a higher frequency of anomalies. Nevertheless, part of these may correspond to developmental delays, with later recovery as suggested by the decrease in total anomalies relative to control found at 80hpf. Most has different results in the appearance of anomalies. The reduction observed at 80hpf was unexpected and seems to point to an antagonistic effect of norfluoxetine and venlafaxine, mainly explained by the absence of significant anomalies in the embryos' spine, contrarily to embryos exposed to venlafaxine. Like previously mentioned, norfluoxetine and venlafaxine act by selectively inhibiting serotonin 5-HT reuptake receptors and adrenergic receptors that on aquatic organisms are present throughout most embryonic development (Airhart *et al.,* 2012; Kastenhuber *et al.*, 2010; Kreke and Dietrich, 2008). Main anomalies found were in pigmentation, vitelline sac and spine. In particular, there was a decrease in pigmentation in embryos, which may be a result of the effect of norfluoxetine and venlafaxine on embryo adrenergic receptors, which are present in the melanophores stimulating pigmentation (Xu and Xie, 2011). This is an indication that both compounds have an effect on adrenergic receptors in the zebrafish embryo that should be studied in more detail. There were also significant anomalies in the vitelline sac (32hpf) and spine (80hpf). If anomalies in the vitelline sac seemed to disappear at 80hpf, minimizing possible effects in populations, spinal anomalies in individuals already in a larval stage can influence the population. The later can result in decreased swimming performance and, consequently, in major difficulty to avoid predators and find food, calling into question the organisms' survival. In the total of anomalies found, higher concentrations of norfluoxetine and venlafaxine are the ones that induce greatest effects. It is also important to notice a visible significant increase in the appearance of anomalies at concentrations that may be

found in the environment for both tested compounds, indicating that aquatic organisms may already been suffering negative impacts of exposure to norfluoxetine and venlafaxine.

Concerning gene expression, a wide range of genes involved in the mode of action of the tested pharmaceuticals, and defence mechanisms of zebrafish in response to xenobiotics, was tested. mRNA expression of three reference genes was also assessed. Recently, the use of a multiple reference gene strategy was suggested as the most adequate to normalise mRNA expression (Urbatzka *et al.,* 2013). In the present study, among the three reference genes tested, the combinations *actb1+rpl8*, *ef1+rpl8* and *actb1+rpl8* were found suitable to normalise mRNA expression following norfluoxetine, venlafaxine and their cocktail exposure, respectively. Expression of these gene combinations was found to be the most stable across treatments in each experiment. Normalisation of mRNA expression of the 34 genes investigated by the respective combination indicated by NormFinder thus allowed to minimise bias in the results obtained (Urbatzka *et al*., 2013). NormFinder software, based on a methodology integrating intra- and intergroup variation of experimental data, proved to be helpful and easy of use to find the most stable combinations of reference genes across treatments.

For the genes involved in the mode of action of the drugs tested, significant differences among treatments were found for both venlafaxine and norfluoxetine. In the single exposure to different concentrations of norfluoxetine, increased expression of serotonin receptor *5-ht2c* at 80ng/L was found. This was unexpected once that norfluoxetine, as SSRI, is expected to act by inhibiting 5-HT receptors (Mennigen *et al.,* 2011). However, in humans mainly in the first weeks of exposure, many antidepressants can cause over activity of the *5-ht2c* receptors, originating some side effects in the organism (Milan, 2005); the same could possibly happen in zebrafish embryos. Expression of adrenergic receptors, in particular *adra2c* at the lowest concentration tested, tended to be decreased while alterations in embryos' pigmentation were found at the highest test concentrations. These results suggest that norfluoxetine interferes with adrenergic receptors. It has been shown previously

that these receptors are involved in the pigmentation in zebrafish embryos (Xu and Xie, 2011). Embryos exposed to different concentrations of venlafaxine, also showed alterations in genes involved in the pharmaceutical mode of action. Venlafaxine acts by inhibiting both serotonin and norepinephrine receptor (Gutierrez *et al.,* 2003; Mennigen *et al.,* 2011), which means that an inhibition in the transcription of these genes would be expected. Serotoninergic genes showed a tendency to inhibition of expression, as expected, though at different test concentrations of venlafaxine: 10000ng/L for *serta* (-60%) and *5-ht1a* (-80%), and 80 and 400ng/L for *5-ht2c* (-70 and 60%, respectively). For adrenergic genes there was an unexpected mixed response. Norepinephrine transporter (*net*) was inhibited in the highest concentration of venlafaxine tested. However, adrenergic receptors did not follow the same tendency. In fact, adrenergic receptor *adra2b* showed strong induction of expression at concentrations ≥400ng/L, concentrations for which anomalies in embryos' pigmentation were also found. Nevertheless, more investigation is needed in order to better understand the relation between modulation of adrenoreceptors and anomalies in pigmentation elicited by exposure to either norfluoxetine or venlafaxine. In mammals, high concentrations of venlafaxine also affect the dopaminergic system, inhibiting the dopaminergic receptors (Sansone and Sansone, 2014).

