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Data Article

Effects of norfluoxetine and venlafaxine in zebrafish larvae: Molecular data

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ABSTRACT

The data presented herein relates to the article entitled "Norfluoxetine and venlafaxine in zebrafish larvae: single and combined toxicity of two pharmaceutical products relevant for risk assessment" [1]. Recent studies have shown the occurrence of active metabolites of human and veterinary pharmaceuticals in surface and wastewaters. Besides their biological activity, some are predicted to interact with the same molecular targets of their parental compounds, thus showing the potential to elicit detrimental effects on animals. Despite this, limited investigation on their effects on aquatic animals has been done. Genomic material resulting from zebrafish (*Danio rerio*) larvae exposed to the psychoactive compounds norfluoxetine (main fluoxetine metabolite), venlafaxine, or their mixture was collected for gene expression analysis of a determined pool of genes potentially involved in their mode-of-action and metabolism. Molecular parameters are a cost-effective and reliable way to understand modes-of-action and the potential risk of micropollutants, such as pharmaceutical products, in non-target organisms. Moreover, gene expression patterns can provide crucial complementary information to improve risk assessment, and monitoring of

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affected systems. The data reported in this article was used to depict the effects of single or combined exposure to norfluoxetine and venlafaxine and identify biomarkers of exposure to these compounds of interest to diagnose exposure and routine monitoring.

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Specifications Table

Subject	Biology
Specific subject area	Environmental Toxicology
Type of data	Tables Figures
How data were acquired	Primers for genes of interest were based on gene sequences available in GenBank and designed in Primer 3 Plus program. Data on stability of exposure concentrations were retrieved from the available literature. Evaluation of primer efficiency, optimal concentration, and gene expression was performed by quantitative real-time reverse transcription PCR (qRT-PCR) in an Eppendorf Mastercycler realplex 4 qPCR system (Eppendorf, Hamburg, Germany).
Data format	Raw data and analyzed data
Parameters for data collection	Embryos were exposed to norfluoxetine, venlafaxine, or a mixture of these compounds for 80 h post-fertilization (hpf). Five norfluoxetine (from 0.64 to 400 ng/L) and five venlafaxine concentrations (from 16 to 10,000 ng/L) were tested, as well as a combination of 3.2 ng/L norfluoxetine + 2000 ng/L venlafaxine. Mortality and malformation rates were recorded. A sensorimotor test was performed in the mixture larvae. Larvae were then collected for molecular analysis.
Description of data collection	Zebrafish embryos were exposed to different, environmentally relevant and higher concentrations of norfluoxetine, venlafaxine or their combination over 80 hpf. At the end of the assays, surviving larvae were collected into RNAlatter for posterior processing (RNA extraction and cDNA synthesis) and evaluation of gene expression by qPCR (SYBRGreen).
Data source location	CIIMAR - Interdisciplinary center of Marine and Environmental Research Matosinhos, Portugal
Data accessibility	Data is with this article and in the Mendeley Data repository (http://dx.doi.org/10.17632/svr9kvsngy.1)
Related research article	Rodrigues P, Cunha V, Oliva-Teles L, Ferreira M, Guimarães L. 2020. Norfluoxetine and venlafaxine in zebrafish larvae: single and combined toxicity of two pharmaceutical products relevant for risk assessment. Journal of Hazardous Materials 400. 123,171. DOI:10.1016/j.jhazmat.2020.123171.

Value of the Data

- Despite their detection in environmental compartments, hazardous effects of pharmaceutical metabolites on aquatic species, either single or in mixture with other bioactive drugs, are unknown limiting our understanding of the need to conduct a risk assessment of these chemicals.
- The data is useful for other professionals who may wish to assess the expression of these genes in zebrafish. It brings understanding about the on modes-of-action of these chemicals useful for academic researchers and complementary data on population-level effects relevant for stakeholders involved in environmental assessment and monitoring.
- The data provided for a wide pool of genes can be used for comparative analysis of these chemicals under different factors or exposure conditions, and detoxification and neurotransmission pathways of other relevant pharmaceuticals in non-target organisms.
- Data about the toxicity of pharmaceutical metabolites is scarce. Though, their mixture with other drugs can have more hazardous effects than the parental compounds alone, alerting for the need to address these chemicals in existing risk assessment guidelines.

