Environment International 146 (2021) 106253

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

# Glyphosate targets fish monoaminergic systems leading to oxidative stress and anxiety

Melissa Faria [a,1], Juliette Bedrossiantz [a,1], Jonathan Ricardo Rosas Ramírez [b], Marta Mayol [c], Gerardo Heredia García [b], Marina Bellot [c], Eva Prats [d], Natàlia Garcia-Reyero [e], Cristian Gómez-Canela [c], Leobardo Manuel Gómez-Oliván [b], Demetrio Raldúa [a,*]

[a] Institute for Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona, 18, 08034 Barcelona, Spain
[b] Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan s/n, Col. Residencial Colón, 50120 Toluca, Estado de México, Mexico
[c] Department of Analytical Chemistry and Applied (Chromatography Section), School of Engineering, Institut Químic de Sarrià-Universitat Ramon Llull, Via Augusta 390, 08017 Barcelona, Spain
[d] Research and Development Center (CID-CSIC), Jordi Girona 18, 08034 Barcelona, Spain
[e] Environmental Laboratory, US Army Engineer Research and Development Center, Vicksburg, MS, USA

ARTICLE INFO

Handling editor: Marti Nadal

*Keywords:*
Glyphosate
Neurotoxicity
Zebrafish
Behavioral impairment
Dopaminergic system
Oxidative stress

ABSTRACT

Glyphosate is the active ingredient of some of the most highly produced and used herbicides worldwide. The intensive applications of glyphosate-based herbicides and its half-life in water lead to its presence in many aquatic ecosystems. Whereas recent studies have reported neurotoxic effects of glyphosate including autism-related effects, most of them used extremely high (mg/L to g/L) concentrations, so it is still unclear if chronic, low environmentally relevant concentrations of this compound (ng/L to μg/L) can induce neurotoxicity. In this study we analyzed the neurotoxicity of glyphosate in adult zebrafish after waterborne exposure to environmentally relevant concentrations (0.3 and 3 μg/L) for two weeks. Our data showed that exposed fish presented a significant impairment of exploratory and social behaviors consistent with increased anxiety. The anterior brain of the exposed fish presented a significant increase in dopamine and serotonin levels, as well as in the DOPAC/dopamine and homovanillic acid/dopamine turnover ratios. Moreover, the expression of genes involved in the dopaminergic system, as *th1*, *th2*, *comtb*, and *scl6a3* was downregulated. Finally, the brain of exposed fish presented a significant increase in the catalase and superoxide dismutase activities, with a concomitant decrease of glutathione stores. These changes in the antioxidant defense system are consistent with the observed increase in oxidative stress, reflected by the increase in the levels of lipid peroxidation in the brain. The presented results show that current glyphosate concentrations commonly found in many aquatic ecosystems may have detrimental consequences on fish survival by decreasing exploration of the environment or altering social interactions. Furthermore, as zebrafish is also a vertebrate model widely used in human neurobehavioral studies, these results are relevant not only for environmental risk assessment, but also for understanding the risk of chronic low-dose exposures on human health.

## 1. Introduction

Glyphosate (N-phosphonomethyl-glycine) is a phosphonate widely used as active ingredient of the most highly produced and used herbicides worldwide (Guyton et al., 2015; Myers et al., 2016). Glyphosate is used in both agriculture and forestry, as well as for weed killing in non-agricultural areas such as water systems (Martínez et al., 2018; Vencill, 2002). The wide range of applications of glyphosate and its several weeks half-life in water lead to its prevalent presence in aquatic ecosystems (Annett et al., 2014; Pohl et al., 2019). Therefore, the detection of glyphosate in water is becoming frequent, with median and maximum concentrations of 0.03 and 73 μg/L, respectively, in large rivers and streams, and <0.02 and 301 μg/L in lakes, ponds and wetlands in the US (Battaglin et al., 2014).

* Corresponding author.
*E-mail address:* drpqam@cid.csic.es (D. Raldúa).
[1] M.F. and J.B. contributed equally to this work.

https://doi.org/10.1016/j.envint.2020.106253
Received 12 August 2020; Received in revised form 23 October 2020; Accepted 27 October 2020
Available online 18 November 2020

Glyphosate-based herbicides were initially considered safe for animals, since glyphosate specifically targets aromatic amino acid synthesis, a metabolic pathway specific of plants (Steinrücken and Amrhein, 1980). In terms of human risk, most reported levels of glyphosate residues in water are well below the accepted maximum contamination levels (MCLs) in drinking water, 700 μg/L in the USA and 1000 μg/L in Australia (Bai and Ogbourne, 2016), and therefore, toxicity of the current environmental levels for humans and animals is assumed to be minimal. However, whereas most of the research used in glyphosate risk assessment to support its safety was conducted more than 30 years ago, more recent reports strongly suggest that current safety standards may fail to protect public health and aquatic ecosystems (Edge et al., 2013; Vandenberg et al., 2017; Webster and Santos, 2015). For instance, there is some controversy regarding the potential neurotoxic effects of glyphosate. While the United States Environmental Protection Agency (USEPA) (EPA, n.d.) has classified this compound as non-neurotoxic and no evidences of neurotoxicity were also found by the European Food Safety Agency (EFSA) (Authority, 2015), many recent studies support the neurotoxic potential of glyphosate, including autism-related effects, in different animal species (Cattani et al., 2014; Gallegos et al., 2018; Pereira et al., 2018; Pu et al., 2020; Roy et al., 2016). Oxidative stress, neuroinflammation, glutamate excitotoxicity, changes in the neurotransmitter profile and changes in behavior are some of the reported effects of glyphosate on the central nervous system (Cattani et al., 2017, 2014; de Souza et al., 2019). However, most of these neurotoxic effects were obtained using extremely high (mg/L to g/L) concentrations of glyphosate (Cattani et al., 2017, 2014), so it is still unclear if environmental relevant concentrations of this compound can induce neurotoxicity.

Zebrafish (*Danio rerio*) is a cyprinid classically used as fish model species in ecotoxicology (Faria et al., 2020, 2019a), as well as a vertebrate model increasingly used in biomedical research, including neurotoxicology studies (Babin et al., 2014; Faria et al., 2015; M. Faria et al., 2018; Melissa Faria et al., 2018; Tingaud-Sequeira et al., 2017), as this animal species exhibits a similar overall nervous system organization to that of humans as well as similar neurotransmitter systems (Horzmann and Freeman, 2016). A few studies using adult zebrafish have already addressed the potential neurotoxicity of glyphosate (Bridi et al., 2017; da Costa Chaulet et al., 2019; Pereira et al., 2018), but most of them used environmentally unrealistic concentrations (≥500 μg/L), so predicting the risk of low level chronic exposures remains a challenge.

In this study we analyzed the neurotoxicity of glyphosate in adult zebrafish by waterborne exposure to environmentally relevant concentrations (0.3 and 3 μg/L) for two weeks. Effects on locomotor, exploratory and social behaviors were thoroughly analyzed, and changes in the expression of key genes and neurochemical profiles in the brain of the exposed fish were determined. Finally, the status of the antioxidant defense systems and the presence of oxidative stress was also analyzed in the brain of the exposed fish.

## 2. Material and methods

### 2.1. Animals and housing

Adult wild-type zebrafish (standard length: 3.1–3.5 cm) were obtained from Piscicultura Superior (Barcelona, Spain) and maintained into a recirculating zebrafish system (Aquaneering Inc., San Diego, USA) at the CID-CSIC zebrafish facilities for at least 4 weeks before starting the exposures. During this adaptation time, fish were housed in 2.8 L tanks (density: 20 fish/tank) with fish water [reverse-osmosis purified water containing 90 mg/L Instant Ocean® (Aquarium Systems, Sarrebourg, France), 0.58 mM $CaSO_4{\cdot}2H_2O$] at 28 ± 1 °C under a 12L:12D photoperiod. The main parameter of the water during the housing of the fish were: Temperature: 28° ± 1° C; pH: 6.5–7.0; Conductivity: 750–900 μS/cm; $NO_3$: 5–10 mg/L ; $NO_2$: ≈0.1 mg/L. Fish were fed twice a day with flake food (TetraMin, Tetra, Germany). All procedures were approved by the Institutional Animal Care and Use Committees at the CID-CSIC and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

### 2.2. Stability of glyphosate in water

Glyphosate (CAS # 1071-83-6) was purchased from ChemCruz (sc-211568; Santa Cruz Biotechnology, Dallas, TX) with a purity of 98%. The stability of glyphosate in fish water was assessed measuring its concentration in two working solutions 0.3 μg/L and 3.0 μg/L freshly prepared in fish water during 48 h. Working solutions were placed under the same temperature and light conditions as fish exposure, measurements were conducted immediately after preparing each nominal concentration and 24 and 48 h later. The experimental concentration was checked by ultra-high performance liquid chromatography with triple quadrupole detector (UHPLC-MS/MS) analysis. Three sample replicates of each concentration were prepared (see Supplementary Methods for additional details).