In this study, obtained results showed inhibition of the dopamine receptor *drd1b* (80 and 400ng/L) in a U-shape response where highest test concentrations elicited responses similar to control and lowest venlafaxine level tested. Concomitant induction of *dat* at 400ng/L was additionally found. For the mixture of 3.2ng/L of norfluoxetine and 2000ng/L venlafaxine, results revealed inhibition of *5-ht2c* but not of *adra2b*. Both low-concentration and non-monotonic responses were previously reported to be caused by exposure of non-target organisms to selective serotonin reuptake inhibitors (Rodrigues *et al.,* 2014, 2015; Cunha *et al.,* 2016). Moreover, according to a recent wide review such responses should be typically expected from substances with endocrine-disrupting activity (Vandenberg *et al.,* 2012). The different effects may be produced because many receptors, or receptor subtypes, are expressed specifically in a single or a few cell types whereas others are found in

multiple cell types. Different coregulators (e.g. specific ions) present in the different cell types may further contribute to differences in behaviour of target genes. On this regard, it is well known that some SSRI and SNRI act on more than one receptor/receptor subtype, which may be expressed in different quantities in different cell types and organs, therefore producing variable effects on gene expression or cellular phenomena. For example, studies have shown that fluoxetine may also interact with acetylcholine receptors (Garcia-Colunga *et al.,* 1997) and that *eat-6* is a component of the pathway that couples 5-HT signaling and acetylcholine neurotransmission in Caenorhabditis elegans (Govorunova *et al.,* 2010). *eat-6* is expressed in ventral cord cholinergic neurons. This gene mediates 5-HT inputs to cholinergic neurons, regulating acetylcholine pre-synaptic neurotransmission, and has a post-synaptic role at the body wall neuromuscular junctions (Govorunova *et al.,* 2010). According to the authors, both stimulatory and inhibitory inputs of serotonergic neurons to cholinergic neurotransmission could occur through regulation of different receptor subtypes at different cellular targets, resulting in both synergistic or antagonistic effects. Such type of cross-regulation could explain divergent response patterns observed in the present study. As recently pointed out by Ford and Fong (2016) in an interesting review about the effects of these psychotropic drugs, SSRI and SNRI exhibit binding affinity also to dopamine reuptake receptors, sigma receptors and enzymes such as nitric oxide synthase and a variety of cytochrome P450 enzymes, so that their specificity has been debated for years.

It is also of note that interactions between serotonergic, adrenergic and dopaminergic systems (Kreke and Dietrich, 2008) should occur through G protein-coupled receptors (GPCRs), which have a variety of physiological roles. GPCRs are a large family of protein receptors able to sense extracellular molecules, subsequently activating intracellular signal transduction pathways, such as the cyclic adenosine monophosphate (cAMP) or the phosphatidylinositol signalling pathways (Filmore, 2004; Trzaskowski *et al.,* 2012). GPCRs are activated by a wide variety of ligands, including light stimulatory molecules, pheromones, hormones and neurotransmitters. Biogenic amines are mainly involved in the regulation of mood and

behaviour. Furthermore, not only cross-talking may occur between GPCRs but also they may become desensitized when exposed to their ligand for a prolonged period of time, either through downregulation of the activated GPCR or through downregulation of a different GPCR (Filmore, 2004; Trzaskowski *et al.,* 2012). According to the present results, for venlafaxine, alterations observed in *5-ht1a* (5-htr1aa) and *adra2b* should act on, inhibitory GPCR, Gi/Go pathway (Kelder *et al.,* 2012; Kutmon *et al.,* 2016) (Figure 52).

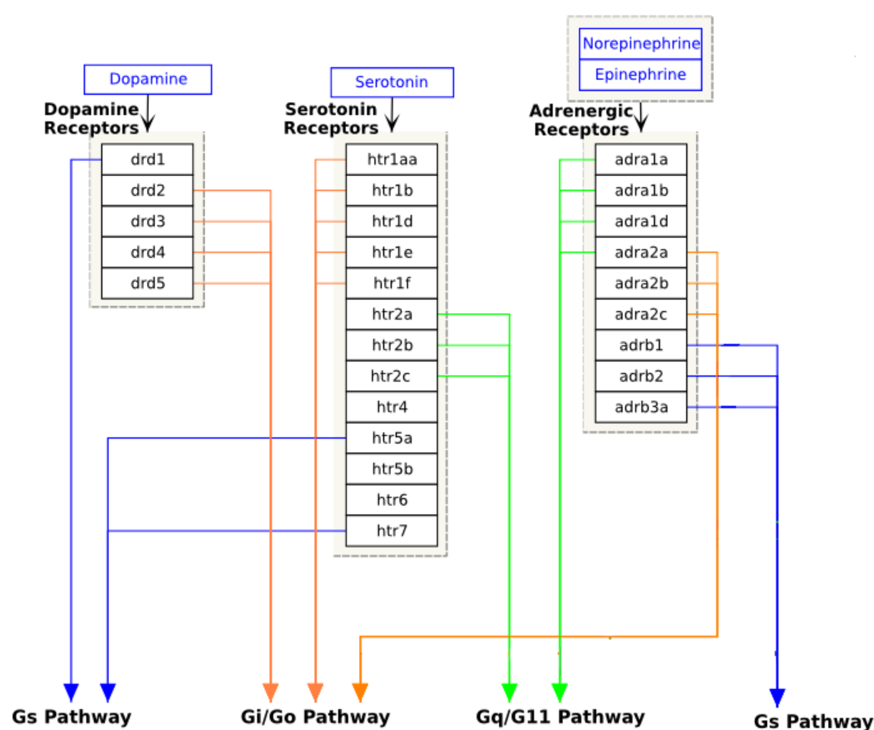


Figure 52. Relations between different monoamine receptors and associated G protein-coupled receptors (GPCRs) pathways in zebrafish. Gs (stimulatory GPCRs) and Gi (inhibitory GPCRs) use the cAMP signal transduction, whereas Gq uses the Phosphatidylinositol signal transduction. Adapted from Kelder et al. (2012) and Kutmon et al. (2016). (http://wikipathways.org/index.php/Pathway:WP1389).

