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” \cite{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
affected systems. The data reported in this article was used to depict the effects of single or combined exposure to norfloxetine 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 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 norfloxetine, venlafaxine, or a mixture of these compounds for 80 h post-fertilization (hpf). Five norfloxetine (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 norfloxetine + 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 norfloxetine, 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/svr9kvsgny.1) |
| Related research article | Rodrigues P, Cunha V, Oliva-Teles L, Ferreira M, Guimarães L. 2020. Norfloxetine 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-ht1a   | NM_001123321.1   | Serotonin receptor                | F: ATGAGGATGAGCGGGGATG            | 300                      | 80                   | 125             |
| 5-ht2c   | NM_001129893.1   | Serotonin receptor                | R: CAAATCGGAGAGACCAGG            | 1000                     | 89                   | 126.4           |
| abc4     | JQ014001         | ABC transporter                   | F: GTACCATCTGCTCTTCAAT            | 300                      | 159                  | 110.6           |
| abcd1a   | JM_002661199     | ABC transporter                   | R: TCTGAGGACCTCCGACG             | 300                      | 99                   | 125.1           |
| abcd2a   | NM_200519       | ABC transporter                   | F: ATGAGGATGAGCGGGGATG           | 300                      | 97                   | 116.5           |
| abcd2b   | NM_206737.2     | ABC transporter                   | R: CAAATCGGAGAGACCAGG            | 1000                     | 86                   | 114             |
| adra2a   | NM_206738.1     | Norepinephrine receptor           | F: GTACCATCTGCTCTTCAAT            | 1000                     | 80                   | 119.7           |
| adra2b   | NM_206739.1     | Norepinephrine receptor           | R: TCTGAGGACCTCCGACG             | 1000                     | 80                   | 133.8           |
| adra2c   | NM_206739.1     | Norepinephrine receptor           | F: ATGAGGATGAGCGGGGATG           | 300                      | 96                   | 113.8           |
| ahr2     | NM_001007789.2  | Aryl hydrocarbon nuclear receptor | R: CAAATCGGAGAGACCAGG            | 300                      | 91                   | 113             |
| cat      | NM_130912.1     | Antioxidant enzyme                | F: ATGAGGATGAGCGGGGATG           | 300                      | 113                  | 110             |
| Cu/Zn sod| Y12236           | Antioxidant enzyme                | R: CAAATCGGAGAGACCAGG            | 300                      | 97                   | 102             |
| cyp1a1   | NM_131,879.1    | Phase I biotransformation enzyme  | F: GTACCATCTGCTCTTCAAT            | 300                      | 82                   | 91              |
| cyp3a65  | NM_001037438.1  | Phase I biotransformation enzyme  | R: TCTGAGGACCTCCGACG             | 300                      | 86                   | 97              |
| dat      | NM_131755.1     | Dopamine transporter              | F: ATGAGGATGAGCGGGGATG           | 150                      | 98                   | 117.2           |
| drd1b    | NM_001135976.2  | Dopamine receptor                 | R: CAAATCGGAGAGACCAGG            | 600                      | 96                   | 110.7           |
| drd2b    | NM_197936.1     | Dopamine receptor                 | F: ATGAGGATGAGCGGGGATG           | 300                      | 105                  | 119             |
| gstπ     | NM_131,734      | Phase II biotransformation enzyme | R: CAAATCGGAGAGACCAGG            | 300                      | 105                  | 119             |