### 2.3. Experimental procedure

Working solutions of 0.3 and 3.0 μg/L glyphosate were prepared the day of the experiment from a 3 mg/L stock solution freshly prepared in fish water. The pH of both working solutions was similar to the fish water (pH: 6.8). Adult zebrafish (50:50 male:female ratio) were randomly selected from the CID-CSIC facilities and exposed for 2 weeks to 0.3 or 3.0 μg/L glyphosate, at 28.5 °C and 12L:12D photoperiod. Control fish were maintained in fish water under identical conditions. Experiments were conducted in duplicate or triplicate, in glass tanks containing 500 mL of water with 3 fish in each. Experimental solutions were renewed every 48 h, 30 min after the first feeding of the day. The total number of fish used in this study for the control, 0.3 μg/L and 3 μg/L groups was 65, 60 and 65, respectively. Tanks were kept in an incubation chamber (POL-EKO APARATURA Climatic chamber KK350, Poland) set to 28.5 °C and 12L:12D photoperiod. For brain sample collection, fish were euthanized by inducing hypothermic shock in ice-chilled water (2–4 °C). Brains were immediately dissected and those for neurotransmitter profiling and gene expression analysis were sectioned in the standard frontal plane at two different levels, the anterior part of the optic tectum and the posterior part of the cerebellum (Supplementary Fig. S1). As a result of this sectioning strategy, anterior, middle and posterior brain regions were obtained. The “anterior brain region” included the olfactory bulbs, telencephalon and the preoptic area, the “middle brain region” included the optic tectum, cerebellum, thalamic and diencephalic structures and locus coeruleus, and the “posterior brain region” included the medulla oblongata and the initial part of the spinal cord. Anterior and middle brain regions, where most of the monoaminergic neurons are located, were individually stored at −80 °C for further analysis.

### 2.4. Behavioral testing

All tests were performed in an isolated behavior room at 27–28 °C. Animals (≈50:50 male:female ratio) were brought to the behavior room one hour before testing began, to acclimate to this new environment, and then, behavioral testing was conducted between 10:00 and 17:00 h. All fish used in this study were experimentally naïve and all behavioral testing was performed in a blind manner, with observers unaware of the experimental group. In order to avoid any potential tank effect, the experimental group assigned to each tank was switched between trials.

The novel tank test (NTT), used to assess geotaxis, freezing and erratic movements of fish, was performed using an experimental setup previously described (M. Faria et al., 2018). This behavioral paradigm was performed on a total of 28, 23 and 29 adult zebrafish from the control, 0.3 and 3 μg/L glyphosate groups, respectively. Each trial was video-recorded with a GigE camera mounted in front of the

experimental tank. Then, videos were analyzed by Ethovision XT 13.0 (Noldus, Wageningen, the Netherlands). First, the front of the tank was divided into two equal virtual zones, top and bottom, and the total distance travelled (cm), distance travelled at the top and at the bottom (cm), the time spent in the top (s) and the latency to top (s) were determined. The Behavioral Observation Research Interactive Software (BORIS) free software (Friard and Gamba, 2016) was used to determine the number and duration (s) of freezing and erratic bouts.

The dark-light test (DLT), used to determine scototaxis of the fish, was performed using an experimental setup previously described (Faria et al., 2019b). No test battery effect has been reported in batteries of NTT and LDT when they are run immediately one after the other (Kysil et al., 2017), so following the 3Rs principle (reduction), LDT was performed on 20, 22, and 21 adult zebrafish from the control, 0.3 and 3 μg/L glyphosate groups, respectively previously tested for NTT. Each trial was video-recorded with a GigE camera mounted on the top of the experimental tank. The recorded videos were analyzed by Ethovision XT 13.0, and the time spent in the white zone (s), number of transitions to the white zone and the latency to enter to the white zone (s) were determined.

The shoaling test, used to assess social and anxiety-like behaviors, was performed using (M. Faria et al., 2018). Two independent trial were performed, with a shoal size of 6 fish per experimental group in each. A total of 20 screenshots per experimental group were used in this study. For each screenshot, the distance between each fish in the group was measured by using the free-processing ImageJ software (National Institutes of Health (NIH), http://rsb.info.nih.gov/ij/). Finally, the average interfish distance (cm), farthest neighbor distance (cm) and nearest neighbor distance (cm) were calculated.

Finally, the novel approach test (NAT), used here to assess boldness, was basically performed as described by Hamilton et al. (2017) (Hamilton et al., 2017; Johnson and Hamilton, 2017). The test was conducted after the shoaling test, in trunked conical white plastic tanks with a LEGO® figurine attached with Velcro to the center of the arena served as the novel object (see Supplementary Methods for additional details).

### 2.5. Analysis of neurochemicals by LC-MS/MS

Monoaminergic neurotransmitters in the anterior and middle regions of individual brains were analyzed by liquid chromatography coupled to a triple quadrupole detector (Xevo TQS, Acquity Waters, Milford, USA) (LC-MS/MS) (Mayol-Cabré et al., 2020). In total 60 analyses were performed corresponding to the two regions (anterior and middle brain) of 30 brains from the control, 0.3 and 3 μg/L groups. The content of monoaminergic neurotransmitters was analyzed in a total of 10 brains per experimental group. Separation was performed with an HILIC column, and a binary mixture based on acetonitrile (A) and water (B) was employed as mobile phase. The injection volume was 10 μL. A gradient elution was performed starting at 100% A, followed by increasing B, and returning to initial conditions. All compounds eluted in 5 min, with a total run time of 10 min. Flow rate was selected at 0.25 mL $min^{-1}$. Neurotransmitters were measured under positive electrospray ionization (ESI+) and MS conditions of each neurochemical were optimized (including cone voltage and collision energy). Acquisition was performed in selective reaction monitoring (SRM) mode, in which two transitions from each compound were selected. The first transition, which corresponded to the most intensive fragment, was used as the quantifier ion, whereas the second as a qualifier ion. The target compounds 5-HTP, 3-MT, DOPAC, L-tryptophan, 5-HIAA and NE were quantified by internal calibration, whereas the remaining 4 neurotransmitters (5-HT, DA, L-tyrosine and HVA) were determined using external calibration. Data were processed using MassLynx v4.1 software package.

### 2.6. Real-time polymerase chain reaction

Since dopaminergic neurons are mainly located in the anterior and middle brain regions (Filippi et al., 2010; Yamamoto et al., 2010), posterior brain was removed during the sampling for transcriptomic analysis of the dopaminergic relevant genes. A total of 8 brains from the control, 0.3 and 3 μg/L groups were analyzed. Total RNA was extracted from each brain using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop™ ND-8000 spectrophotometer (Fisher Scientific) and the quality checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RIN (RNA Integrity Number) values ranged between 9 and 10. First strand cDNA was synthesized from 1 μg of total RNA previously treated with DNaseI (Ambion, Austin, TX), using First Strand cDNA synthesis Kit (Roche Diagnostics, Mannheim, Germany) and oligo(dT) according to manufacturer's instructions.

Real Time qRT-PCR was performed in a LightCycler® 480 Real-Time PCR System with SYBR Green PCR Master Mix (Roche Diagnostics, Mannheim, Germany). Cycling parameters: 95 °C for 15 min and 45 cycles of 95 °C, 10 s and 60 °C, 30 s. A dissociation curve analysis was added to ensure the specificity of the reaction. Eight biological replicates were assayed for each treatment, and 3 technical replicates were run for each sample.

Primer sequences for the selected genes (*th1*, *th2*, *comta*, *comtb*, *mao*, *scl6a3, tph1a*, *tph1b*, *scl6a4a*, *scl18a2 and gfap*) are listed in Table S1. Specificity of primers was previously confirmed by the presence of a single peak in the melting curves of PCR reactions.

The mRNA expression of each target gene was normalized to the housekeeping *ppia2* as reference gene. Relative quantification of mRNA abundance for the selected genes among treatments with respect to the control group were calculated following the ΔΔCt method (Livak and Schmittgen, 2001) deriving fold-change ratios from these values.

### 2.7. Biochemical determination

All biochemical determinations were conducted in the subcellular fraction of zebrafish whole brain tissue. Antioxidant enzyme activities, catalase (CAT) and superoxide dismutase (SOD), and levels of lipid peroxidation (LPO) were determined according to Faria et al. (2019a, 2019b) (Faria et al., 2019b), while glutathione levels (GSH) and glutamate cysteine ligase (GCL) activity was measured following the method described by White et al. (2003) (White et al., 2003). Briefly, CAT was measured following the decrease in absorbance of $H_2O_2$ decomposition; SOD activity was determined based on the measurement of the degree of inhibition, caused by the presence of SOD in the sample, of the reduction of Cytocrome c by free oxygen radicals released by the reaction xanthine/xanthine oxidase; LPO was determined by quantifying the levels of malondialdehyde (MDA) in the presence of 1-methyl-2-phenylindole under acidic conditions; finally GSH and GCL activity was measured by detecting the fluorescence signal generated by the complex formed between the conjugation of naphthalene dicarboxyaldehyde (NDA) and γ-glutamylcysteine (γ-GC). In total, 15 individual brains per experimental condition were analyzed for CAT and SOD activities, 12, 8 and 12 pools of two brains, for control, 0.3 μg/L and 3 μg/L groups, respectively, were analyzed for LPO, and 8, 11 and 8 brains, for control, 0.3 μg/L and 3 μg/L groups, respectively, for GSH and γ-GCL. A more detailed description of the biochemical determinations is given in the Supplementary Information.