vmat2 and *mao* interact with monoamines like serotonin, norepinephrine and dopamine. *vmat2* is responsible for the intracellular transport of these monoamines, while *mao* is responsible for their oxidative deamination (Wimalasena, 2010; Sallinen *et al.,* 2009). Both venlafaxine and norfluoxetine cause increase in the levels of serotonin and norepinephrine in the synaptic cleft (Kreke and Dietrich, 2008; Fent *et al.,* 2006) and because of that, mao is expected to reduce its activity in the presence

of these compounds. In fact, in the highest concentration of venlafaxine tested (10000ng/L) inhibition of mao expression was observed, suggesting that only concentrations above 2000 to 10000ng/L of venlafaxine would be able to cause intracellular monoamine depletion at a level high enough to downregulate *mao* activity. For *vmat2*, decreased expression inhibition was observed at 80 and 400ng/L venlafaxine and in embryos exposed to the mixture of 3.2ng/L of norfluoxetine and 2000ng/L of venlafaxine. In fact, inhibition of monoamine reuptake is expected to reshape presynaptic and postsynaptic responses causing long-term depletion of vesicular stores (Kristensen *et al.,* 2011). Such a mechanism could cause a decrease in both *mao* and *vmat2*. Indeed, in rats there are indications that SSRIs have an inhibitory effect in *vmat2* (Yasumoto *et al.,* 2009). Also inhibition of *vmat2* can contribute to an increase in extracellular levels of catecholamines, like serotonin and norepinephrine (Wimalasena, 2011). The mode of action of venlafaxine is precisely associated with increased levels of these catecholamines in the synaptic cleft, which supports a possible inhibition of *vmat2*. Obtained results therefore suggest that an inhibitory effect of antidepressants in *vmat2* may also occur in fish species, but more work is needed, to confirm this hypothesis.

Nuclear receptors are involved in the regulation of different phases of xenobiotics metabolisation (Xu *et al.,* 2005). For all different nuclear receptors tested, embryos exposed to norfluoxetine showed significant differences only in the expression of rarga that was strongly increased at 3.2ng/L. Nuclear receptors like rarga are responsible for the regulation of genes involved in xenobiotic metabolism. It is thus possible that norfluoxetine stimulates this receptor at 3.2ng/L, but more tests are needed to support this. In embryos exposed to different concentrations of venlafaxine wider alterations in nuclear receptors were visible, namely inhibition of rxrab, rxrga and rxrgb expression (400ng/L). rxr nuclear receptors can form heterodimers with other nuclear receptors, regulating their main targets (Xu *et al,* 2005). Thanks to that binding ability, rxr are involved in the regulation of a high amount of drug metabolising enzymes (Xu *et al.*, 2005). This means that the decreased expression found in some rxr forms can compromise the regulation of drug metabolising enzymes which can

influence negatively the organisms' homeostasis. In another study, it was found that rxrg signalling can influence dopamine receptor d2 in mice (Krzyzosiak *et al.,* 2010). In this study, drd2b showed no decreased gene expression. However, expression of drd1b was found to be lower than control at same concentration found for rxrg (400ng/L), suggesting zebrafish rxrg expression may influence dopamine receptor d1. All *ppar* nuclear receptors showed decreased expression at 400ng/L of venlafaxine and some even in the range 80-2000ng/L. Previous studies in fish, demonstrated that *ppar* are involved in lipid and xenobiotic metabolism (Maradonna *et al.,* 2015; Wang *et al.,* 2008). Thereby, the observed alterations suggest that zebrafish capacity to metabolise lipids and xenobiotics is compromised in the presence of venlafaxine even at environmentally relevant concentrations. In addition to their primary action as lipid sensors and regulators of lipid metabolism, *ppar* are also expressed in the brain where they have anti-inflammatory and neuroprotective roles in the central nervous system. *ppar-α* loss of function, for example, has been shown to promote repetitive behavior and cognitive inflexibility (D'Agostino *et al.,* 2015). Moreover, another important issue that has been raised is the role of *pparα* and *pparγ* agonists in drug addiction and dopaminergic neurons. *ppar* agonists showed an important role in reducing self-intake of drugs in mammals, as well as reduced stimulation of dopaminergic neurons involved in mesolimbic dopamine pathway of the brain reward system (Panlilio *et al.,* 2012; Melis *et al.,* 2008). Activation of *pparα* and *pparγ* was shown to suppress sensitization that develops the effects of psychotropic drugs when they are administered chronically; these effects could be reversed by *ppar* antagonists that had no effect on drug uptake on their own. Because behavioral sensitization is thought to contribute to the development of drug dependence, this came to support interest of *ppar* agonists as treatments for drug dependence (Mascia *et al.,* 2012; Foll *et al.,* 2013). The underexpression of *ppar* elicited by venlafaxine exposure in the present study, could suggest the occurrence of drug dependence. It may also explain the induction of *dat* at 400ng/L. However, more research is needed to better understand this question, even because lack of physiological dependence in humans is one the advantages in the use of venlafaxine (Kelsey, 2000). *ppar* genes expression was also