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: ACCAATCTAAAAACCCGAATTC R: GTAGCCAAAGGGTTCACACA | 300                      | 151                  | 105           |
| net    | XM_689046.5      | Norepinephrine transporter                    | F: AGTCCAGGCTTCTCTGTGTT R: TCTGCCCAATATGGAAAAAC | 300                      | 92                   | 117           |
| pparα  | NM_001161333.1   | Peroxisome proliferator activated nuclear receptor | F:CATCTTGCTTCGCAGACATT R:CAGGTCACTCCTTTACAC | 600                      | 204                  | 81.6          |
| pparβ  | AF342937.1       | Peroxisome proliferator activated nuclear receptor | F:GCGTAAGCTAGTCGAGGTGTC R:TGCACCAGAGAGTCCATGTC | 600                      | 250                  | 87            |
| pparγ  | DQ839547.1       | Peroxisome proliferator activated nuclear receptor | F:GTGCCATTACGGGCAGTCAC R:TGGGCCAGTACGGGAA | 600                      | 94                   | 112.7         |
| pnr    | DQ069792.1       | Predn X nuclear receptor                      | F: CTTTTCACAGTCGCGATGAR R:TTGGACTGTCCTCTGTTCG | 300                      | 118                  | 108.7         |
| raraa  | NM_131406.2      | Retinoic acid nuclear receptor                | F:GTAGTGAGTGGATGGATGTTA R:GTGCTGCTGTGATGGATGA | 300                      | 115                  | 109.4         |
| rarab  | NM_131399.1      | Retinoic acid nuclear receptor                | F:ATGATTATCTACCCACAGAC R:TCCTCAAGAGATGGTGCAG | 300                      | 79                   | 117.6         |
| rarga  | NM_131339.1      | Retinoic acid nuclear receptor                | F:GGTCCAGTCAGCAAGATAAAR R:GGGATCATGTCAGCAAG | 600                      | 99                   | 101.8         |
| rxraa  | NM_001161551.1   | Retinoid X receptor                           | F:ATCCATGGGATCTCTCTG R:GGGCTTCACTCAGCAAGTCA | 300                      | 87                   | 109.4         |
| rxrab  | NM_131153.1      | Retinoid X receptor                           | F:CCGCCATCAATACCCATAAAC R:TGAATGCTGCTGATGGATGTA | 300                      | 105                  | 100.7         |
| rxrb   | NM_131238.1      | Retinoid X receptor                           | F:TCAACACTGGGGCAGTGACC R:CCATCTGCAGACAGCTCAT | 300                      | 105                  | 99.6          |
| rxrga  | NM_131217.2      | Retinoid X receptor                           | F:ATCTCACATTCTGGTCAGCTAG R:CGTTGATGAGCTGAGGGTTG | 300                      | 114                  | 97.7          |
| rxrgb  | NM_001002345.1   | Retinoid X receptor                           | F:CGGAGAATGGATGCTACTAG R:GCTGATGAGGCGACGATG | 300                      | 73                   | 100           |
| sert  / slc6a4a | NM_001039972.1 | Serotonin transporter                         | F: CATCTATCAGGGCTATGTT R: AAGAATGTAAGGCGAGAAC | 150                      | 231                  | 133           |
| vmat2  | NM_001256225.2   | Vesicular monoamine transporter               | F:CTAAAAAGCTCCGCATCCAG R: TGGCCAAAGGCAAAGAATG | 10                       | 147                  | 100.5         |
| actb1  | NM_131031.1      | Reference gene                                | F: TCCAAACCCCAACAGAGAAG R: GTCACACCATCACAGAGT | 300                      | 84                   | 116.8         |
| efl    | NM_131263.1      | Reference gene                                | F:GGACACAGAGACTCATCACAGAAC R: ACCAACCAGCCCAGCAAC | 10                       | 136                  | 96            |
| rpl8   | NM_200713.1      | Reference gene                                | F:CAATGACACCCCGACG R: GCCACAGACTCCTGCACT | 10                       | 136                  | 96            |
Table 2
Nominal and exposure concentrations, or recovery%, reported for venlafaxine and norfluoxetine in previous works.