### 2.8. Data analysis

Data were analyzed with IBM SPSS v25, and data were plotted with GraphPad Prism 8.31 for Windows (GraphPad software Inc, La Jolla, CA). Normality was assessed using Kolmogorov-Smirnov and Shapiro-Wilk tests. One-way ANOVA followed by Dunnett's multiple

comparison test was used to test differences between normally distributed groups, whereas the Kruskal-Wallis test followed by a pairwise comparison using the Bonferroni correction was used to test differences between groups that did not meet parametric assumptions. Data are presented in Figs. 1–4 as scatter plots, with a line indicating the median, in order to provide consistent information for both normally and non-normally distributed data. For the same reason, neurotransmitter results are presented as the median and interquartile ranges in the Supplementary Tables S3 and S4. Since biochemical results obtained for the control group in the different independent experiments exhibited significant differences, in order to compare control vs treated groups data from each experiment were previously normalized to % of the respective control. Significance was set at $P < 0.05$. To perform the statistical analyses, NTs concentrations between method detection limit (MDL) and the method quantification limit (MQL) were used unaltered for calculations (Joerss et al., 2019), whereas values below MDL were treated as MDL normalized per square root of two. Analysis of the qRT-PCR data was performed using the ΔΔCt method, and then differences among control and treated groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test or Kruskal-Wallis test followed by Bonferroni correction, when appropriate.

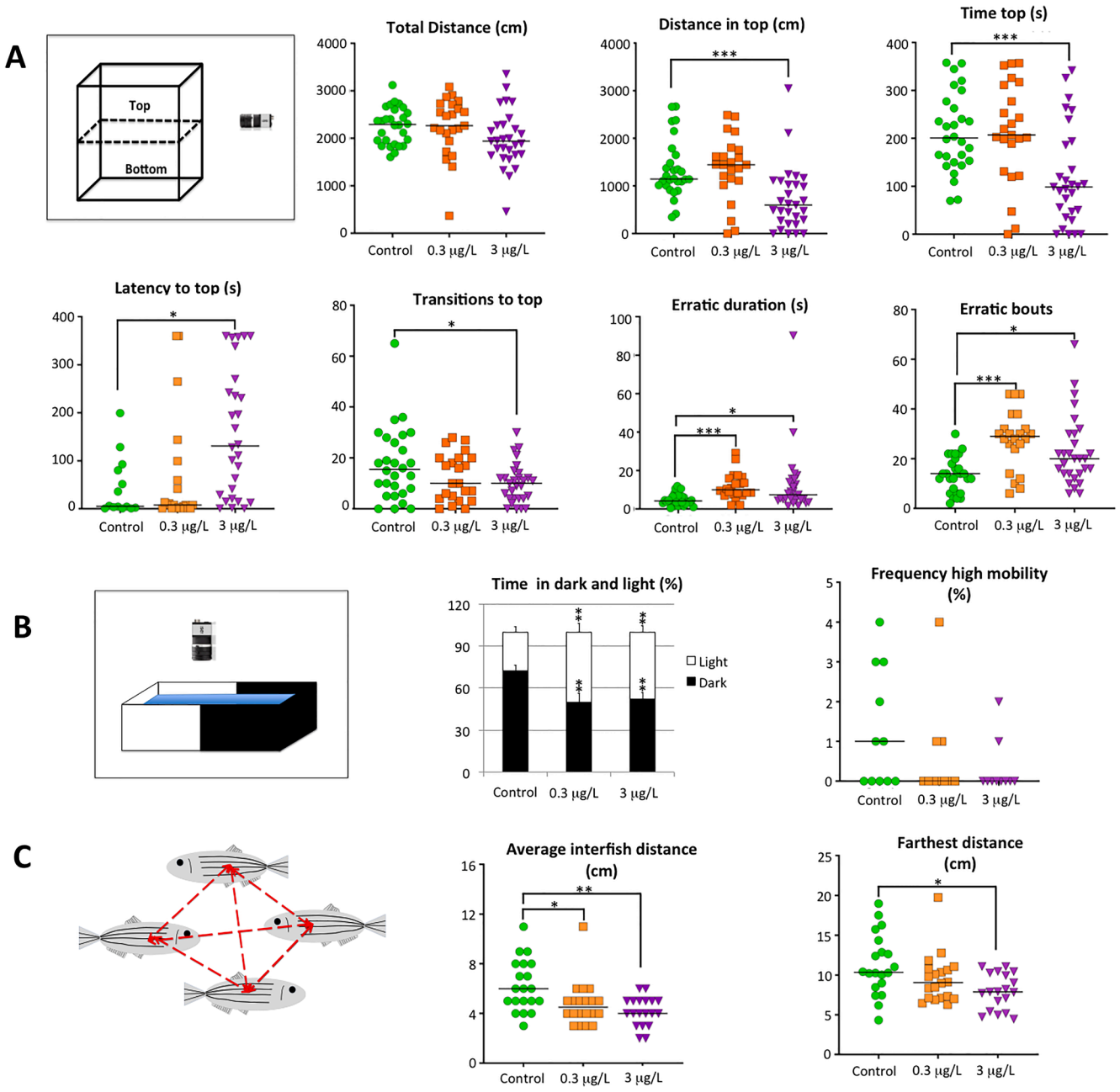


**Fig. 1.** Behavioral changes in adult zebrafish waterborne exposed to 0.3 and 3 µg/L glyphosate for 2 weeks. (A) Behavioral parameters assessed in standard 6-min novel tank test (NTT), as well as a cartoon of the experimental tank divided into two equal virtual zones, top and bottom; (B) Behavioral parameters assessed in standard 6-min dark-light test (DLT), as well as a cartoon of the experimental tank divided into two equal zones, white and black; (C) Behavioral parameters of zebrafish shoaling behavior in control and glyphosate-treated zebrafish. Data reported as scatter plot with the median (n = 23–29 for NTT, n = 20–22 for DLT and n = 19–20 for shoaling test), *p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA with Dunnett's multiple comparison test (Total distance, Distance in top, Time in top, Transitions to top, Erratic bouts, High mobility frequeny, Average interfish distance and Farthest distance) or Kruskal Wallis test with Bonferroni correction (Latency to the top, Erratic duration) Data from 2 to 4 independent experiments.