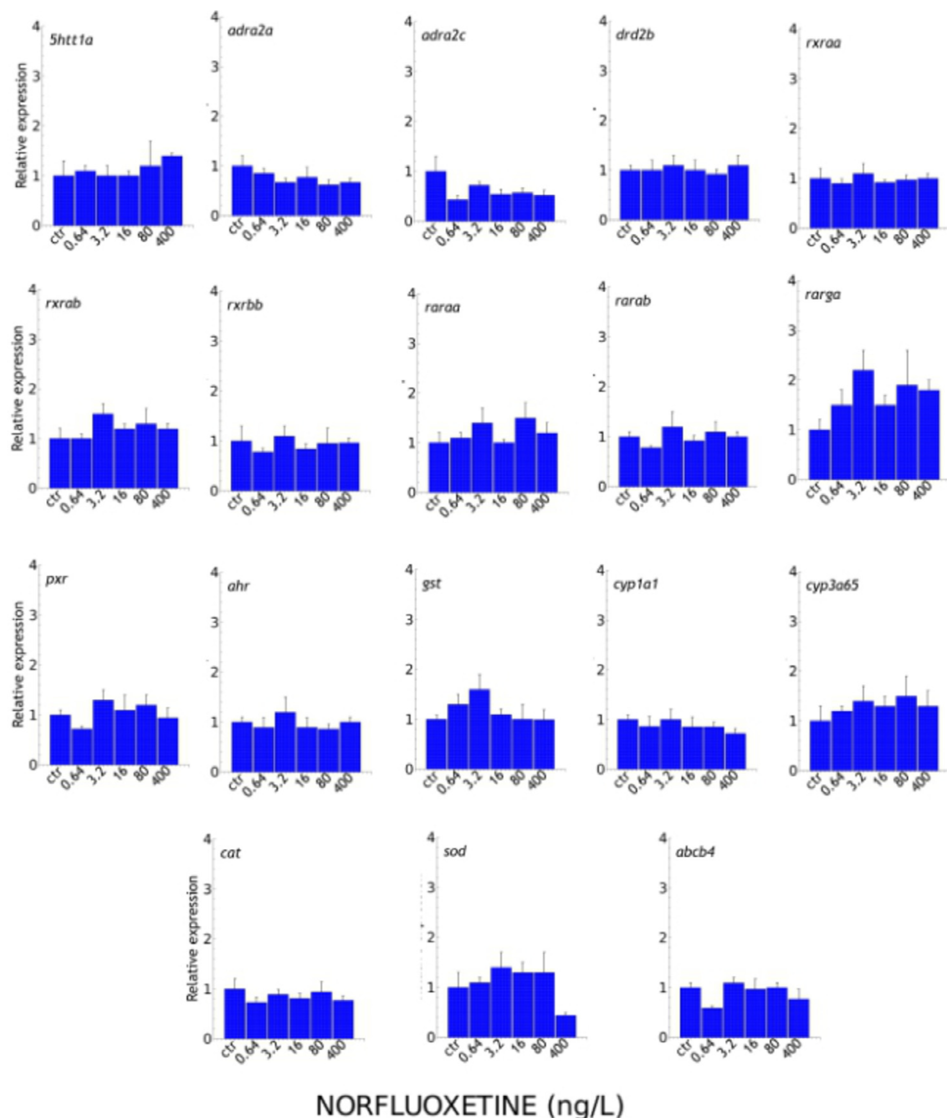

Fig. 1. Gene expression levels determined in zebrafish larvae exposed to norfluoxetine for 80 hpf and in controls.

1. Data Description

This data article presents a table where technical information about 37 genes with different functions in zebrafish, which expression was evaluated in larvae exposed for 80 hpf (hours post fertilization) to norfluoxetine, venlafaxine and a mixture of both (Table 1). Data illustrating the low degradation and stability of norfluoxetine and venlafaxine in test solutions is presented in Table 2. Raw data are provided [2] and the results of the ANOVA tests performed for genes quantified following exposure to the pharmaceutical compounds under evaluation are shown (Tables 3–5). Presented figures (Figs. 1–3) are the graphical outcome of calculated gene expression after normalization for the reference genes, not displayed in the original co-submitted article [1]. The

Table 1
Accession numbers (Genbank), function and primer information for the target genes investigated in this study and the three reference genes used.