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

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 chromatography mass spectrometry; SPE-HPLC-FD, Solid-phase extraction with high-performance liquid chromatography 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|
| rara    | 0.822    | 5        | 1.050       | 18          | 0.783| 0.575|
| rarb    | 0.683    | 5        | 1.088       | 18          | 0.628| 0.681|
| rarga   | 1.346    | 5        | 0.904       | 18          | 1.489| 0.242|
| rxaa    | 0.446    | 5        | 1.154       | 18          | 0.387| 0.851|
| rrxb    | 1.090    | 5        | 0.975       | 18          | 1.118| 0.386|
| rrxb    | 0.376    | 5        | 1.173       | 18          | 0.321| 0.894|
| rrxb    | 0.747    | 5        | 1.070       | 18          | 0.698| 0.632|
| rrxa    | 0.465    | 5        | 1.149       | 18          | 0.405| 0.839|
| ppasa   | 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|
| ah2     | 0.545    | 5        | 1.126       | 18          | 0.484| 0.784|
| 5-htr2c | 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|
| sarta   | 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-htr1a | 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. Reproductors 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 highlighted in bold.

| Gene    | MS Model | df Model | MS Residual | df Residual | F       | p      |
|---------|----------|----------|-------------|-------------|---------|--------|
| abcc2   | 1.332    | 5        | 0.908       | 18          | 1.467   | 0.249  |
| abc2a   | 2.358    | 5        | 0.623       | 18          | 3.786   | 0.016  |
| abc4    | 1.655    | 5        | 0.818       | 18          | 2.024   | 0.124  |
| abcc1   | 2.460    | 5        | 0.594       | 18          | 4.140   | 0.011  |
| gest    | 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  |
| rarg    | 1.365    | 5        | 0.898       | 18          | 1.520   | 0.233  |
| rxaar   | 0.822    | 5        | 1.049       | 18          | 0.784   | 0.575  |
| rxb    | 1.727    | 5        | 0.798       | 18          | 2.165   | 0.104  |
| rxrb    | 1.185    | 5        | 0.946       | 18          | 1.264   | 0.322  |
| rxrgb   | 3.101    | 5        | 0.416       | 18          | 7.446   | -0.001 |
| rrga    | 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  |
| sert    | 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-hT2a  | 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 highlighted in bold.

| Gene    | MS Model | df Model | MS Residual | df Residual | F       | p      |
|---------|----------|----------|-------------|-------------|---------|--------|
| abcc2   | 3.366    | 3        | 0.355       | 11          | 9.493   | 0.002  |
| abc2a   | 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,
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 18s band and by measuring the optical density ratio at λ 260/280 nm. RNA was quantified using Take3 micro-volume plates (2μL) in a BioTek spectrophotometer. After confirming RNA quantities, 1μg 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 β1 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 2μL 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, 2μL 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 drd1b, 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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References

[1] P. Rodrigues, V. Cunha, L. Oliva-Teles, M. Ferreira, L. Guimarães, Norfluoxetine and venlafaxine in zebrafish larvae: single and combined toxicity of two pharmaceutical products relevant for risk assessment, J. Hazard. Mater. 400 (2020) 123171.
[2] P. Rodrigues, L. Oliva-Teles, L Guimarães, “norven”, Mendeley Data, v1, 2020 http://dx.doi.org/10.17632/svr9kvsngy.1.
[3] V. Cunha, P. Rodrigues, M.M. Santos, P. Moradas-Ferreira, M. Ferreira, Danio rerio embryos on Prozac - effects on the detoxification mechanism and embryo development, Aquatic. Toxicol. 178 (2016) 182–189.
[4] A.T. Ford, P.P. Fong, The effects of antidepressants appear to be rapid and at environmentally relevant concentrations, Environ. Toxicol. Chem. 35 (2016) 794–798.
[5] A.P. Rodrigues, L.H.M.L.M. Santos, M.J. Ramalhosa, C. Delerue-Matos, L. Guimarães, Sertraline accumulation and effects in the estuarine decapod Carcinus maenas: importance of the history of exposure to chemical stress, J. Hazard. Mater. 283 (2015) 350–358.
[6] J. Costa, M.A. Reis-Henriques, L.F. Castro, M. Ferreira, Gene expression analysis of ABC efflux transporters, CYP1A and GSTalpha in Nile tilapia after exposure to benzo(a)pyrene, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155 (2013) 469–482.
[7] C.L. Andersen, J. Ledet-Jensen, T. Ørntoft, Normalization of real-time quantitative RT-PCR data: a model based variance estimation approach to identify genes suited for normalization - applied to bladder- and colon-cancer data-sets, Cancer Res. 64 (2004) 5245–5250.
[8] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) 2002–2007.