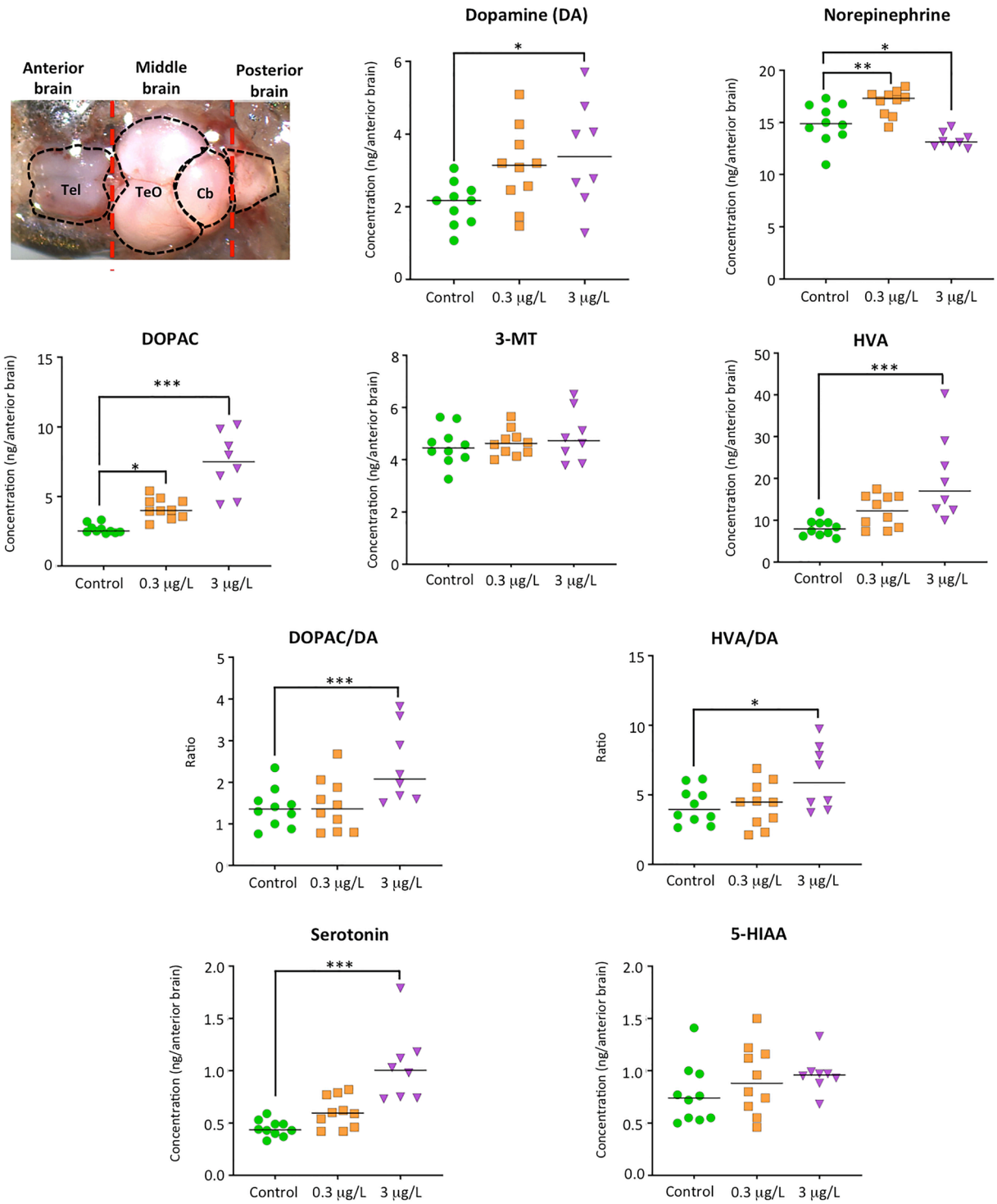


**Fig. 2.** Increased dopaminergic activity in the anterior brain region of zebrafish exposed to 0.3 and 3 µg/L glyphosate for 2 weeks. Levels of L-tyrosine, DOPAC, HVA, and the ratios DOPAC/DA and HVA/DA increased significantly in the anterior brain of zebrafish exposed to 3 µg/L glyphosate for 2 weeks. The cartoon on the upper left shows a dorsal view of an adult zebrafish brain, with the three regions collected for the neurotransmitter analysis. Data reported as scatter plot with the median. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA with Dunnett's multiple comparison test (NE) or Kruskal Wallis test with Bonferroni correction (L-tyrosine, DA, DOPAC, HVA, 3-MT, DOPAC/ DA, HVA/ DA). Data from 2 independent experiments.

## 3. Results

### 3.1. Stability of glyphosate in fish water

To select the right glyphosate exposure system for this study, stability of glyphosate in fish water was analyzed by UHPLC-MS/MS. First, when glyphosate working solutions of 0.3 µg/L and 3.0 µg/L were freshly prepared in fish water, measured and nominal concentrations were very close (Supplementary Table S1). Moreover, when these solutions were incubated under experimental conditions (28 °C and 12 L:12D photoperiod), glyphosate concentrations remained quite stable (±13% of the values at time 0) for at least 48 h (Supplementary Table S2). Therefore, a 48 h semi-static exposure system was selected for the exposure experiments.

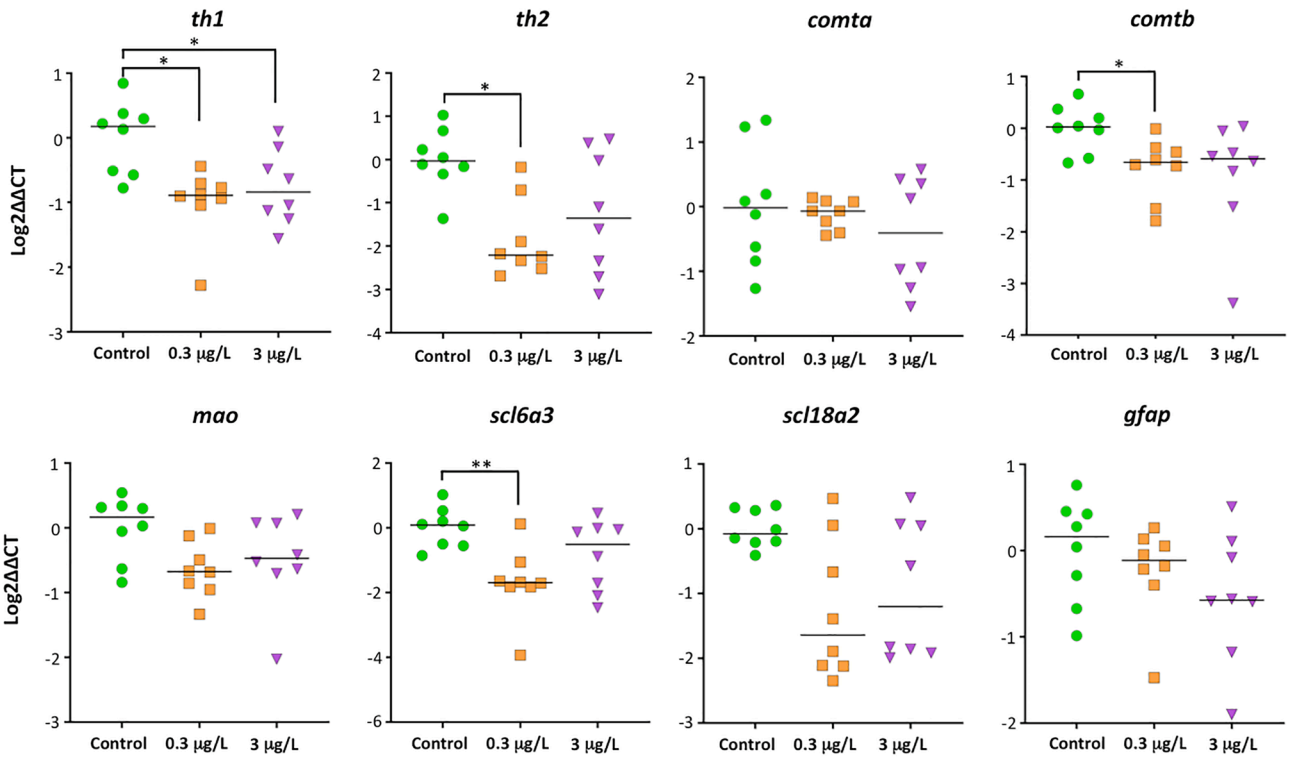


**Fig. 3.** Effect of two weeks exposure to 0.3 and 3 µg/L glyphosate on the expression of genes associated with the dopaminergic system (*th1*, *th2*, *comta*, *comtb*, *mao*, *scl6a3*, *scl18a2 and gfap*) in the anterior-middle brain regions of adult zebrafish. Data reported as scatter plot with the median. *$P < 0.05$, **$P < 0.01$; one-way ANOVA with Dunnett's multiple comparison test (*th1*, *th2*, *comta*, *mao*, *scl6a3*, *gfap*) or Kruskal Wallis test with Bonferroni correction (*comtb*, *scl18a2*). Data from 2 independent experiments (n = 8).

### 3.2. Glyphosate alters exploratory behavior in adult zebrafish

In order to assess if subchronic exposure to environmentally relevant concentrations of glyphosate has an ecologically relevant effect on adult zebrafish, two experimental paradigms related to the exploratory behavior were initially used, novel tank and dark-light paradigms. In both paradigms, fish face a mildly stressful novel situation, triggering the expression of characteristic avoidance behaviors, geotaxis and scototaxis, respectively. When the exploratory behavior of the fish was assessed in the novel tank test (NTT) (Fig. 1A), exposure to 3 µg/L glyphosate resulted in a positive geotaxis, with a significant increase in the latency to visit the top of the tank ($H(2) = 9.628$, $P = 0.008$) and a decrease in the distance travelled at the top ($F_{2,77} = 9.118$, $P = 0.0003$), the time in top ($F_{2,77} = 8.744$, $P = 0.0004$) and the number of transitions to the top ($F_{2,77} = 3.441$, $P = 0.037$). The effect of glyphosate on the erratic and freezing behaviors of the animals was then explored in the novel tank (Fig. 1A and Supplementary Fig. S2). Glyphosate significantly increased the duration ($H(2) = 17.261$, $P = 0.025$) and number of erratic bouts ($F_{2,76} = 10.073$, $P = 0.0001$; Fig. 1A). No differences in freezing were found between groups in this task (Pearson Chi-Square (2) = 2.964, $P = 0.253$; Supplementary Fig. S2).

Interestingly, when the behavior was tested on the dark-light test (DLT) (Fig. 1B), a negative scototaxis was found in animals exposed to both glyphosate concentrations, with a significant increase in the time spent in the white zone of the tank ($F_{2,60} = 6.038$, $P = 0.004$). No differences in freezing were found between groups in this task (Pearson Chi-Square (2) = 5.538, $P = 0.062$; Supplementary Fig. S3).