Gene	Accession number	Function	Primers Sequence (5′→3′)	Final Concentration (nM)	Amplicon Length (bp)	Efficiency (%)
5-ht1aa	NM_001123321.1	Serotonin receptor	F: ATGAGGATGAGCGGGATGTAG R: CAATCAGCCAGGACCACG	300	80	125
5-ht2c	NM_001129893.1	Serotonin receptor	F: GCGCTCTCTGTCCTATTTGG R: GTAGCGGTCGAGAGAAATGG	1000	89	126.4
abcb4	JQ014001	ABC transporter	F: TACTGATGATGCTTGGCTTAATC R: TCTCTGGAAAGGTGAAGTTAGG	300	159	110.6
abcc1	XM_002661199	ABC transporter	F: GCTCGAGCTCTCCTCAGAAA R:TCGGATGGTGGACTGTATCA	300	99	125.1
abcc2	NM_200589	ABC transporter	F: GCACAGCATCAAGGGAAACA R: CCTCATCCACTGAAGAACCGA	300	87	116.5
abcg2a	NM_001042775.1	ABC transporter	F: AAGGGTATCGAGGACCGTCT R: AATCCTGACCCTGAACGATG	300	97	113.1
adra2a	NM_207637.2	Norepinephrine receptor	F: AGCGTTTTGTGACTGCTGTG R: TAATGGGATTGAGGGAGCTG	300	86	114
adra2b	NM_207638.1	Norepinephrine receptor	F: GTCTGCCTGGCCACACTAAT R: GTACGGGGCGAGTTTTATCA	1000	80	119.7
adra2c	NM_207639.1	Norepinephrine receptor	F: CTATTCTCCGGCCACCATTA R: CCAGCACATTCCCCACTATT	1000	80	133.8
ahr2	NM_001007789.2	Aryl hydrocarbon nuclear receptor	F:TTCTGTTGCCGATTCAGATG R:CTTGTTTTGCCCATGGAGAT	300	96	113.8
cat	NM_130912.1	Antioxidant enzyme	F: CAGGAGCGTTTGGCTACTTC R: ATCGGTGTCGTCTTTCCAAC	300	91	113
Cu/Zn sod	Y12236	Antioxidant enzyme	F: GTCGTCTGGCTTGTGGAGTG R: TGTCAGCGGGCTAGTGCTT	300	113	110
cyp1a1	NM_131,879.1	Phase I biotransformation enzyme	F: AACTCTTCGCAGGTGCTCAT R: ACAAACTGCCATTGGAGACC	300	97	102
cyp3a65	NM_001037438.1	Phase I biotransformation enzyme	F: TGACCTGCTGAACCCTCTCT R: AAGGGCGAAATCCATCTTCT	300	82	91
dat	NM_131755.1	Dopamine transporter	F:ACGTCAATTCTCTTTGGAGT R:TCCTCGATATCATCACTGAA	150	86	97
drd1b	NM_001135976.2	Dopamine receptor	F: CTGCGACTCCAGCCTTAATC R: AGATGCGGGTGTAAGTGACC	600	98	117.2
drd2b	NM_197936.1	Dopamine receptor	F: ACGCCGAATATCAGTCCAAC R: GCAGTGCCTGAGTTTCAACA	300	96	110.7
gstπ	NM_131,734	Phase II biotransformation enzyme	F: TCTGGACTCTTTCCCGTCTCTCAA R: ATTCACTGTTGCCGTTGCCGT	300	105	119

(*continued on next page*)

Table 1 (continued)

Gene	Accession number	Function	Primers Sequence (5′→3′)	Final Concentration (nM)	Amplicon Length (bp)	Efficiency (%)
mao	NM_212827.2	Monoamine oxidase	F: ACCAACTCAAAACCGCATTC R: GTAGGCAAAAGGGTTCCACA	300	151	105
net	XM_689046.5	Norepinephrine transporter	F: AGTCCAGCGTTCTTGCTGTT R: TCTGCCCAGTATGGGAAAAC	300	92	117
pparα	NM_001161333.1	Peroxisome proliferator activated nuclear receptor	F:CATCTTGCCTTGCAGACATT R:CACGCTCACTTTTCATTTCAC	600	81	88.3
pparβ	AF342937.1	Peroxisome proliferator activated nuclear receptor	F:GCGTAAGCTAGTCGCAGGTC R:TGCACCAGAGAGTCCATGTC	600	204	81.6
pparγ	DQ839547.1	Peroxisome proliferator activated nuclear receptor	F:GGTTTCATTACGGCGTTCAC F:TGGTTCACGTCACTGGAGAA	600	250	87
pxr	DQ069792.1	Pregnane X nuclear receptor	F: CTTTTTCAGACGTGCGATGA R:TTGGCACTGTCTTCTGTTGC	300	94	112.7
raraa	NM_131406.2	Retinoic acid nuclear receptor	F:GTAGTGGAGTGTGGATGTGAA R:GTGCTGATGTCTGATGGATGA	300	118	108.7
rarab	NM_131399.1	Retinoic acid nuclear receptor	F:ATGGATTACTACCACCAGAAC R:TCTCCACAGAGTGATTCGAGC	300	115	109.4
rarga	NM_131339.1	Retinoic acid nuclear receptor	F:CCCGCCAACTGTACGATGTCA R:GGGTCCAGTCCAGCATAGAAA	300	79	117.6
rxraa	NM_001161551.1	Retinoid X receptor	F:ATTCAATGGCATCTCCTG R:GCGGCTTAATATCCTCTG	600	99	101.8
rxrab	NM_131153.1	Retinoid X receptor	F:CGCCGCATCAAATCACATAAAC R:TGAATGGGTTGGACAGTATTTAGC	300	87	109.4
rxrbb	NM_131238.1	Retinoid X receptor	F:TCACAACTTGGGCGTGGAGGC R:CGCATCTTGCAGACCAGCTCAG	300	105	100.7
rxrga	NM_131217.2	Retinoid X receptor	F:ATCTCAGTTCTTCGTTGCAGGTAG R:CGTTGATGATGGATGGGTGATGG	300	105	99.6
rxrgb	NM_001002345.1	Retinoid X receptor	F:CGCGGAATGGATACTCACG R:GCTGATGACGGACGGATGAC	300	114	97.7
serta / slc6a4a	NM_001039972.1	Serotonin transporter	F: CATCTATGCTGAGGCTATTG R: AAGAATATGATGGCGAAGA	300	73	100
vmat2	NM_001256225.2	Vesicular monoamine transporter	F: CTAAAAAGCTCCGCATCCAG R: TGTCCAAGAGCAAAGCAATG	150	231	133
actb1	NM_131031.1	Reference gene	F: TCCCAAAGCCAACAGAGAGAAG R: GTCACACCATCACCAGAGTCC	10	147	100.5
ef1	NM_131263.1	Reference gene	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAACGT	300	84	116.8
rpl8	NM_200713.1	Reference gene	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	10	136	96