tested in the cocktail assay, but no alterations were found in exposed larvae, meaning that the mixture did not change significantly the action of this nuclear receptor. Given that no change in *ppar* expression was obtained in the presence of norfluoxetine, the results further indicate an antagonistic effect of the two psychotropic drugs on these nuclear receptors.

Members of the superfamily ABC in aquatic species are part of the mechanism denominated MXR (multixenobiotic resistance) (Kurelec, 1992) and are considered the first line of defence against toxic substances and its metabolites at the cellular level (Bard, 2000). For ABC transporters tested, embryos exposed to norfluoxetine showed diminished expression of abcb4 (0.64ng/L) and activation of abcc1 (3.2ng/L). In embryos exposed to venlafaxine there was decreased expression of abcb4 (2000ng/L), abcc1 (400ng/L) and abcg2a (80 and 400ng/L). The abcb4 gene encodes the functional P-glycoprotein in zebrafish, playing the role of first line of defence against toxic substances in the organism, incorporating the phase 0 of biotransformation (Fischer *et al.,* 2013). The role this gene plays in the organism is critical as it provides great protection to fish against toxic compounds. Expression of this gene, therefore, should not exhibit large variation in order to not compromise individuals' survival (Epel *et al.,* 2008). Thereby decreased abcb4 caused by norfluoxetine or venlafaxine can be life-threatening to embryos living under high chemical stress conditions. *abcc1* and *abcg2a* are responsible for the efflux of metabolites outside the cell (phase III of biotransformation) (Xu *et al.,* 2005). This suggests that low concentrations of norfluoxetine may result in abcc1 stimulation to efflux norfluoxetine or its metabolites out of the cell to defend the organism against their potential damage. In contrast, low concentrations of venlafaxine appears to impair cells' ability to efflux metabolites; aquatic organisms may thus lose efflux capacity of potentially harmful compounds for cell function, suffering injury to the homeostasis of the organism. It is also important to refer that the decreased expression of ABC transporters reported herein occurred at concentrations levels found in the aquatic environment. Therefore, populations of aquatic fish may be already suffering these effects, and their consequences, in systems where one or the

other of these psychotropic drugs have been detected. Expression of ABC transporters abcc1, abcg2 and abcc2 was also tested in the cocktail assay but no significant alterations were found, indicating the mixture does not affect the action of these transporters. The expression levels obtained for the mixture had intermediate values in relation to the exposure to venlafaxine and norfluoxetine alone, again pointing out an antagonistic relationship between the compounds, in this case for the action of ABC transporters.

For norfluoxetine, no differences in gene expression could be found for the biotransformation enzymes tested: *cyp1a1*, *cyp3a65* and *gstπ*. In some part, this is not unexpected once that nuclear receptors *pxr* and *ahr2*, which regulate the expression of *cyp3a* and *cyp1a*, respectively (Lehman *et al.,* 1998; van der Oost, 2003), did not show relevant differences in their expression in the presence of the tested drugs. For venlafaxine there was visible inhibition of *cyp3a65* at 10000ng/L, though *pxr* and *ahr2* were not affected.

An interesting characteristic of cyp enzymes is that a drug can be metabolised by one enzyme and inhibited by that same enzyme (Lynch and Price, 2007). In humans *CYP2D6* has a major role in the metabolisation of norfluoxetine and venlafaxine (Ring *et al.,* 2001; Margolis *et al.,* 2000; Shams *et al.,* 2006). Nevertheless, norfluoxetine is also able to inhibit the action of this enzyme (Lynch and Price, 2007). The same is true for fluoxetine. Under these circumstances *CYP2C19*, *CYP2C9*, *CYP3A4* and *CYP3A5* become more relevant for fluoxetine metabolism especially during chronic administration of the drug, when metabolisation by *CYP2D6* is limited through the parent SSRI and its major metabolite (Lynch and Price, 2007). On the other hand, *CYP2D6* is conserved in chimpanzee, Rhesus monkey, rat, chicken, and frog (Pan *et al.,* 2016). However, in the National Centre for Biotechnology Information (NCBI) *CYP2D6* has 8 homologs in 5 species, but none of them is a fish species (Pan *et al.,* 2016). In Ensembl 84, it has 97 orthologs from 49 species of chordates, none of them is a fish species either, suggesting that in zebrafish *CYP2D6* role could be fulfilled by different cyp enzymes. Of interest is also the gap occurring between dosing and drug decrease due to cyp activation. While inhibition of cyp enzymes is very rapid, their

induction usually takes more time to occur. For some drugs (*e.g.* phenobarbital) there can be a delay up to one week between the beginning of dosing and the decrease in concentration of the drug due to cyp activation (Lynch and Price, 2007).