Zebrafish have a natural trend to form shoals, relatively non-polarized groups of conspecifics held together by social pressure. Anxiety or fear causes the shoal to "tighten", and this effect can be easily identified in the shoaling test. Thus, when the shoaling test was used, the shoal of fish exposed to 3 µg/L glyphosate showed a significant decrease in the average interfish distance ($F_{2,56} = 5.664$, $P = 0.006$) and the farthest distance ($F_{2,56} = 7.413$, $P = 0.001$) with respect to the control, results consistent with an increase in anxiety.

Finally, when the behavior of control and treated fish was evaluated in the novel approach test, no differences were found in the time spent in the center zone, near the figurine ($F_{2,32} = 1.470$, $P = 0.245$; Supplementary Fig. S4). As the time spent in the center and the thigmotaxis zones is a common index of boldness, this result suggests that in the experimental conditions used, the boldness of the fish was not altered by glyphosate exposure. Furthermore, no differences were found in the time spent by control and treated fish in the thigmotaxis zone ($F_{2,32} = 0.612$, $P = 0.548$), a result indicating that glyphosate has no effect on thigmotaxis.

### 3.3. Glyphosate increases dopamine metabolism in the brain

The profile of the monoaminergic neurotransmitters dopamine (DA), serotonin (5-HT) and norepinephrine (NE), as well as their main precursors [L-tryptophan, 5-hydroxy-L-tryptophan (5-HTP), L-tyrosine] and degradation products [5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanilic acid (HVA)], was determined in the anterior, middle and posterior brain regions of controls and glyphosate-treated zebrafish (Supplementary Table S3-4). Moreover, DOPAC/DA, HVA/DA and 5-HIAA/5-HT turnover ratios were also calculated.

In the anterior brain (Fig. 2), exposure to 0.3 µg/L glyphosate resulted in a significant increase in DOPAC ($P = 0.014$; Independent-samples Kruskal Wallis test followed by Bonferroni correction for multiple tests) and NE ($P = 0.044$; one-way ANOVA followed by Dunnett's test) levels. Exposure to 3 µg/L glyphosate resulted in a significant decrease in NE levels ($F_{2,27} = 14.832$, $P = 0.000057$) and an increase of the dopaminergic activity (Fig. 2), with increased levels of dopamine ($F_{2,27} = 3.920$, $P = 0.033$) and the dopamine metabolites DOPAC ($H(2) = 21.405$, $P = 0.000022$) and HVA ($F_{2,27} = 8.975$, $P = 0.001$) but not 3-

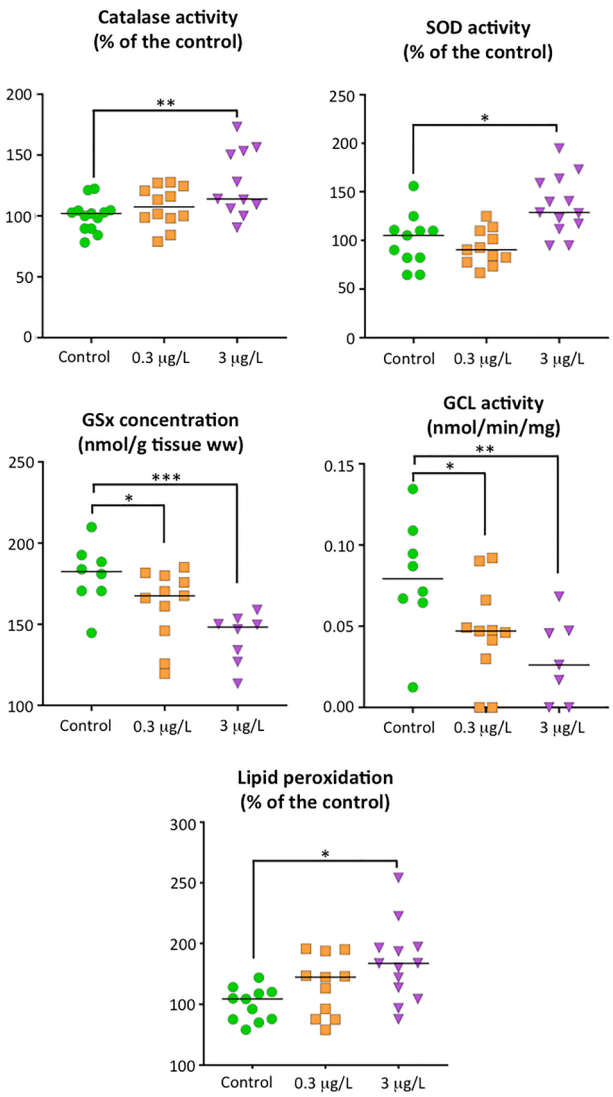


**Fig. 4.** Effect of two weeks exposure to 0.3 and 3 µg/L glyphosate on the antioxidant status and lipid peroxidation levels in the brain of adult zebrafish. Whereas catalase and superoxide dismutase (SOD) activities increased, the gamma-glutamate cysteine ligase (γ-GCL) activity was strongly inhibited in the brain of glyphosate-treated fish. Moreover, total glutathione stores decreased and lipid peroxidation levels increased in the treated fish. Data reported as scatter plot with the median. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA with Dunnett's multiple comparison test (catalase, γ-GCL, GSx) or Kruskal Wallis test with Bonferroni correction (SOD, LPO). Data from 2 independent experiments (n = 11–13).

MT($F_{2,27} = 0.557$, $P = 0.0.580$). .Levels of the DA precursor L-tyrosine increased also with glyphosate concentration, but as a result of the high intragroup variability these differences were no statistically significant ($H(2) = 5.563$, $P = 0.062$). DOPAC/DA ($F_{2,27} = 6.230$, $P = 0.006$) and HVA/DA ($F_{2,27} = 3.679$, $P = 0.040$) turnover ratios reflecting the metabolism and/or release of dopamine, were also increased in the anterior brain of animals exposed to 3 µg/L glyphosate. Finally, when the effect of glyphosate on the serotonergic system was analyzed, a significant increase in the levels of 5-HT ($F_{2,27} = 18.162$, $P = 0.000013$), but not effect on 5-HIAA ($F_{2,27} = 1.133$, $P = 0.338$), were found.

At the middle brain level (Supplementary Table S4), NE was significantly reduced by both glyphosate concentrations ($F_{2,27} = 9.952$, $P = 0.001$). Moreover, exposure to 0.3 µg/L glyphosate resulted in a significant increase in DOPAC ($H(2) = 15.158$, $P = 0.001$), HVA ($H(2) = 10.798$, $P = 0.005$) levels, with no changes in the DA ($F_{2,27} = 1.083$, $P = 0.353$) levels. No effect was found in 5-HT levels ($H(2) = 0.062$, $P = 0.970$) in this brain region at any glyphosate concentrations.

### 3.4. Transcription of genes involved in the dopaminergic system are down-regulated by glyphosate

In order to better understand the changes observed in the dopaminergic system in the brain of glyphosate-treated zebrafish, the expression of seven genes involved in the synthesis (*th1*, *th2*), degradation (*mao*, *comta*, *comtb*) and transport [*scl6a3* (DAT), *scl18a2* (VMAT2)] of dopamine was analyzed (Fig. 3). The expression of both *th1* and *th2* in the brain of glyphosate-treated animals was down-regulated with respect to that of controls ($F_{2,21} = 6.888$, $P = 0.005$ for *th1*, and $F_{2,21} = 6.352$, $P = 0.007$ for *th2*). Moreover, expression of *scl6a3*, but not *scl18a2* was also found down-regulated in the brain of zebrafish after 2 weeks exposure to the lowest concentration of glyphosate ($F_{2,21} = 6.061$, $P = 0.008$ for *scl6a3* and $H(2) = 4.745$, $P = 0.093$ for *scl18a2*). Kruskal-Wallis test with Bonferroni correction for multiple comparison test also showed a significant down-regulation in *comtb* expression ($P = 0.0400$; n = 8) in the group exposed to the lowest concentration. In contrast, glyphosate exposure didn't change the expression of *comta*, and *mao* in the brain. No differences ($p > 0.05$) in the expression of genes specifically involved in the synthesis (*tph1a*, *tph1b*) and transport (*scl6a4a*) of serotonin were found in the brain of fish exposed to both glyphosate concentrations. Finally, when the expression of *gfap*, a gene commonly used as a neuroinflamation marker, was determined (Fig. 3), no differences were found with the control values either.