Table 2
Nominal and exposure concentrations, or recovery%, reported for venlafaxine and norfluoxetine in previous works.

Venlafaxine						
Nominal concentration (ng/L)	Real concentration (ng/L)	Recovery (%)	Sampling time	Media replacement	Quantification method	Reference
300 30,000	Not reported	96	24, 96 and 144h	Daily	UHPLC-TQMS	Study conducted with zebrafish embryo Hodcovikova et al., 2019
200 1000	260 ± 8 1020 ± 14	106 to 117	2 to 7 days after exposure	Daily (40%)	SPE-QTRAP	Study conducted with immature rainbow trout Melnyk-Lamont et al., 2014
(*s*)-norfluoxetine						
3500 15,000 28,000	Not reported	62.5 84.1 91.1	day 1, 2 and 3	Not reported	SPE-HPLC-FD	Study conducted with extracts obtained from wastewater effluents Ribeiro et al., 2014
(*r*)-norfluoxetine						
3500 15,000 28,000	Not reported	99.1 102 103	day 1, 2 and 3	Not reported	SPE-HPLC-FD	Study conducted with extracts obtained from wastewater effluents Ribeiro et al., 2014

The above mentioned studies were conducted under controlled laboratorial conditions. UHPLC-TQMS, ultra-high-performance liquid chromatography coupled with mass spectrometry; SPE-QTRAP, Solid-phase extraction-liquid chromatrography mass spectrometry; SPE-HPLC-FD, Solid-phase extraction with high-performance liquid cromatography coupled with Chirobiotic V and fluorescence detection.

Table 3
ANOVA results for the exposure of zebrafish larvae to norfluoxetine for 80 hpf.

Gene	MS Model	df Model	MS Residual	df Residual	*F*	*p*
abcc2	1.290	5	0.919	18	1.403	0.270
abcg2a	0.736	5	1.074	18	0.685	0.641
abcb4	1.519	5	0.856	18	1.776	0.169
abcc1	1.981	5	0.727	18	2.725	0.053
gst	1.169	5	0.953	18	1.226	0.337
Cu/Zn sod	1.796	5	0.779	18	2.305	0.087
cyp1a1	0.637	5	1.101	18	0.579	0.716
cyp3a65	0.441	5	1.155	18	0.382	0.855
cat	0.511	5	1.136	18	0.450	0.808
raraa	0.822	5	1.050	18	0.783	0.575
rarab	0.683	5	1.088	18	0.628	0.681
rarga	1.346	5	0.904	18	1.489	0.242
rxraa	0.446	5	1.154	18	0.387	0.851
rxrab	1.090	5	0.975	18	1.118	0.386
rxrbb	0.376	5	1.173	18	0.321	0.894
rxrgb	0.747	5	1.070	18	0.698	0.632
rxrga	0.465	5	1.149	18	0.405	0.839
ppara	1.052	5	0.985	18	1.068	0.410
pparb	0.683	5	1.088	18	0.627	0.681
pparg	0.742	5	1.072	18	0.693	0.635
pxr	1.274	5	0.924	18	1.380	0.278
ahr2	0.545	5	1.126	18	0.484	0.784
5-ht2c	1.565	5	0.843	18	1.856	0.152
drd1b	1.172	5	0.952	18	1.230	0.336
drd2b	0.220	5	1.217	18	0.181	0.966
adra2b	1.207	5	0.942	18	1.281	0.315
adra2c	1.472	5	0.869	18	1.694	0.187
adra2a	0.905	5	1.026	18	0.882	0.513
dat	1.590	5	0.836	18	1.902	0.144
serta	0.262	5	1.205	18	0.217	0.950
net	0.412	5	1.163	18	0.354	0.875
vmat2	1.327	5	0.909	18	1.460	0.251
mao	1.143	5	0.960	18	1.190	0.353
5-ht1a	0.393	5	1.169	18	0.336	0.884