A panoply of possibilities may therefore be advanced for the results obtained in this investigation, including that for norfluoxetine the test concentrations may be below the threshold for cyp activation, the time window analysed is still within the gap phase of cyp increase or that these drugs are metabolised by other cyp enzymes. Worthy to mention is that 80hpf larvae exposed to fluoxetine, under similar conditions, exhibited inhibition of *cyp1a1*, *cyp3a65* and *gstπ* expression, as well as of *sod*, though at comparatively higher test concentrations (Cunha *et al.,* 2016). In addition, *pxr* was increased compared to the control and *ahr2* showed a biphasic response characterised by activation of expression in the lower exposure levels and diminished expression in the higher fluoxetine treatments.

HCA, with heat maps, and PCA were subsequently carried out with the results obtained for gene expression in order to better understand associations between genes and the effects of the different concentrations tested for each compound. For norfluoxetine the HCA separated larvae exposed to 3.2ng/L from those exposed to the remaining tested concentrations, probably due to the higher degree of induction found in that concentration. The heat map helps to understand the general panorama of gene expression obtained. Indeed, this showed a general tendency for induction in gene expression, although in most cases with values close to those of control and only sporadic differences detected. The PCA does not support such distinction, indicating that there were no differences between the tested concentrations and the control. This was expected once that, as already mentioned, no relevant alterations were detected for the majority of genes and no particular pattern of expression could be noted for any test concentration.

One interesting result comes from the comparison between norfluoxetine and the parental compound (fluoxetine). Hiemke and Hartter (2000), work indicates that norfluoxetine is more potent than parental compound to inhibit neurotransmitter uptake. However, results obtained in this work do not support this. In fact, the present

study indicates for the first time that influence of norfluoxetine over development of a teleost embryo appears to be, notably, less severe than that of fluoxetine. Comparing the results of this investigation with the ones obtained in a recent study (Cunha *et al.,* 2016), norfluoxetine showed comparatively lower mortality and gross damage to the organism than fluoxetine. In the present work the highest concentration of norfluoxetine tested (0.0014μM) is similar to the lowest concentration of fluoxetine tested in Cunha, *et al* (2016) (0.0015μM). Comparing these two concentrations, it can be observed the rate of anomalies caused by fluoxetine exposure was well above the one caused by norfluoxetine in embryonic development of zebrafish at 32hpf. Table 3 summarises gene expression alterations obtained after exposure of zebrafish embryos for 80hpf to similar concentrations of fluoxetine, norfluoxetine, venlafanixe and the cocktail. At the molecular level, more significant differences in the expression of genes involved in the detoxification mechanism were visible for fluoxetine than for norfluoxetine at similar concentrations.

Table 3. Summary of the gene expression changes observed in zebrafish embryos exposed to relevant concentrations of fluoxetine, norfluoxetine, venlafaxine and cocktail. The symbols denote significant induction (↑), inhibition (↓) or no alteration (~) relative to the respective control group.

Gene	Fluoxetine	Norfluoxetine		Venlafaxine			Cocktail
	512ng/L (0.0015µM)	3.2ng/L	400ng/L	80ng/L	400ng/L	2000ng/L	
serta	↓**	~	~	~	~	~	§
5-ht2c	↓**	~	~	↓	↓	~	↓
5-ht1a	~**	~	~	~	~	~	§
dat	↓**	~	~	~	↑	~	§
drd1b	~**	~	~	↓	↓	~	↓
drd2b	↓**	~	~	~	~	~	§
net	~**	~	~	~	~	~	§
adra2a	~**	~	~	~	~	~	§
adra2b	~**	~	~	~	↑	↑	~
adra2c	↓**	~	~	~	~	~	§
vmat2	↓**	~	~	↓	↓	~	↓
mao	~**	~	~	~	~	~	§
pxr	↑*	~	~	~	~	~	§
ahr2	~*	~	~	~	~	~	§
raraa	~*	~	~	~	~	~	§
rarab	~*	~	~	~	~	~	§
rarga	↓*	↑	~	~	~	~	§
rxraa	↓*	~	~	~	~	~	§
rxrab	~*	~	~	~	↓	~	§
rxrbb	~*	~	~	~	~	~	§
rxrga	↑*	~	~	~	↓	~	§
rxrgb	~*	~	~	~	↓	~	§
pparα	↓*	~	~	~	↓	~	~
pparβ	↓*	~	~	↓	↓	~	~
pparγ	↓*	~	~	↓	↓	↓	~
abcb4	~*	~	~	~	~	↓	§
abcc2	↓*	~	~	~	~	~	~
abcc1	~*	↑	~	~	↓	~	~
abcg2a	↓*	~	~	↓	↓	~	~
cyp1a1	~*	~	~	~	~	~	§
cyp3a65	~*	~	~	~	~	~	§
gstπ	~*	~	~	~	~	~	§
sod	~*	~	↓	~	~	~	§
cat	↑*	~	~	~	~	~	§

§ - not determined; * (Cunha *et al.,* 2016); ** (Cunha *et al.,* unpublished)

For venlafaxine more intense alterations were observable, resulting in differences in the tested concentrations in relation to control. Heat map projection showed a general tendency to inhibition in the genes' transcription. The inhibition was higher in the range 80 to 2000ng/L; these concentrations were grouped by HCA against the remaining treatments, supporting the results showing a tendency for a U-shaped response. Based on the PCA, embryos exposed to 80, 40 and 10000ng/L of venlafaxine show differences in relation to the control. Moreover, it is evident that 400ng/L venlafaxine is the concentration with an unique expression response (mainly inhibition of *rxr* and *ppar* nuclear receptors, and *abc* transporters, as well as induction of dopamine and adrenergic receptors), most distant from that of the control. In the following concentrations recuperation to near control values appears to start occurring.