### 3.5. Glyphosate impairs the antioxidant defense system, increasing lipid peroxidation in the brain

A direct effect of glyphosate on glutathione (GSH) homeostasis resulting in GSH depletion and ROS generation has been suggested to be involved in the neurotoxic effect of this herbicide (Cattani et al., 2014). Thus, catalase, superoxide dismutase (SOD) and gamma-glutamate cysteine ligase (γ-GCL) activities, as well as total glutathione (GSx) and lipid peroxidation (LPO) levels were determined in the brain of the control and glyphosate-treated zebrafish (Fig. 4). First, a significant increase in catalase ($F_{2,33} = 5.917$, $P = 0.006$) and SOD ($H(2) = 14.076$, $P = 0.001$) activities was found in the brain of animals exposed to the highest concentration of glyphosate. Moreover, a concentration-dependent inhibition of γ-GCL was found in the brain of glyphosate-treated fish ($F_{2,23} = 5.087$, $P = 0.015$), an effect that was paralleled by a significant decrease in the GSx levels in these animals ($F_{2,24} = 7.815$, $P = 0.002$). This altered profile of the antioxidant defense system was finally reflected in a significant increase in the lipid peroxidation ($H(2) = 8.692$, $P = 0.013$) found in the brain of exposed animals to the highest concentration of glyphosate.

## 4. Discussion

While recent studies have increased the concern about the potential neurotoxicity of glyphosate, most of them used environmentally unrealistic concentrations of this compound, so the relevance of this effect for aquatic organisms and human health is still unclear. In this study we exposed adult zebrafish for 2 weeks to two environmentally relevant concentrations of glyphosate, 0.3 and 3 µg/L, and changes in an ecologically relevant apical endpoint, behavior, were analyzed. NTT and DLT are experimental paradigms based on the analysis of the exploratory behavior of animals when they are in front of a mild stressful stimulus, the novelty. These two paradigms are widely used to study anxiety-like behaviors in adult zebrafish (Meshalkina et al., 2017). Whereas NTT is based on bottom-dwelling behavior (geotaxis), the innate escape diving behavior of fish in novel environments, DLT is based on scototaxis, the avoidance by adult fish of brightly lit areas (Kysil et al., 2017). Typically, anxiogenic substances are predicted to

increase geotaxis in the NTT and scototaxis in the DLT. In the present study, however, exposure to 3 μg/L glyphosate exhibited an anxiogenic effect in geotaxis and anxiolytic in scototaxis. A similar behavioral phenotype, with positive geotaxis and negative scototaxis, has been previously reported for VMAT2$^{-/-}$ mutants (Wang et al., 2016) and adult zebrafish treated with tryptophan hydroxylase inhibitor *para*-chlorophenylalanine (PCPA) (Maximino et al., 2013) and with the neurotoxicant acrylamide (Faria et al., 2019b; M. Faria et al., 2018). In all these cases, this behavioral phenotype was associated with reduced levels of monoaminergic neurotransmitters. The lack of consistency between NTT and DLT probably reflects that these paradigms produce measures of different aspects of anxiety, from the exploration/avoidance axis for the former and from the shyness-boldness axis for the latter (Maximino et al., 2012). Interestingly, the behavioral phenotype found in zebrafish exposed for 2 weeks to 3 μg/L glyphosate is a phenocopy of that observed in zebrafish exposed to 53 mg/L (0.75 mM) acrylamide for 3 days, including the anxiogenic-like effect observed on the shoal size (Faria et al., 2019b; M. Faria et al., 2018).

While the precise mechanism by which glyphosate produces the observed behavioral phenotype remains unknown, the effects induced by glyphosate on adult zebrafish behavior are highly ecologically relevant since they affect predation risk, foraging efficiency and other variables important for survival (Kellner et al., 2016).

In mammals, neuroanatomically, responses to the typical potential threat of anxiety are mediated by a network of telencephalic structures including the medial prefrontal cortex, frontotemporal amygdala, ventral hippocampus and lateral habenula, whereas hypothalamus seems to play an essential role in defensive behaviors (Maximino et al., 2012). Whereas zebrafish lack the expanded telencephalon with the typical mammalian laminar cortex, zebrafish telencephalic cells have formed a ventral pallium that topologically corresponds to the mammalian basolateral amygdala, a lateral pallium corresponding to the mammalian piriform cortex, a dorsal pallium corresponding to the (iso-)cortex, and a medial pallium corresponding to the hippocampus (Mueller et al., 2011). Thus, in order to understand the potential link between changes in the neurochemicals profile and the observed anxiety-like behavior found in glyphosate-exposed fish, monoaminergic neurotransmitters were analyzed separately in the anterior (pallium) and middle (hypothalamus) brain regions. Similarly to the behavioral results, the most relevant changes in the neurotransmitter profile were found in animals exposed to 3 μg/L glyphosate, and specifically in the anterior brain, the region containing the pallium. Exposure to the highest concentration of glyphosate resulted in a significant decrease, in the anterior and middle brain regions, of NE levels, a catecholamine involved in mood and anxiety disorders (Brunello et al., 2003). Levels of DA and 5-HT were specifically increased in the anterior brain. Whereas it is generally accepted that both monoaminergic neurotransmitters are involved in anxiety disorders (Gordon and Hen, 2004; Reis et al., 2004; Zweifel et al., 2011), their action is extremely complex and conflicting data from human and animal studies argue for both anxiolytic and anxiogenic roles (Gordon and Hen, 2004; Moratalla et al., 2017). Therefore, the increased levels of DA and 5-HT found in the anterior brain of 3 μg/L glyphosate exposed fish might be directly related with the observed changes in exploratory and social behaviors. Anterior brain of fish exposed to the highest glyphosate concentration exhibited also increased levels of DA metabolites DOPAC and HVA, as well as DOPAC/HVA and HVA/DA turnover ratios, but unchanged levels of 3-MT. Whereas DOPAC primarily reflects intraneuronal dopamine metabolism, HVA is a product of intra-and extra neuronal metabolism and 3-MT is the only exclusive metabolite reflecting the released DA (Godefroy et al., 1989; Roffler-Tarlov et al., 1971; Westerink, 1985). Thus, the observed changes in DA metabolites found in this study strongly suggest that exposure to 3 μg/L of glyphosate results in an increase in the intraneuronal metabolism of DA and not in an increased release of this neurotransmitter. An potential mechanism consistent with the observed increase in the dopaminergic activity would be the inhibition of the vesicular monoamine transporter 2 (VMAT2;*slc18a2*) increasing the metabolism of the intraneuronal free DA, where the decrease in DA is compensated by an increase in *de novo* synthesis. Interestingly, zebrafish treated for 7 days with reserpine, an irreversible inhibitor of VMAT2, exhibit positive geotaxis and negative scototaxis (Kyzar et al., 2013), a behavioral profile consistent with that found in glyphosate-treated zebrafish.

One important limitation of this study is that the neurotransmitter analysis has been performed on the total pool of brain neurotransmitters. Unfortunately, currently no microdialysis sampling systems adapted for the zebrafish brain size are available, and therefore it is not possible to determine changes in the neurotransmitters released to the synaptic clefts. Consequently, an specific effect of glyphosate decreasing the dopamine or serotonin released to the dopaminergic or serotonergic synapses cannot be ruled out.

Another of the most relevant results found in this study is the altered antioxidant status and the presence of lipid peroxidation in the brain of glyphosate-exposed fish, especially for those exposed to the highest concentration. In mammals, the metabolism of DA to DOPAC is mediated by the monoamine oxidase isoforms MAO-A and/or MAO-B and, as such, linked to the production of free radicals with one $H_2O_2$ molecule for each molecule of dopamine metabolized (Casarejos et al., 2013; Hermida-Ameijeiras et al., 2004). Therefore, the increased dopaminergic activity in the anterior brain of zebrafish exposed to 3 μg/L glyphosate should lead to an increase in reactive oxygen species (ROS) generation, an effect consistent with the observed increase in the oxidative stress found in their brains. However, the potential involvement of additional mechanisms of ROS generation, such as the inhibition of the mitochondrial complex enzymatic activity (Pereira et al., 2018) or the glutamate excitotoxicity (Cattani et al., 2014), should not be discarded.

The results presented in this manuscript demonstrate that exposure to environmentally relevant concentrations of glyphosate are able to impair exploratory and social behaviors in adult fish, a highly relevant ecological effect. Moreover, the neurotoxic effect of glyphosate on fish behavior was also reflected at lower levels of organization, with an increase in the dopaminergic activity in the anterior brain region, altered expression of genes involved in the dopaminergic system, and impairment of the antioxidant system with a concomitant increase in lipid peroxidation in the brain of the exposed fish. Further efforts should be made to determine whether the observed adverse effects of glyphosate are directly produced by this compound in the CNS, or if, on the contrary, they are produced indirectly through other mechanisms such as previously reported effects of glyphosate on gut microbiome (Aitbali et al., 2018; Motta et al., 2018; Pu et al., 2020), acting through the gut-brain axis (Aitbali et al., 2018). Considering that adult zebrafish is a vertebrate model widely used not only in ecotoxicology but also to study complex human brain disorders (Kalueff et al., 2014), our results emphasize the need of further studies addressing the potential risks of low-level exposures on human CNS.