data presented herein gives detailed support to the methodology applied by Rodrigues et al. [1]. The aim was to provide a complete set of data shedding light on the modes-of-action of the tested pharmaceutical products in zebrafish larvae, as well, as provide useful data to infer about the need to carry out an environmental risk assessment of drug metabolites.

2. Experimental Design, Materials and Methods

2.1. Test organisms

Zebrafish (*Danio rerio*) specimens, were maintained in the certified facilities for aquatic animals of CIIMAR, Matosinhos, Portugal. Reproducers were maintained in 70 L tanks with continuous air flow and water circulation at 27 ± 1 °C. The photoperiod was 14/10 h (light/dark) and the animals were fed twice a day.

2.2. Experimental design

Ecotoxicological assays were performed as described by Cunha and colleagues [3]. Briefly, embryos (0–1 hpf) were collected and exposed in 24-well plates for 80 h, to different norfluoxetine (0.64, 3.2, 16, 80 and 400 ng/L) and venlafaxine (16, 80, 400, 2000 and 10,000 ng/L)

Table 4
ANOVA results for the exposure of zebrafish larvae to venlafaxine for 80 hpf. Genes showing significant differences among test treatments are highligthed in bold.

Gene	MS Model	*df*Model	MS Residual	*df* Residual	*F*	*p*
abcc2	1.332	5	0.908	18	1.467	0.249
abcg2a	**2.358**	**5**	**0.623**	**18**	**3.786**	**0.016**
abcb4	1.655	5	0.818	18	2.024	0.124
abcc1	**2.460**	**5**	**0.594**	**18**	**4.140**	**0.011**
gst	0.708	5	1.081	18	0.654	0.662
Cu/Zn sod	1.386	5	0.893	18	1.552	0.224
cyp1a1	0.797	5	1.056	18	0.754	0.594
cyp3a65	1.524	5	0.854	18	1.784	0.167
cat	1.760	5	0.789	18	2.231	0.096
raraa	1.368	5	0.898	18	1.523	0.232
rarab	1.617	5	0.828	18	1.952	0.135
rarga	1.365	5	0.898	18	1.520	0.233
rxraa	0.822	5	1.049	18	0.784	0.575
rxrab	1.727	5	0.798	18	2.165	0.104
rxrbb	1.195	5	0.946	18	1.264	0.322
rxrgb	**3.101**	**5**	**0.416**	**18**	**7.446**	**<0.001**
rxrga	**2.090**	**5**	**0.697**	**18**	**2.998**	**0.039**
ppara	**2.465**	**5**	**0.593**	**18**	**4.157**	**0.011**
pparb	**2.486**	**5**	**0.587**	**18**	**4.232**	**0.010**
pparg	**3.127**	**5**	**0.409**	**18**	**7.641**	**<0.001**
pxr	1.988	5	0.726	18	2.740	0.052
ahr2	0.946	5	1.015	18	0.932	0.487
5-ht2c	**2.282**	**5**	**0.644**	**18**	**3.545**	**0.021**
drd1b	**2.385**	**5**	**0.615**	**18**	**3.877**	**0.015**
drd2b	0.538	5	1.128	18	0.477	0.789
adra2b	**2.902**	**5**	**0.472**	**18**	**6.155**	**0.002**
adra2c	0.878	5	1.034	18	0.849	0.533
adra2a	1.925	5	0.743	18	2.591	0.062
dat	1.918	5	0.745	18	2.573	0.063
serta	1.997	5	0.723	18	2.763	0.051
net	**2.217**	**5**	**0.662**	**18**	**3.348**	**0.026**
vmat2	**2.570**	**5**	**0.564**	**18**	**4.556**	**0.007**
mao	**2.881**	**5**	**0.478**	**18**	**6.033**	**0.002**
5-ht1a	1.616	5	0.829	18	1.949	0.136

Table 5
ANOVA results for the exposure of zebrafish larvae to a mixture of norfluoxetine and venlafaxine for 80 hpf. Genes showing significant differences among test treatments are highligthed in bold.