The differences in expression were mild, however, fairly comparable to those obtained previously for fluoxetine (Cunha *et al.,* 2016). The set of genes selected for investigation of the cocktail effects, is promising as biomarker of exposure to venlafaxine during embryonic development. The non-linear dose-response observed poses challenges to common risk assessment approaches, in what concerns risk calculation. Risk estimation based on conventional toxicological parameters (e.g. No Observed Effect Concentration, NOEC, or low Effect Concentrations, EC) cannot be easily applied here, similar to previous observations for other substances with endocrine disrupting activity (Vandenburg *et al.,* 2012).

PCA showed curious correlations patterns among some genes altered by venlafaxine exposure. *ppar* receptors, *abc* transporters, *drd1b, 5-ht2c* and *vmat2* were well correlated to each other, in a positive manner. The correlation between ppar receptors and abc transporters may indicate that venlafaxine acts in the lipidic metabolism of zebrafish embryos. Like previously mentioned, in fish, *ppar* receptors are involved in lipid sensing and metabolism (Maradonna *et al.,* 2015; Wang *et al.,* 2008), while abc transporters are known for their efflux capacity of cholesterol in mammals, mainly *abca1* and *abcg1* (Phillips, 2014). Also, human patients treated with venlafaxine show significant alterations of cholesterol levels in the serum (Allgulander *et al.,* 2004; Simon *et al.,* 2004; Hummels *et al.,* 2011). Venlafaxine thus appears to interfere with cholesterol levels, also in fish by affecting the expression of *abc* transporters and *ppar* receptors. Figure 53 shows the known lipid metabolism pathway in zebrafish. Here it is possible to see the action of *ppar* receptors and a myriad of *abc* transporters. Several lipid ligands for some of these nuclear receptors, and their regulated genes are shown, including *ppar* receptors for fatty acids. These receptors act by forming heterodimers with retinoid X receptors (*RXR*). Upon binding to various ligands, three classes of proteins are synthesised including lipid binding proteins, the ATP-binding cassette (ABC) transporters and cytochrome P450 member proteins which catalyze lipid anabolism, metabolism and elimination. In addition to lipid metabolism, some members of the cytochrome P450 family genes are responsible for activation of procarcinogens, detoxification of environmental toxins and metabolism of drugs and

xenobiotics (Lynch and Price, 2007). Here *abcc2* is associated to the lipid metabolism, namely with steroids resulting from cholesterol metabolism. Alterations of *abcb4* expression are also associated with the lipid metabolism, specifically with fatty acids, through regulation by *ppara*. In order to understand if other *abc* transporters are activated by this metabolism in the presence of venlafaxine, further evaluation of those target genes would be needed.

Figure 53. Nuclear receptors in lipid metabolism and toxicity of zebrafish**.** PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptors; RAR, retinoic acid receptors; VDR, vitamin D receptor; NR1, nuclear hormone receptor subfamily 1; ABC, ATP-binding cassette transporters; cyp, cytochrome P450 gene family. Adapted from: Kelder *et al.* (2012) and Kutmon *et al.* (2016) (http://wikipathways.org/index.php/Pathway:WP1326).

The results also showed that major alterations in the gene expression of zebrafish larvae exposed to venlafaxine occurred at low environmentally relevant concentrations, rather than at high test concentrations of this psychotropic drug. This is an aspect that has been reported in the literature (Fong and Ford, 2014) and is further supported by the results of the present work.

For the cocktail assays, HCA, showed higher similarity of its expression profile to norfluoxetine than venlafaxine. Nevertheless, the combination of both compounds resulted in a response pattern different from those of the single drugs. *abc* transporters and *ppar* receptors showed intermediate expression levels between norfluoxetine and venlafaxine whereas neurohormonal genes showed higher downregulation degree, compared to single compounds. PCA showed the clear separation between the neurohormonal genes tested from nuclear receptors and detoxification transporters. Overall, data suggests an antagonistic effect between the two psychotropic drugs. Surprisingly, a very low rate of norfluoxetine to venlafaxine (with differences over 2 orders of magnitude between the two compounds) was found to produce milder effects than venlafaxine. This was particularly evident in the number of spinal anomalies and some target genes, mainly the ones involved in both drugs metabolism. Taken together they suggest that a small concentration of norfluoxetine would be enough to reverse detrimental effects elicited by venlafaxine. Moreover, considering this, it is possible that the environmental impact of venlafaxine over teleost embryos may be attenuated in aquatic ecosystems where the two drugs co-occur. Although there are some cases, like the anomalies in embryos pigmentation at 32hpf, where synergetic effects are found. According to the rates of mortality and abnormalities obtained, and the changes highlighted in Table 3 the toxicity of the compounds can be ranked as follows, from highest to lowest: fluoxetine>venlafaxine>cocktail~norfluoxetine.