## CRediT authorship contribution statement

**Melissa Faria:** Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Formal analysis. **Juliette Bedrossiantz:** Methodology, Validation, Investigation, Visualization. **Jonathan Ricardo Rosas Ramírez:** Investigation. **Marta Mayol:** Investigation. **Gerardo Heredia García:** Investigation. **Marina Bellot:** Investigation. **Eva Prats:** Investigation, Writing - original draft, Writing - review & editing, Resources. **Natàlia Garcia-Reyero:** Conceptualization, Resources, Writing - original draft, Writing - review & editing. **Cristian Gómez-Canela:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing. **Leobardo Manuel Gómez-Oliván:** Conceptualization, Resources, Supervision. **Demetrio Raldúa:** Supervision, Conceptualization, Formal analysis, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This work was supported by the Spanish Government with FEDER Funds (CTM2017-83242-R; D.R.) and the network of recognized research groups by the Catalan Government (2017 SGR_902). J.B. was supported by a Spanish fellowship PRE2018-083513. Mention of specific products or trade names does not indicate endorsement by the US federal government.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106253.

## References

Aitbali, Y., Ba-M'hamed, S., Elhidar, N., Nafis, A., Soraa, N., Bennis, M., 2018. Glyphosate based- herbicide exposure affects gut microbiota, anxiety and depression-like behaviors in mice. Neurotoxicol. Teratol. 67, 44–49. https://doi.org/10.1016/j.ntt.2018.04.002.

Annett, R., Habibi, H.R., Hontela, A., 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. J. Appl. Toxicol. 34, 458–479.

Authority, E.F.S., 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA J. 13, 4302.

Babin, P.P.J., Goizet, C., Raldúa, D., 2014. Zebrafish models of human motor neuron diseases: advantages and limitations. Prog. Neurobiol. 118, 36–58.

Bai, S.H., Ogbourne, S.M., 2016. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ. Sci. Pollut. Res. 23, 18988–19001.

Battaglin, W.A., Meyer, M.T., Kuivila, K.M., Dietze, J.E., 2014. Glyphosate and its degradation product AMPA occur frequently and widely in US soils, surface water, groundwater, and precipitation. JAWRA J. Am. Water Resour. Assoc. 50, 275–290.

Bridi, D., Altenhofen, S., Gonzalez, J.B., Reolon, G.K., Bonan, C.D., 2017. Glyphosate and Roundup® alter morphology and behavior in zebrafish. Toxicology 392, 32–39.

Brunello, N., Blier, P., Judd, L.L., Mendlewicz, J., Nelson, C.J., Souery, D., Zohar, J., Racagni, G., 2003. Noradrenaline in mood and anxiety disorders: Basic and clinical studies. Int. Clin. Psychopharmacol. https://doi.org/10.1097/00004850-200307000-00001.

Casarejos, M.J., Perucho, J., Gomez, A., Muñoz, M.P., Fernandez-Estevez, M., Sagredo, O., Fernandez Ruiz, J., Guzman, M., De Yebenes, J.G., Mena, M.A., 2013. Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. J. Alzheimer's Dis. 35, 525–539. https://doi.org/10.3233/JAD-130050.

Cattani, D., Cavalli, V.L. de L.O., Rieg, C.E.H., Domingues, J.T., Dal-Cim, T., Tasca, C.I., Silva, F.R.M.B., Zamoner, A., 2014. Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: involvement of glutamate excitotoxicity. Toxicology 320, 34–45.

Cattani, D., Cesconetto, P.A., Tavares, M.K., Parisotto, E.B., De Oliveira, P.A., Rieg, C.E.H., Leite, M.C., Prediger, R.D.S., Wendt, N.C., Razzera, G., 2017. Developmental exposure to glyphosate-based herbicide and depressive-like behavior in adult offspring: implication of glutamate excitotoxicity and oxidative stress. Toxicology 387, 67–80.

da Costa Chaulet, F., de Alcantara Barcellos, H.H., Fior, D., Pompermaier, A., Koakoski, G., da Rosa, J.G.S., Fagundes, M., Barcellos, L.J.G., 2019. Glyphosate-and fipronil-based agrochemicals and their mixtures change zebrafish behavior. Arch. Environ. Contam. Toxicol. 1–9.

de Souza, J.S., Laureano-Melo, R., Herai, R.H., da Conceição, R.R., Oliveira, K.C., da Silva, I.D.C.G., Dias-da-Silva, M.R., Romano, R.M., Romano, M.A., de Barros Maciel, R.M., 2019. Maternal glyphosate-based herbicide exposure alters antioxidant-related genes in the brain and serum metabolites of male rat offspring. Neurotoxicology.

Edge, C.B., Gahl, M.K., Thompson, D.G., Houlahan, J.E., 2013. Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and Batrachochytrium dendrobatidis. Sci. Total Environ. 444, 145–152.

EPA, U.S., n.d. Re-registration Eligibility Decision (RED) Glyphosate. EPA-738-R-93-014. Washington, DC: US Environmental Protection Agency, Office.

Faria, M., Bedrossiantz, J., Prats, E., Garcia, X.R., Gómez-Canela, C., Piña, B., Raldúa, D., 2019a. Deciphering the mode of action of pollutants impairing the fish larvae escape response with the vibrational startle response assay. Sci. Total Environ. 672, 121–128.

Faria, Melissa, Fuertes, I., Prats, E., Abad, J.L., Padrós, F., Gomez-Canela, C., Casas, J., Estevez, J., Vilanova, E., Piña, B., Raldúa, D., 2018. Analysis of the neurotoxic effects of neuropathic organophosphorus compounds in adult zebrafish. Sci. Rep. 8, 4844. https://doi.org/10.1038/s41598-018-22977-4.

Faria, M., Garcia-Reyero, N., Padrós, F., Babin, P.J., Sebastián, D., Cachot, J., Prats, E., Arick Ii, M., Rial, E., Knoll-Gellida, A., Mathieu, G., Le Bihanic, F., Escalon, B.L., Zorzano, A., Soares, A.M.V.M., Ralduá, D., 2015. Zebrafish models for human acute organophosphorus poisoning. Sci. Rep. 5 https://doi.org/10.1038/srep15591.

Faria, M., Valls, A., Prats, E., Bedrossiantz, J., Orozco, M., Porta, J.M., Gómez-Oliván, L.M., Raldúa, D., 2019b. Further characterization of the zebrafish model of acrylamide acute neurotoxicity: gait abnormalities and oxidative stress. Sci. Rep. 9, 1–7.

Faria, M., Wu, X., Luja-Mondragón, M., Prats, E., Gómez-Oliván, L.M., Piña, B., Raldúa, D., 2020. Screening anti-predator behaviour in fish larvae exposed to environmental pollutants. Sci. Total Environ. 714, 136759 https://doi.org/10.1016/j.scitotenv.2020.136759.

Faria, M., Ziv, T., Gómez-Canela, C., Ben-Lulu, S., Prats, E., Novoa-Luna, K.A., Admon, A., Piña, B., Tauler, R., Gómez-Oliván, L.M., Raldúa, D., 2018. Acrylamide acute neurotoxicity in adult zebrafish. Sci. Rep. 8 https://doi.org/10.1038/s41598-018-26343-2.

Filippi, A., Mahler, J., Schweitzer, J., Driever, W., 2010. Expression of the paralogous tyrosine hydroxylase encoding genes th1 and th2 reveals the full complement of dopaminergic and noradrenergic neurons in zebrafish larval and juvenile brain. J. Comp. Neurol. 518, 423–438. https://doi.org/10.1002/cne.22213.

Friard, O., Gamba, M., 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol. Evol. 7, 1325–1330.

Gallegos, C.E., Baier, C.J., Bartos, M., Bras, C., Domínguez, S., Mónaco, N., Gumilar, F., Giménez, M.S., Minetti, A., 2018. Perinatal glyphosate-based herbicide exposure in rats alters brain antioxidant status, glutamate and acetylcholine metabolism and affects recognition memory. Neurotox. Res. 34, 363–374.

Godefroy, F., Bassant, M.H., Weil-Fugazza, J., Lamour, Y., 1989. Age-related changes in dopaminergic and serotonergic indices in the rat forebrain. Neurobiol. Aging 10, 187–190. https://doi.org/10.1016/0197-4580(89)90029-8.

Gordon, J.A., Hen, R., 2004. The serotonergic system and anxiety. NeuroMolecular Med. 5, 27–40. https://doi.org/10.1385/nmm:5:1:027.

Guyton, K.Z., Loomis, D., Grosse, Y., El Ghissassi, F., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Mattock, H., Straif, K., 2015. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. Lancet Oncol. 16, 490–491.

Hamilton, T.J., Morrill, A., Lucas, K., Gallup, J., Harris, M., Healey, M., Pitman, T., Schalomon, M., Digweed, S., Tresguerres, M., 2017. Establishing zebrafish as a model to study the anxiolytic effects of scopolamine. Sci. Rep. 7, 1–9.

Hermida-Ameijeiras, Á., Méndez-Álvarez, E., Sánchez-Iglesias, S., Sanmartín-Suárez, C., Soto-Otero, R., 2004. Autoxidation and MAO-mediated metabolism of dopamine as a potential cause of oxidative stress: role of ferrous and ferric ions. Neurochem. Int. 45, 103–116. https://doi.org/10.1016/j.neuint.2003.11.018.

Horzmann, K.A., Freeman, J.L., 2016. Zebrafish get connected: investigating neurotransmission targets and alterations in chemical toxicity. Toxics 4, 19.

Joerss, H., Apel, C., Ebinghaus, R., 2019. Science of the Total Environment Emerging per- and poly fl uoroalkyl substances (PFASs) in surface water and sediment of the North and Baltic Seas. Sci. Total Environ. 686, 360–369. https://doi.org/10.1016/j.scitotenv.2019.05.363.

Johnson, A., Hamilton, T.J., 2017. Modafinil decreases anxiety-like behaviour in zebrafish. PeerJ 5, e2994.

Kalueff, A.V., Stewart, A.M., Gerlai, R., 2014. Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol. Sci. 35, 63–75.

Kellner, M., Porseryd, T., Hallgren, S., Porsch-Hällström, I., Hansen, S.H., Olsén, K.H., 2016. Waterborne citalopram has anxiolytic effects and increases locomotor activity in the three-spine stickleback (Gasterosteus aculeatus). Aquat. Toxicol. 173, 19–28.

Kysil, E.V., Meshalkina, D.A., Frick, E.E., Echevarria, D.J., Rosemberg, D.B., Maximino, C., Lima, M.G., Abreu, M.S., Giacomini, A.C., Barcellos, L.J.G., 2017. Comparative analyses of zebrafish anxiety-like behavior using conflict-based novelty tests. Zebrafish 14, 197–208.

Kyzar, E., Stewart, A.M., Landsman, S., Collins, C., Gebhardt, M., Robinson, K., Kalueff, A.V., 2013. Behavioral effects of bidirectional modulators of brain monoamines reserpine and d-amphetamine in zebrafish. Brain Res. 1527, 108–116. https://doi.org/10.1016/j.brainres.2013.06.033.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 C T method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Martínez, M.-A., Ares, I., Rodríguez, J.-L., Martínez, M., Martínez-Larrañaga, M.-R., Anadón, A., 2018. Neurotransmitter changes in rat brain regions following glyphosate exposure. Environ. Res. 161, 212–219.

Maximino, C., deOliveira, D.L., Rosemberg, D.B., Batista, E.J. de O., Herculano, A.M., Oliveira, K.R.M., Benzecry, R., Blaser, R., 2012. A comparison of the light/dark and novel tank tests in zebrafish. Behaviour 149, 1099–1123.

Maximino, C., Puty, B., Benzecry, R., Araújo, J., Lima, M.G., Batista, E.J. de O., de Matos Oliveira, K.R., Crespo-Lopez, M.E., Herculano, A.M., 2013. Role of serotonin in zebrafish (Danio rerio) anxiety: relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and para-chlorophenylalanine (pCPA) in two behavioral models. Neuropharmacology 71, 83–97.

Mayol-Cabré, M., Prats, E., Raldúa, D., Gómez-Canela, C., 2020. Characterization of monoaminergic neurochemicals in the different brain regions of adult zebrafish. Sci. Total Environ. 141205 https://doi.org/10.1016/j.scitotenv.2020.141205.

Meshalkina, D.A., Kysil, E.V., Warnick, J.E., Demin, K.A., Kalueff, A.V., 2017. Adult zebrafish in CNS disease modeling: a tank that's half-full, not half-empty, and still filling. Lab Anim. (NY) 46, 378–387.

Moratalla, R., Khairnar, A., Simola, N., Granado, N., García-Montes, J.R., Porceddu, P.F., Tizabi, Y., Costa, G., Morelli, M., 2017. Amphetamine-related drugs neurotoxicity in

humans and in experimental animals: Main mechanisms. Prog. Neurobiol. 155, 149–170. https://doi.org/10.1016/j.pneurobio.2015.09.011.
Motta, E.V.S., Raymann, K., Moran, N.A., 2018. Glyphosate perturbs the gut microbiota of honey bees. Proc. Natl. Acad. Sci. U. S. A. 115, 10305–10310. https://doi.org/10.1073/pnas.1803880115.
Mueller, T., Dong, Z., Berberoglu, M.A., Guo, S., 2011. The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei). Brain Res. 1381, 95–105. https://doi.org/10.1016/j.brainres.2010.12.089.
Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., 2016. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environ. Heal. 15, 19.
Pereira, A.G., Jaramillo, M.L., Remor, A.P., Latini, A., Davico, C.E., da Silva, M.L., Müller, Y.M.R., Ammar, D., Nazari, E.M., 2018. Low-concentration exposure to glyphosate-based herbicide modulates the complexes of the mitochondrial respiratory chain and induces mitochondrial hyperpolarization in the Danio rerio brain. Chemosphere 209, 353–362.
Pohl, H.R., Wohlers, D., Chou, C.-H., Citra, M.J., Fay, M., Coley, C., Harper, C., Ingerman, L., Buser, M.C., Ingber, S.Z., 2019. Toxiclogical profile for glyphosate: draft for public comment.
Pu, Y., Yang, J., Chang, L., Qu, Y., Wang, S., Zhang, K., Xiong, Z., Zhang, J., Tan, Y., Wang, X., Fujita, Y., Ishima, T., Wang, D., Hwang, S.H., Hammock, B.D., Hashimoto, K., 2020. Maternal glyphosate exposure causes autism-like behaviors in offspring through increased expression of soluble epoxide hydrolase. Proc. Natl. Acad. Sci. U. S. A. 117, 11753–11759. https://doi.org/10.1073/pnas.1922287117.
Reis, F.L.V., Masson, S., De Oliveira, A.R., Brandão, M.L., 2004. Dopaminergic mechanisms in the conditioned and unconditioned fear as assessed by the two-way avoidance and light switch-off tests. Pharmacol. Biochem. Behav. 79, 359–365. https://doi.org/10.1016/j.pbb.2004.08.006.
Roffler-Tarlov, S., Sharman, D.F., Tegerdine, P., 1971. 3,4-Dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the mouse striatum: a reflection of intra- and extra-neuronal metabolism of dopamine? Br. J. Pharmacol. 42, 343–351. https://doi.org/10.1111/j.1476-5381.1971.tb07118.x.
Roy, N.M., Carneiro, B., Ochs, J., 2016. Glyphosate induces neurotoxicity in zebrafish. Environ. Toxicol. Pharmacol. 42, 45–54.
Steinrücken, H.C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94, 1207–1212.
Tingaud-Sequeira, A., Raldúa, D., Lavie, J., Mathieu, G., Bordier, M., Knoll-Gellida, A., Rambeau, P., Coupry, I., André, M., Malm, E., Möller, C., Andreasson, S., Rendtorff, N.D., Tranebjærg, L., Koenig, M., Lacombe, D., Goizet, C., Babin, P.J., 2017. Functional validation of ABHD12 mutations in the neurodegenerative disease PHARC. Neurobiol. Dis. 98 https://doi.org/10.1016/j.nbd.2016.11.008.
Vandenberg, L.N., Blumberg, B., Antoniou, M.N., Benbrook, C.M., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., 2017. Is it time to reassess current safety standards for glyphosate-based herbicides? J Epidemiol. Community Heal. 71, 613–618.
Vencill, W.K., 2002. Herbicide Handbook. Weed Science Society of America.
Wang, Y., Li, S., Liu, W., Wang, F., Hu, L.-F., Zhong, Z., Wang, H., Liu, C.-F., 2016. Vesicular monoamine transporter 2 (Vmat2) knockdown elicits anxiety-like behavior in zebrafish. Biochem. Biophys. Res. Commun. 470, 792–797.
Webster, T.M.U., Santos, E.M., 2015. Global transcriptomic profiling demonstrates induction of oxidative stress and of compensatory cellular stress responses in brown trout exposed to glyphosate and Roundup. BMC Genomics 16, 32.
Westerink, B.H.C., 1985. Sequence and significance of dopamine metabolism in the rat brain. Neurochem. Int. 7, 221–227. https://doi.org/10.1016/0197-0186(85)90108-1.
White, C.C., Viernes, H., Krejsa, C.M., Botta, D., Kavanagh, T.J., 2003. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. Anal. Biochem. 318, 175–180. https://doi.org/10.1016/S0003-2697(03)00143-X.
Yamamoto, K., Ruuskanen, J.O., Wullimann, M.F., Vernier, P., 2010. Two tyrosine hydroxylase genes in vertebrates. New dopaminergic territories revealed in the zebrafish brain. Mol. Cell. Neurosci. 43, 394–402. https://doi.org/10.1016/j.mcn.2010.01.006.
Zweifel, L.S., Fadok, J.P., Argilli, E., Garelick, M.G., Jones, G.L., Dickerson, T.M.K., Allen, J.M., Mizumori, S.J.Y., Bonci, A., Palmiter, R.D., 2011. Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety. Nat. Neurosci. 14, 620–628. https://doi.org/10.1038/nn.2808.