Gene	MS Model	df Model	MS Residual	df Residual	*F*	*p*
abcc2	**3.366**	**3**	**0.355**	**11**	**9.493**	**0.002**
abcg2a	1.751	3	0.795	11	2.201	0.145
abcc1	**2.344**	**3**	**0.633**	**11**	**3.702**	**0.046**
ppara	1.402	3	0.890	11	1.575	0.251
pparb	2.305	3	0.644	11	3.579	0.050
pparg	**2.365**	**3**	**0.628**	**11**	**3.767**	**0.044**
5-ht2c	**3.113**	**3**	**0.424**	**11**	**7.348**	**0.006**
drd1b	**2.640**	**3**	**0.553**	**11**	**4.775**	**0.023**
adra2b	**3.425**	**3**	**0.339**	**11**	**10.116**	**0.002**
vmat2	**3.557**	**3**	**0.303**	**11**	**11.756**	**0.001**

concentrations. Norfluoxetine (CAS Number 57226–68–3) was purchased from Cayman Chemical Company® and venlafaxine (CAS Number 99300–78–4) was from the European Pharmacopoeia Reference Standard®. Ten embryos were exposed per well in 2 mL of the test solutions. The tested concentrations were planned to span from levels detected in aquatic systems and higher to account for differential responses elicited by low and high exposure [3–5]. In mixture assays,

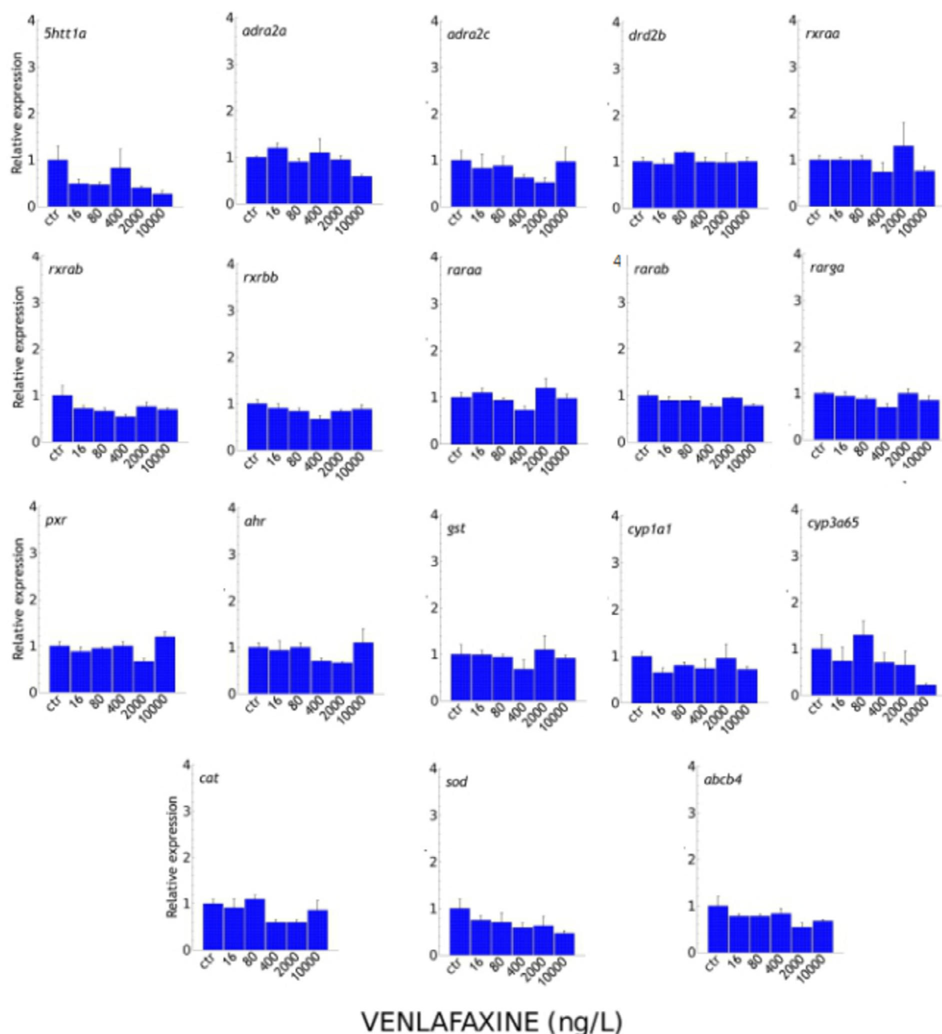

Fig. 2. Gene expression levels determined in zebrafish larvae exposed to venlafaxine for 80 hpf and in controls.