In conclusion, the present work contributed to increase the knowledge base on effects of low concentrations of psychotropic drugs on non-target aquatic organisms, by evaluating sensitive endpoints in a developmental model for which alterations found may reflect at the population level. The data provided comes to support the need for alternate approaches to the risk assessment of substances with endocrine disrupting activity. It also points out the importance of developing routine biomonitoring of contamination of aquatic systems by these compounds. The research performed provided an expression profile, based on a set of affected genes, of potential interest to be employed as biomarker of exposure to venlafaxine and mixtures of norfluoxetine

and venlafaxine. Finally, it calls the attention for the need to better assess effects of exposure to drug metabolites in non-target species and of combinations of chemicals that may co-occur in the aquatic environment.

Chapter 5: Conclusions and future perspectives

5. Conclusions and future perspectives

With this study, it was concluded that the presence of norfluoxetine, venlafaxine and the combination of both substances in the aquatic environment have effects on zebrafish embryos, in particular embryonic development, gene expression of serotonergic, dopaminergic and adrenergic systems as well as in the gene expression involved in detoxification mechanisms.

In the presence of different concentrations of norfluoxetine and venlafaxine mortality was not affected. The same did not happen for the cocktail exposure, where significant mortality was observed. Exposure to single compounds or to the cocktail resulted in increased rate of gross anomalies during embryonic development, mainly at the highest concentrations tested. The anomalies found most often were alteration in pigmentation and vitelline sac at 32hpf, and pigmentation and spinal anomalies at 80hpf in the case of venlafaxine.

Concerning gene expression of neurohormonal and metabolisation mechanisms, different responses were visible, according to the compound(s) to which embryos were exposed. For norfluoxetine there was a tendency for a slight induction of gene expression and a small number of genes presented significant alterations in relation to control. There were no remarkable correlations among genes; main patterns observed associated nuclear receptors known to dimerise to exert their action, such as *ppar*, *rxr* and *rar*. No differences among norfluoxetine concentrations were found in relation to the control. The results also, appear to counteract the idea that norfluoxetine is more potent than the parental compound although test concentrations were low. For venlafaxine general inhibition of gene expression was visible and a wider range of genes was affected, compared to norfluoxetine. Inhibition was higher in the 400ng/L concentration, that is environmentally relevant. Visible correlations were noted between ppar's, abc transporters, *vmat2, drd1b* and *5-ht2c*, suggesting that venlafaxine can interfere with lipid metabolism and the activity of dopamine neurons linked to brain reward systems. Alterations in *adra2b* and *5-ht2c* suggested a disturbance in the Gi/Go pathway. The general dose-response pattern obtained for

venlafaxine showed a U-shape, where 80, 400 and 2000ng/L were the most affected; an apparently typical response for these drugs. Differences found in adrenergic receptors can explain anomalies in the pigmentation of embryos and larvae. In addition, *abcb4* expression was also significantly inhibited at environmentally relevant concentrations, meaning that populations may already be at risk. The pool of genes tested for the cocktail exposure showed different responses for neurohormonal receptors and genes responsible for metabolisation. Neurohormonal receptors were highly inhibited, while metabolisation genes presented an intermediate response in relation to exposure to single compounds.

In conclusion, aquatic organisms may suffer adverse effects due to the presence of norfluoxetine and venlafaxine in ecosystems. However, future developments in this area are needed for better understanding the effects of these compounds on aquatic organisms. It would be important to address possible effects of exposure during the embryonic phase in swimming behaviour and the reproductive level, as well as establish behavioral tests to assess preference or avoidance for contaminated medium. Deeper studies about the mechanisms that can be affected by exposure to these compounds, specially lipid metabolism, dopaminergic neurons activity and Gi/Go pathway in the case of venlafaxine. It would also be important extend the pool of genes tested for the cocktail, in order to better understand the potential antagonistic effects between the two drugs and their implications to aquatic organisms.

Chapter 6: References

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APPENDIX A1

Genbank accession numbers, primer sequences and concentrations, amplicon lengths and efficiency of reaction for the target genes investigated in this study.