a combination of 2000 ng/L venlafaxine plus 3.2 ng/L norfluoxetine was tested. These assays also included single treatments of norfluoxetine and venlafaxine at the concentrations in the mixture for comparative purposes and better data interpretation. Test solutions were prepared from a stock solution (2 mg/L), followed by a dilution series. A control group (water) was also included in each assay. Twenty-four hours prior each assay the 24-well plates were filled with 2 mL of the corresponding test solution, to avoid losses by adsorption to the test recipient, minimizing possible differences between nominal and real concentrations. Test solutions were renewed daily. At 80 hpf hatched larvae were collected and preserved in RNAlater for molecular analysis.

2.3. Molecular analysis

RNA was extracted, using Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the kit standardized protocol. RNA quality was verified by electrophoresis on an agarose gel

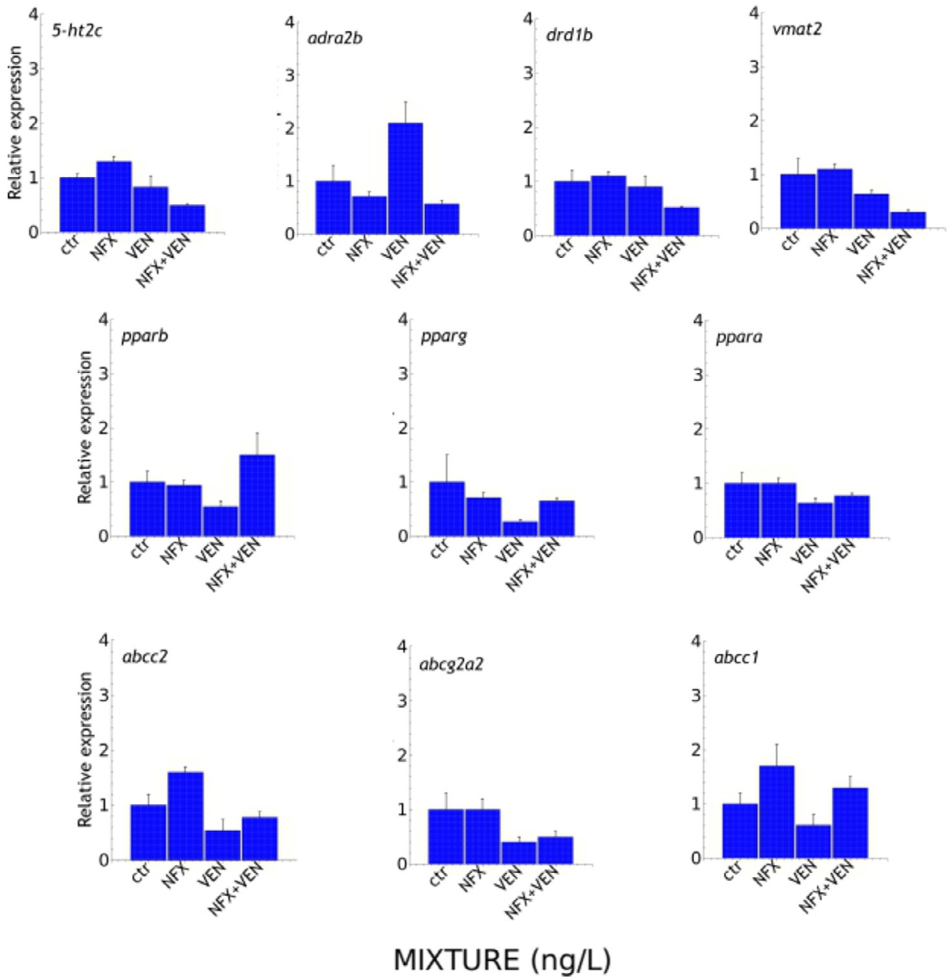

Fig. 3. Gene expression levels determined in zebrafish larvae exposed to venlafaxine, norfluoxetine and their mixture for 80 hpf and in controls.

of the 18 s band and by measuring the optical density ratio at λ260/280 nm. RNA was quantified using Take3 micro-volume plates (2ηL) in a BioTek spectrophotometer. After confirming RNA quantities, 1 µg of total RNA was subjected to the digestion of genomic DNA using deoxyribonuclease I Amplification Grade (Invitrogen) and cDNA synthesis was subsequently performed using iScript cDNA Synthesis Kit (Biorad) following the kit protocol.

Serotonin, dopamine, and noradrenaline receptors and transporters, the vesicular monoamine transporter and oxidase genes, several nuclear receptors, ABC transporters, biotransformation and antioxidant enzymes, and reference genes elongation factor 1, actin $\beta 1$ and ribosomal protein L8, were assessed. Pairs of primers (forward and reverse) were based on gene sequences available in public databases and were designed in Primer 3 Plus program. To confirm sequences identity, PCR (polymerase chain reaction) reactions were performed in a Biometra thermocycler with a mixture of $2\mu L$ cDNA per sample. Each PCR reaction was performed with the following parameters, in a final volume of 20µL per reaction: 4µL of 5x buffer, 2µL MgCl2, 1µL of each forward and reverse primer, 0.4 µL of DNTP's, 9.5µL water, 0.1µL of TaqPolimerase (Promega)

and 2µL of cDNA template. Reaction protocol was the following one: 2 min of denaturation at 94 °C; 40 cycles of denaturation for 30 s, 30 s of annealing at 51 °C, 54 °C, 55 °C (51 °C for *vmat2*, 55 °C for receptor of serotonin *5-ht2c* and dopamine *drd1b*; 54 °C for the remaining genes), 30 s of polymerization at 72 °C and 10 min at 72 °C for a final elongation. The size of the bands was evaluated on a 2% agarose gel with 1µL of Gel Red and visualized under direct UV light. Cloning and identification of sequence identity was made according to Costa et al. [6]. The fragments were inserted into pGEM (pGEM(R) - T Easy Vector Systems - Promega) and then into *E. coli* using New Blue Competent Cells (Novagen). Colonies of interest were selected and developed on LB solid medium with, ampicillin 0.1 mg/ml, IPTG 0.1 mM and X-gal 100 mM, at 37 °C overnight. Plasmids were isolated from 5 mL of culture medium and incubated overnight with 5µL ampicillin at 37 °C, with constant agitation. DNA was extracted with Wizard Kit Plus SV Minipreps DNA Purification System (Promega), according to the kit instructions. Products were sequenced by Stabvida (Portugal) and the identity of the sequences was verified with the Blast tool available at the National center for Biotechnology Information (NCBI).

Quantitative real-time PCR (qRT-PCR) was employed to assess the expression of thirty-seven genes in larvae obtained from single exposures to norfluoxetine or venlafaxine. After the initial pool of tests, ten genes with larger differences (at least 50%) in expression relative to the water control were selected for evaluation in the mixture assays. The highest fluorescence signal reached for the lower Cycle threshold (Ct) was used to dictate ideal primer concentrations for qRT-PCR. Primer efficiency was assessed by a series of eight cDNA dilutions ranging from 0.05 to 50 ng/μL. The qRT-PCR reactions (10µL of SybrGreen (Biorad), 4µL of water, 2µL of forward primer, 2 µl of reverse primer and 2µL of cDNA, in a 20 µL reaction volume) were run in an Eppendorf Mastercycler realplex 4. Each reaction was run in duplicate. The reaction parameters were set as follows: 94 °C for 2 min; 40 cycles for 30 s at 94 °C for denaturation, for 30 s at respective annealing temperatures, and for another 30 s at 72 °C for extension; a final extension cycle of 10 min at 72 °C was applied. Annealing temperatures were 51 °C for *vmat2*, 55 °C for *5-ht2c* and *drdb1,* 54 °C for the remaining genes. Blank samples, as well as, melting curves were run for each of the genes assessed. Normalization for quantification of the gene expression was done using *actb1* and *rpl8* as reference genes for norfluoxetine, and *ef1* and *rpl8* as reference genes for venlafaxine, according to the outcome of Normfinder algorithm [7]. The mathematical template of Pfaffl [8], which incorporates the primer efficiencies, was used to calculate the relative gene expression. The expression of each tested gene was determined in four independent exposure replicates.

2.4. Statistical analysis

Differences in mRNA expression were evaluated by means of a one-way analysis of variance (ANOVA), followed by the Tukey HSD at a 5% significance level. When deemed necessary, data were log-transformed in order to fit ANOVA assumptions.

Ethics Statement

The present study involves no experiments covered by the acts on welfare of laboratory animals.

CRediT Author Statement

PR, MF and LG conceived and designed the study and experiments. PR performed the experiments and all analytical measurements with the support of VC. PR and LOT performed the formal data analysis. LG supervised the research activities carried out. All authors contributed to the writing of the manuscript, the reviewing and approval of its final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have, or could be perceived to have, influenced the work reported in this article.

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