Gene	Accession number	Primers Sequence (5'→3')	Final Conc. (nM)	Amplicon length (bp)	Efficiency (%)
abcb4	JQ014001	F: TACTGATGATGCTTGGCTTAATC R: TCTCTGGAAAGGTGAAGTTAGG	300	159	110.6
abcc1	XM_002661199	F: GCTCGAGCTCTCCTCAGAAA R:TCGGATGGTGGACTGTATCA	300	99	125.1
abcc2	NM_200589	F: GCACAGCATCAAGGGAAACA R: CCTCATCCACTGAAGAACCGA	300	87	116.5
abcg2a	NM_001042775.1	F: AAGGGTATCGAGGACCGTCT R: AATCCTGACCCTGAACGATG	300	97	113.1
cyp1a1	NM_131879.1	F: AACTCTTCGCAGGTGCTCAT R: ACAAACTGCCATTGGAGACC	300	97	102
cyp3a65	NM_001037438.1	F: TGACCTGCTGAACCCTCTCT R: AAGGGCGAAATCCATCTTCT	300	82	91
gstπ	NM_131734	F: TCTGGACTCTTTCCCGTCTCTCAA R: ATTCACTGTTGCCGTTGCCGT	300	105	119
Cu/Zn sod	Y12236	F: GTCGTCTGGCTTGTGGAGTG R: TGTCAGCGGGCTAGTGCTT	300	113	110
cat	NM_130912.1	F: CAGGAGCGTTTGGCTACTTC R: ATCGGTGTCGTCTTTCCAAC	300	91	113
pxr	DQ069792.1	F: CTTTTTCAGACGTGCGATGA R:TTGGCACTGTCTTCTGTTGC	300	94	112.7
rxraa	NM_001161551.1	F:ATTCAATGGCATCTCCTG R:GCGGCTTAATATCCTCTG	600	99	101.8
rxrab	NM_131153.1	F:CGCCGCATCAAATCACATAAAC R:TGAATGGGTTGGACAGTATTTAGC	300	87	109.4
rxrbb	NM_131238.1	F:TCACAACTTGGGCGTGGAGGC R:CGCATCTTGCAGACCAGCTCAG	300	105	100.7
rxrga	NM_131217.2	F:ATCTCAGTTCTTCGTTGCAGGTAG R:CGTTGATGATGGATGGGTGATGG	300	105	99.6
rxrgb	NM_001002345.1	F:CGCGGAATGGATACTCACG R:GCTGATGACGGACGGATGAC	300	114	97.7
raraa	NM_131406.2	F:GTAGTGGAGTGTGGATGTGAA R:GTGCTGATGTCTGATGGATGA	300	118	108.7
rarab	NM_131399.1	F:ATGGATTACTACCACCAGAAC R:TCTCCACAGAGTGATTCGAGC	300	115	109.4
rarga	NM_131339.1	F:CCCGCCAACTGTACGATGTCA R:GGGTCCAGTCCAGCATAGAAA	300	79	117.6
pparα	NM_001161333.1	F:CATCTTGCCTTGCAGACATT R:CACGCTCACTTTTCATTTCAC	600	81	88.3

Genbank accession numbers, primer sequences and concentrations, amplicon lengths and efficiency of reaction for the target genes investigated in this study.

Gene	Accession number	Primers Sequence (5'→3')	Final Conc. (nM)	Amplicon length (bp)	Efficiency (%)
pparβ	AF342937.1	F:GCGTAAGCTAGTCGCAGGTC R:TGCACCAGAGAGTCCATGTC	600	204	81.6
pparγ	DQ839547.1	F:GGTTTCATTACGGCGTTCAC F:TGGTTCACGTCACTGGAGAA	600	250	87
ahr2	NM_001007789.2	F:TTCTGTTGCCGATTCAGATG R:CTTGTTTTGCCCATGGAGAT	300	96	113,8
ef1	NM_131263.1	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAACGT	300	84	116.8
vmat2	NM_001256225.2	F: CTAAAAAGCTCCGCATCCAG R: TGTCCAAGAGCAAAGCAATG	150	231	133
mao	NM_212827.2	F: ACCAACTCAAAACCGCATTC R: GTAGGCAAAAGGGTTCCACA	300	151	105
serta	NM_001039972.1	F: CATCTATGCTGAGGCTATTG R: AAGAATATGATGGCGAAGA	300	73	100
5-ht1a	NM_001123321.1	F: ATGAGGATGAGCGGGATGTAG R: CAATCAGCCAGGACCACG	300	80	125
5-ht2c	NM_001129893.1	F: GCGCTCTCTGTCCTATTTGG R: GTAGCGGTCGAGAGAAATGG	1000	89	126.4
dat (transp dop)	NM_131755.1	F:ACGTCAATTCTCTTTGGAGT R:TCCTCGATATCATCACTGAA	150	86	97
drd1b	NM_001135976.2	F: CTGCGACTCCAGCCTTAATC R: AGATGCGGGTGTAAGTGACC	600	98	117.2
drd2b	NM_197936.1	F: ACGCCGAATATCAGTCCAAC R: GCAGTGCCTGAGTTTCAACA	300	96	110.7
Net (transp norepin)	XM_689046.5	F: AGTCCAGCGTTCTTGCTGTT R: TCTGCCCAGTATGGGAAAAC	300	92	117
adra2a	NM_207637.2	F: AGCGTTTTGTGACTGCTGTG R: TAATGGGATTGAGGGAGCTG	300	86	114
adra2b	NM_207638.1	F: GTCTGCCTGGCCACACTAAT R: GTACGGGGCGAGTTTTATCA	1000	80	119.7
adra2c	NM_207639.1	F: CTATTCTCCGGCCACCATTA R: CCAGCACATTCCCCACTATT	1000	80	133.8
actb1	NM_131031.1	F: TCCCAAAGCCAACAGAGAGAAG R: GTCACACCATCACCAGAGTCC	10	147	100.5
Rpl8	NM_200713.1	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	10	136	96

Autor: Pedro Miguel Carneiro Rodrigues

Título: Embryotoxicity and molecular alterations of monoamine reuptake inhibitory drugs in a teleost fish

SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR