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# Environmental concentrations of tire rubber-derived 6PPD-quinone alter CNS function in zebrafish larvae

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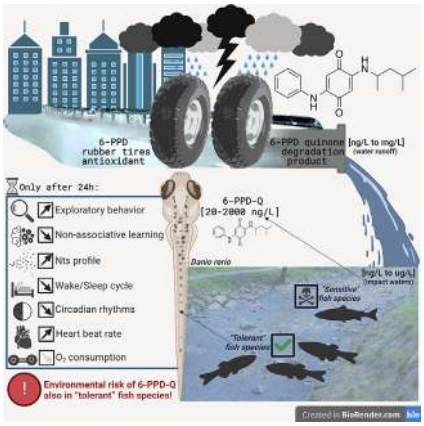
## HIGHLIGHTS

- Environmental levels of 6-PPD quinone impair essential behaviors in zebrafish larvae.
- Environmental levels of 6-PPD quinone impair circadian rhythms in zebrafish larvae.
- Environmental levels of 6-PPD quinone has positive chronotrophy in zebrafish larvae.
- Some of the effects of 6-PPD quinone are non-monotonic.

## GRAPHICAL ABSTRACT

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## ABSTRACT

N-(1,3-Dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine quinone (6PPD-quinone) is a degradation product of 6PPD, an antioxidant widely used in rubber tires. 6PPD-quinone enters aquatic ecosystems through urban stormwater runoff and has been identified as the chemical behind the urban runoff mortality syndrome in coho salmon. However, the available data suggest that the acute effects of 6PPD-quinone are restricted to a few salmonid species and that the environmental levels of this chemical should be safe for most fish. In this study, larvae of a "tolerant" fish species, *Danio rerio*, were exposed to three environmental concentrations of 6PPD-quinone for only 24 h, and the effects on exploratory behavior, escape response, nonassociative learning (habituation), neurotransmitter profile, wake/sleep cycle, circadian rhythm, heart rate and oxygen consumption rate were analyzed. Exposure to the two lowest concentrations of 6PPD-quinone resulted in altered exploratory behavior and habituation, an effect consistent with some of the observed changes in the neurotransmitter profile, including increased levels of acetylcholine, norepinephrine, epinephrine and serotonin. Moreover, exposure to the highest concentration tested altered the wake/sleep cycle and the expression of *per1a*, *per3* and *cry3a*, circadian clock genes involved in the negative feedback loop. Finally, a positive

*Abbreviations:* BLA, basal locomotor activity; CNS, central nervous system; dpf, days postfertilization; ESI, electrospray ionization; IACUC, Institutional Animal Care and Use Committees; LC50, lethal concentration 50; MDL, method detection limit; MQL, method quantification limit; MRM, multiple reaction monitoring; NMCR, nonmonotonic concentration response; MS, mass spectrometry; OCR, oxygen consumption rate; S/N, signal-to-noise ratio; URMS, urban runoff mortality syndrome (URMS); VMR, visual motor response; VSR, vibrational startle response; ZT, Zeitberger time.

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chronotropic effect of 6PPD-quinone was observed in the hearts of the exposed fish. The results of this study emphasize the need for further studies analyzing the effects of 6PPD-quinone in "tolerant" fish species.

## 1. Introduction

N-(1,3-Dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine (6PPD) is a chemical widely used as an antiozonant and antioxidant in rubber tires (Li and Koenig, 2003). As a result of rubber tire abrasion on the road surface, 6PPD enters the urban environment through tire wear particles (TWPs) (Tian et al., 2022). TWPs accumulate on road surfaces, where 6PPD can be oxidized to 6PPD-quinone (Hu et al., 2022). After heavy rainfall, TWPs containing 6PPD-quinone enter aquatic ecosystems in urban stormwater runoff. Levels of 6PPD-quinone in runoff water are commonly in the ng/L to low mg/L range. For instance, levels of 6PPD-quinone in stormwater of 0.086–1.4 μg/L were reported in Canada (Challis et al., 2021), 0.21–2.43 μg/L in China (Cao et al., 2022) and 0.81–20 μg/L in the USA (Tian et al., 2022). Reported levels of 6PPD-quinone in runoff impacting receiving waters are commonly in the ng/L range. Of note, 20–720 ng/L 6PPD-quinone was reported in receiving waters in Canada (Johannessen et al., 2021). However, concentrations up to 3.5 μg/L have been recently found in receiving waters during storm events (Johannessen et al., 2022; Tian et al., 2022).

6PPD-quinone is highly toxic to coho salmon (*Oncorhynchus kisutch*), and it has been identified as the chemical behind urban runoff mortality syndrome (URMS) in this species (Tian et al., 2022). However, the acute toxicity of 6PPD-quinone in fish appears to be species specific. In addition to coho salmon, sensitive species include brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) (Brinkmann et al., 2022). Tolerant species include a wide range of species, including Arctic char (*Salvelinus alpinus*), white sturgeon (*Acipenser transmontanus*), zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) (Brinkmann et al., 2022; Hiki et al., 2021). In these tolerant species, no sign of systemic toxicity has been found even at concentrations approximately 100-fold higher than the coho salmon 24 h LC50. The absence of systemic toxicity after acute exposure in tolerant fish species might suggest that environmental levels of 6PPD-quinone commonly found in aquatic ecosystems are hazardous to only some salmonids but not to other fish species. However, in a recent study, Varshney et al. (2022) reported that exposure for 116 h to 10–20 μg/L 6PPD-quinone, concentrations in the high range of those reported in urban roadway runoff, led to neurotoxic effects, including changes in motor behavior and bradycardia (Varshney et al., 2022). More information is needed to determine whether 6PPD-quinone specifically targets the CNS functions of fish at environmental concentrations.

Fish are expected to be exposed to 6PPD-quinone after urban runoff is diluted in the receiving water. Moreover, the levels of 6PPD-quinone are the highest in urban runoff approximately 14 h after the start of a rain event, remaining at a plateau (low μg/L) for approximately 18 h before returning to basal levels (Johannessen et al., 2022). Considering that the half-life of 6PPD-quinone is approximately 33 h (Hiki et al., 2021), the levels of this chemical should also decrease in the receiving water shortly after a rain event. Therefore, it is necessary to assess the sublethal toxicity of 6PPD-quinone under more realistic conditions: short-term exposure to concentrations in the range of those commonly reported in receiving water.

To this end, 7 days postfertilization (dpf) zebrafish larvae were exposed to 6PPD-quinone using realistic conditions: 24 h of exposure to 20–2000 ng/L 6PPD-quinone, concentrations commonly found in receiving waters during storm events. The neurotoxicity of this compound was initially assessed by analyzing the effects on basal locomotor activity, the visual motor response, the vibrational startle response and habituation, as well as by determining changes in the neurotransmitter profile in larval heads. Moreover, the effects on the circadian rhythm network of the larvae were analyzed. Finally, the effects on the heart rate and oxygen consumption rate were also determined.

## 2. Materials and methods

### 2.1. Fish husbandry and larvae production

Adult wild-type zebrafish were purchased from Pisciber (Barcelona, Spain) and maintained in fish water [reverse-osmosis purified water containing 90 mg/L Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4{\cdot}2H_2O$] at 28 ± 1 °C on a 12 L:12D photoperiod in the CID-CSIC facilities under standard conditions. Embryos were obtained by natural mating and maintained in fish water at 28.5 °C on a 12 L:12 D photoperiod, with lights on at 8 am and off at 8 pm. Larvae were not fed during the experimental period. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Research and Development Center from the Spanish Research Council (CID-CSIC) and conducted under a license from the local government (agreement number 9027).

### 2.2. Experimental concentrations and stability of 6PPD-quinone in fish water

The chemicals and reagents used for the 6PPD-quinone analyses are listed in the Supplementary Methods. A stock solution of 6PPD-quinone was prepared by dissolving 5 mg of standard in 50 mL of HPLC grade ethanol (96 %, Merck Darmstadt, Germany) and stored at 4 °C to minimize degradation (Tian et al., 2022). For the stability test, serial dilutions were prepared from the stock. Spiked fish water was prepared with 6PPD-quinone concentrations of 20, 200, and 2000 ng/L, and the stability of the spiked water was evaluated by direct injection at 0, 6, and 24 h in triplicate. Aliquots of 950 μL of spiked water were transferred to a 2 mL vial to which 50 μL of MeOH had been previously added. All vials were then vortexed for exactly 2 min using a BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, USA).

A 12-point external matrix-matched calibration curve was prepared over a concentration range from 0 (matrix blank) to 10,000 ng/L (0, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, and 10,000 ng/L) by adding the corresponding amount of standard to 950 μL of fish water (final ratio fish water:MeOH, 95:5 (*v*/v)). The linearity of the data was evaluated with a matrix-matched calibration curve constructed between 5 and 10,000 ng/L. Calibration curves were constructed using linear weighted least-squares regression (using 1/*x* as the weighting factor) by plotting the analyte signal to that of its corresponding standard concentration.

Chromatographic separation was carried out with a Waters ACQUITY UPLC system (Waters, Milford, MA) interfaced with a Waters XEVO TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA) operating in positive electrospray ionization (ESI) mode. Mass spectrometry (MS) acquisition was performed in multiple reaction monitoring (MRM) mode by acquiring three MRM transitions per target analyte: 299.2 > 241.1, 299.2 > 215.1, and 299.2 > 187.1. The optimal cone voltage was 40 eV, whereas the collision energies were 30, 30, and 25 eV. The system was controlled by MassLynx 4.1. LC separation was performed with a Waters® ACQUITY UPLC® HSS T3 PREMIER column (100 mm × 2.1 mm i.d., 1.8 μm particle size). The mobile phase (used at a flow rate of 0.4 mL/min) consisted of MeOH with 5 mM ammonium formate and 0.01 % formic acid (solvent A) and water/MeOH (99:1) with 5 mM ammonium formate and 0.01 % formic acid (solvent B). Each chromatographic run was completed in 8 min. The elution gradient was as follows: hold at 5 % A for 0.05 min, increase to 100 % A over 4 min, hold at 100 % A for 2 min, decrease to 5 % A over 1 min, and hold at 5 % A for 1 min. The column temperature was set to 40 °C, the injection volume was 10 μL, and the autosampler temperature was 10 °C. The tuning methods and parameters used for all MS acquisitions were optimized under the conditions described

below. The capillary voltage was set to 3.2 kV (positive), the desolvation temperature was 500 °C, and nitrogen was used as desolvation gas (flow rate 800 L/h) with a cone gas of 150 L/h and a nebulizer pressure of 7 L/h.

To quantify 6PPD-quinone in fish water samples, data were processed using the Waters software QuanLynx tool 4.1 (Waters, Milford, MA). The calibration curve was matrix-matched with fish water in the concentration range of 5 ng to 10,000 ng/L. The $R^2$ value obtained from the calibration curve was 0.9996. The 6PPD-quinone precursor (299.2 *m/z*) was detected at a retention time of 5.15 min. The compound was quantified with the most abundant ion (299.2 > 241.1 m/z) and confirmed by the two minor product ions (299.2 > 215.1 and 299.2 > 187.1). The method detection limit (MDL) and method quantification limit (MQL) were determined as the minimum detectable amount of analyte with a signal-to-noise ratio (S/N) of 3 and 10, respectively, and calculated using the S/N ratio obtained from the matrix-matched calibration curve corresponding to 0.6 and 2 ng/L, respectively.

Quality controls were prepared by spiking fish water and HPLC water at a concentration of 200 ng/L in triplicate and analyzed at each test point (0, 6, and 24 h) throughout the analysis batch experiments. Blanks consisting of HPLC grade water and vials containing only MeOH were also injected during the sample sequence analysis to ensure reliable determination and to confirm the absence of carryover or memory effects.

### 2.3. Experimental protocol

Three concentrations of 6PPD-quinone were tested in this study: 20, 200, and 2000 ng/L. These concentrations were selected because they are in the range of those commonly reported in receiving waters during storm events (Johannessen et al., 2022; Johannessen et al., 2021; Tian et al., 2022). Stock concentrations of 6PPD-quinone were prepared in ethanol, with a final carrier concentration of 0.002 % across all treatments. None of the three selected concentrations caused systemic toxicity (altered gross morphology and/or lethality).

Exposures were conducted in 48-well microplates with 1 larva per well and 1 mL of medium. After 24 h of exposure (from 7 to 8 dpf), the behavior of the larvae was directly evaluated without further manipulation, or larvae were collected for different assays. All exposures were performed in a climatic chamber (POL-EKO APARATURA KK350, Poland) at 28.5 °C on a 12 L:12 D photoperiod. For each assay, samples were collected from 2 to 3 independent exposure experiments.

### 2.4. Behavioral analysis

Basal locomotor activity (BLA), visual motor response (VMR), and vibrational startle response (VSR) assays, as well as the analysis of habituation to a series of vibrational stimuli, were performed and analyzed in a DanioVision system using EthoVision XT 13 software (Noldus, Wageningen, The Netherlands), as previously described (Faria et al., 2021; Faria et al., 2019) (see the Supplementary Methods for additional details).

To study the wake/sleep cycle, 7 dpf larvae were transferred individually to 48-well microplates (1 larva per well) at 11:00 am, and they were acclimated in the DanioVision Observation Chamber under light conditions (1600 lx) for 3 h before starting the experiments. Immediately before starting the experiment, the fish water was replaced by the experimental solutions: solvent control (0.002 % ethanol) or 20, 200 or 2000 ng/L 6PPD-quinone treatments. The locomotor activity was then continuously tracked over a 24 h period, which included three phases matching the photoperiod used for growing the larvae: D1: 6 h of light (lights on from 2 pm to 8 pm, 1600 lx)/N: 12 h of dark (lights off from 8 pm to 8 am)/D2: 6 h of light (lights on from 8 am to 2 pm, 1600 lx). For each day, zeitgeber time 0 (ZT0) corresponds with the time that the lights were switched on, whereas zeitgeber 12 (ZT12) corresponds with the time at which the light was switched off. Therefore, locomotor activity was recorded from ZT6 of 7 dpf larvae to ZT6 of the day are 8 dpf (see Supplementary Fig. S1). These experiments were also performed in a DanioVision system using EthoVision XT 13 software (Noldus, Wageningen, The Netherlands). Locomotor activity (distance moved per hour or period) was determined for each larva. Moreover, the sleep state of the larvae, defined as at least 1 min of immobility (Prober et al., 2006), was also determined as minutes of sleep per hour, number of sleep bouts and the length of the sleep bout. Finally, the sleep latency at night (time between lights off and the first sleep bout (Prober et al., 2006)) was also determined.

### 2.5. Heart rate

The heartbeat activity of the zebrafish larvae, anesthetized with tricaine methanesulfonate (MS222, 17 mg/L), was videorecorded for 30 s with a GigE camera mounted onto a stereomicroscope, as described elsewhere (Bedrossiantz et al., 2023; Faria et al., 2022a) (see the Supplementary Methods for additional details). Analysis of the videos was performed using DanioScope software (Noldus, Wageningen, the Netherlands).

### 2.6. Real-time qPCR

Changes in the expression of some of the most relevant genes of the core circadian rhythm network were confirmed by performing qRT–PCR and further analysis of the obtained data using the ΔΔCT method (see the Supplementary Methods for additional details). The sequences of primers for the nine selected genes (*clock a, clock b, arntl1b, per1a, per1b, per2, per3, cry1a, and cry3a*) are reported in Supplementary Table ST1.

### 2.7. Analysis of neurotransmitters by UHPLC–MS/MS

The chemicals and reagents used for neurotransmitter analysis are listed in the Supplementary Methods. Metabolites of interest were extracted from samples containing pools of 15 heads of control or treated zebrafish larvae as described elsewhere (Gómez-Canela et al., 2018; Prats et al., 2017). Basically, pools of heads were homogenized using a bead mill (TissueLyser LT, Quiagen, Hilden, Germany). Samples were then centrifuged to allow protein precipitation. Finally, the resultant supernatant was filtered through 0.22 μm nylon filters and kept at −20 °C until ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry UHPLC–MS/MS analysis.

An UHPLC–MS/MS detector was used for metabolite characterization using chromatographic and MS conditions previously reported (Gómez-Canela et al., 2018; Prats et al., 2017). Separation and elution were achieved using a BEH Amide column (suitable for polar compounds such as metabolites). Detection was performed in MRM mode with ESI+ to ensure specificity during detection and quantification. More details about the extraction method and instrumental analysis are described in the Supplementary Methods.

### 2.8. Oxygen consumption ($MO_2$) analysis

The oxygen consumption ($MO_2$) of the control and exposed larvae was determined in a Loligo® Microplate Respirometer System (Loligo Systems, Viborg, Denmark). The experiments were performed in a thermostatized room at 28 ± 1 °C. Each experiment was started with calibration of the system with oxygen-saturated water and oxygen-free water (20 g/L $Na_2SO_3$). Larvae were then transferred to a 24-well glass microplate (well volume: 80 μL) fitted with an oxygen sensor in each well, dispensing one larva per well in oxygen-saturated fish water. The microplate was then sealed with PCR film, and the oxygen concentration in each well was recorded for 90 min and analyzed by MicroResp software version 1.0.5 (Loligo Systems, Viborg, Denmark). The oxygen consumption results are expressed in mg $O_2$/kg/h.

### 2.9. Statistical analysis

Data were analyzed with IBM SPSS v29 (Statistical Package 2010, Chicago, IL) and plotted with GraphPad Prism 8.31 for Windows (GraphPad

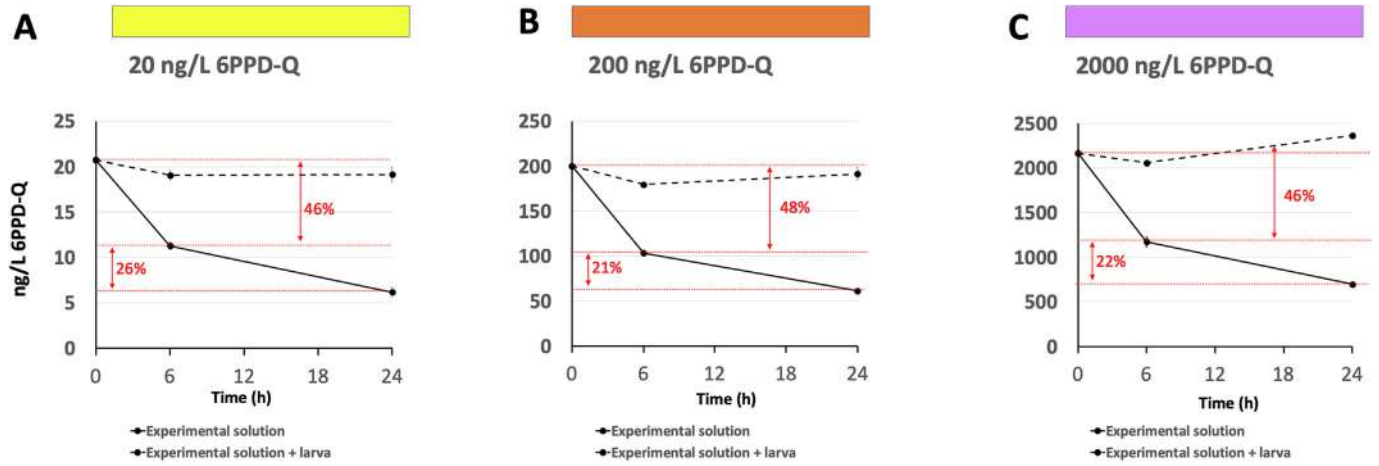



**Fig. 1.** Stability of 6PPD-quinone in fish water. The stability of the 20 ng/L (A), 200 ng/L (B) and 2000 ng/L (C) experimental solutions was determined after 6 and 24 h of incubation at 28 °C with a 12D:12 L photoperiod. The dashed lines show the stability of the solutions over time, while solid show the decrease in 6PPD-quinone during the 24 h of exposure (larva + experimental solution). Percent decreases in the 6PPD-quinone concentration during the first 6 h and the following 18 h are indicated.

Software Inc., La Jolla, CA) and Microsoft Excel for Mac 2011 (v16.69.1; Microsoft Corp., Redmond, WA, USA). Normality was assessed using Shapiro–Wilk tests. For normally distributed groups, Student's *t*-test was used to determine pairwise statistical significance, and one-way ANOVA followed by Dunnett's multiple comparison test was used. When groups did not meet parametric assumptions, the Mann–Whitney *U* test was used to determine pairwise statistical significance, and the Kruskal–Wallis test followed by Dunn-Bonferronis post hoc test was used to test for differences between more than two groups. Data are presented as the mean ± SEM or median and 25-75th percentile of 2–3 independent experiments, unless otherwise stated. A value of $P < 0.05$ was considered to indicate statistical significance.

## 3. Results

### 3.1. Actual concentrations and stability of 6PPD-quinone

The actual concentrations of 6PPD-quinone in the experimental solutions of 20, 200 and 2000 ng/L were 91–106 % of the nominal values (Fig. 1, Supplementary Table S2). In addition, when the stability of 6PPD-quinone was determined under the experimental conditions used in this study (24 h at 28 °C on a 12 L:12D photoperiod), the final concentrations ranged between 92 and 109 % of the initial values (Fig. 1, Supplementary Table S2).

Finally, to estimate the uptake of this chemical by the zebrafish larvae during the exposure time, larvae were exposed to 20, 200 and 2000 ng/L 6PPD-quinone, and the concentrations taken up by the larvae were determined after 6 and 24 h of exposure. Fig. 1 shows that for all three concentrations, a 46–48 % decrease occurred during the first 6 h of exposure and then a 21–25 % decrease was observed during the following 18 h, a result consistent with a first-order kinetics process.

### 3.2. Neurobehavioral effects

The effects of 6PPD-quinone on zebrafish larval behavior were tested by analyzing four experimental paradigms. Fig. 2A shows a nonmonotonic concentration–response (NMCR) relationship between 6PPD-quinone and basal locomotor activity, with larvae exposed to 20–200 ng/L, but not 2000 ng/L, exhibiting an increase in motor activity compared to the controls ($H(3) = 47.211$, $P = 5.1 \times 10^{-10}$). In contrast, 6PPD-quinone had no effects on the visual (Fig. 2B) or vibrationally (Fig. 2C) evoked escape responses in zebrafish larvae. Finally, Fig. 2D shows that a significant NMCR relationship was also found between 6PPD-quinone and habituation to a series of vibrational stimuli ($H(3) = 30.277$, $P = 1.2 \times 10^{-6}$), since the larvae exposed to 20 and 200 ng/L, but not those exposed to 2000 ng/L, habituated earlier to these stimuli than the corresponding controls ($P = 8.1 \times 10^{-5}$, $P = 9.4 \times 10^{-10}$ and $P = 0.34$ for 20, 200 and 2000 ng/L 6PPD-quinone, respectively; Kruskal–Wallis test with the Dunn-Bonferroni post hoc test).

### 3.3. Effects on neurotransmitter profiles

To better understand the behavioral effects induced by exposure to 20–200 ng/L 6PPD-quinone, the effects of this chemical on the neurotransmitter profile were determined in larval heads. As shown in Fig. 2E, 24 h of exposure to 6PPD-quinone resulted in a significant increase in the levels of acetylcholine ($F_{3,28} = 25.544$, $P = 3.6 \times 10^{-8}$), serotonin ($F_{3,27} = 4.519$, $P = 0.011$), norepinephrine ($F_{3,28} = 4.491$, $P = 0.011$), and epinephrine ($F_{3,28} = 13.879$, $P = 9.0 \times 10^{-6}$). However, while all three tested concentrations of 6PPD-quinone resulted in a similar increase in the serotonin content, a NMCR relationship was found between this compound and the acetylcholine, norepinephrine and epinephrine levels. No changes in dopamine, GABA or glycine levels were found in the heads of the exposed larvae compared to the controls.

### 3.4. Circadian rhythm network

The next step was to determine the potential effect of 6PPD-quinone on the sleep-wake cycle in diurnal zebrafish larvae. With this aim, the locomotor activity of the larvae was determined throughout the 24 h exposure period starting at 2 pm, recording a light/dark/light cycle of 6 h (2–8 pm)/12 h (8 pm-8 am)/6 h (8 am-2 pm). As shown in Fig. 3A, larvae from the control and 6PPD-quinone-exposed groups exhibited a similar general profile, with high locomotor activity during the daytime periods (wake behavior) and very low motor activity during the nighttime period (sleep behavior). No significant effects of 6PPD-quinone on locomotor activity were found during the night period (ZT12–24 h). However, 6PPD-quinone exhibited a significant effect on locomotor activity ($F_{3,133} = 3.800$, $P = 0.012$) when the whole daytime period was considered (D1 (ZT6-ZT12 h) + D2 (ZT0–6 h)), with a significant decrease in locomotor activity in larvae exposed to 2000 ng/L 6PPD-quinone (Fig. 3B; $P = 0.014$; one-way ANOVA with Dunnett's test). When the effect of 6PPD-quinone on larval activity was assessed during each daylight period, D1 and D2, the decrease in locomotor activity observed after the highest 6PPD-quinone concentration was significant during D2 only ($H(3) = 8.456$, $P = 0.037$). Although the activity of the larvae exposed to this 6PPD-quinone concentration exhibited a similar trend during D1, the period corresponding to the first 6 h of exposure, this effect was not statistically significant ($H(3) = 6.874$, $P = 0.076$).

To determine if the decrease in locomotor activity observed during the daylight period in larvae exposed for 24 h to 2000 ng/L 6PPD-quinone

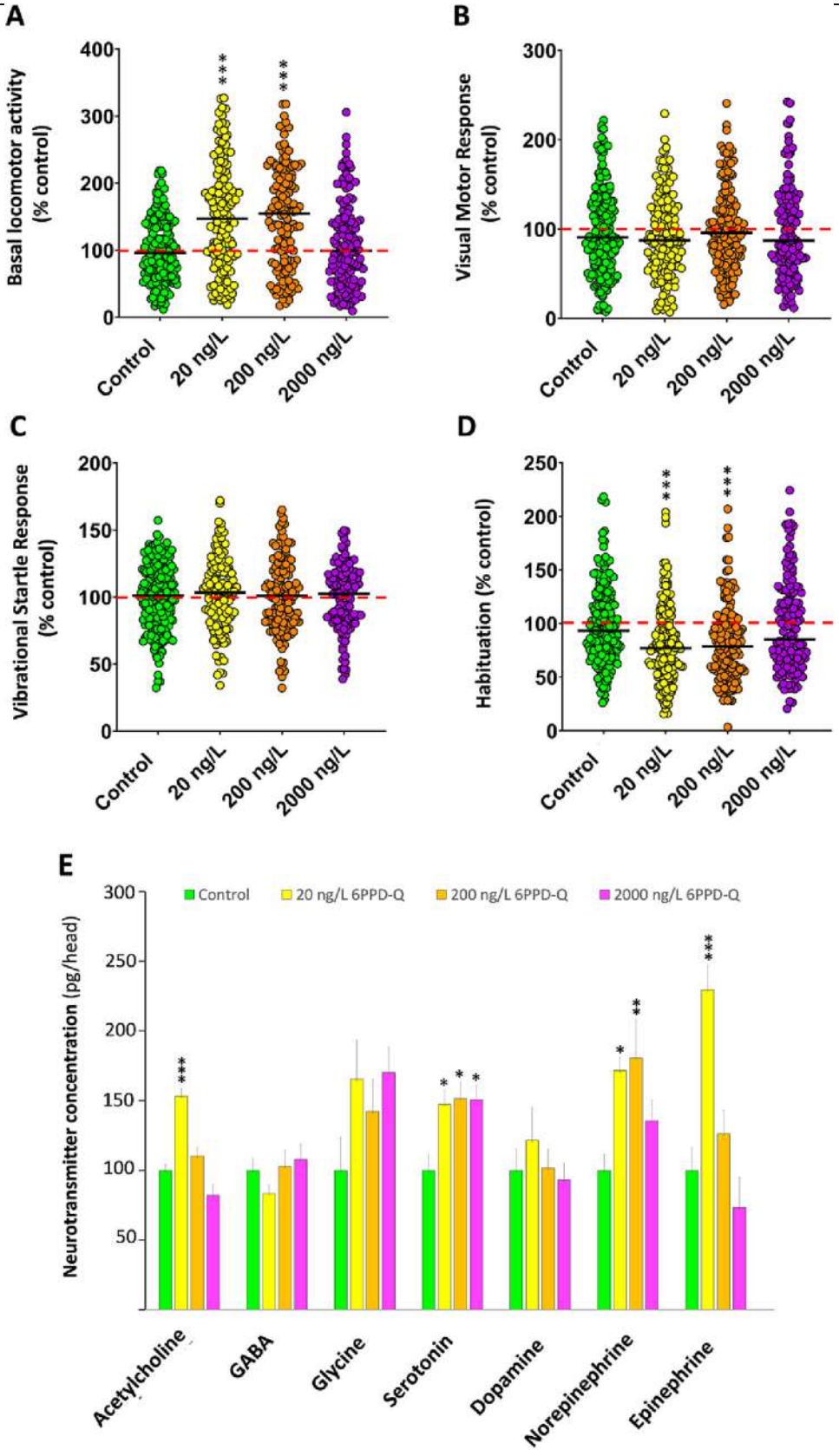
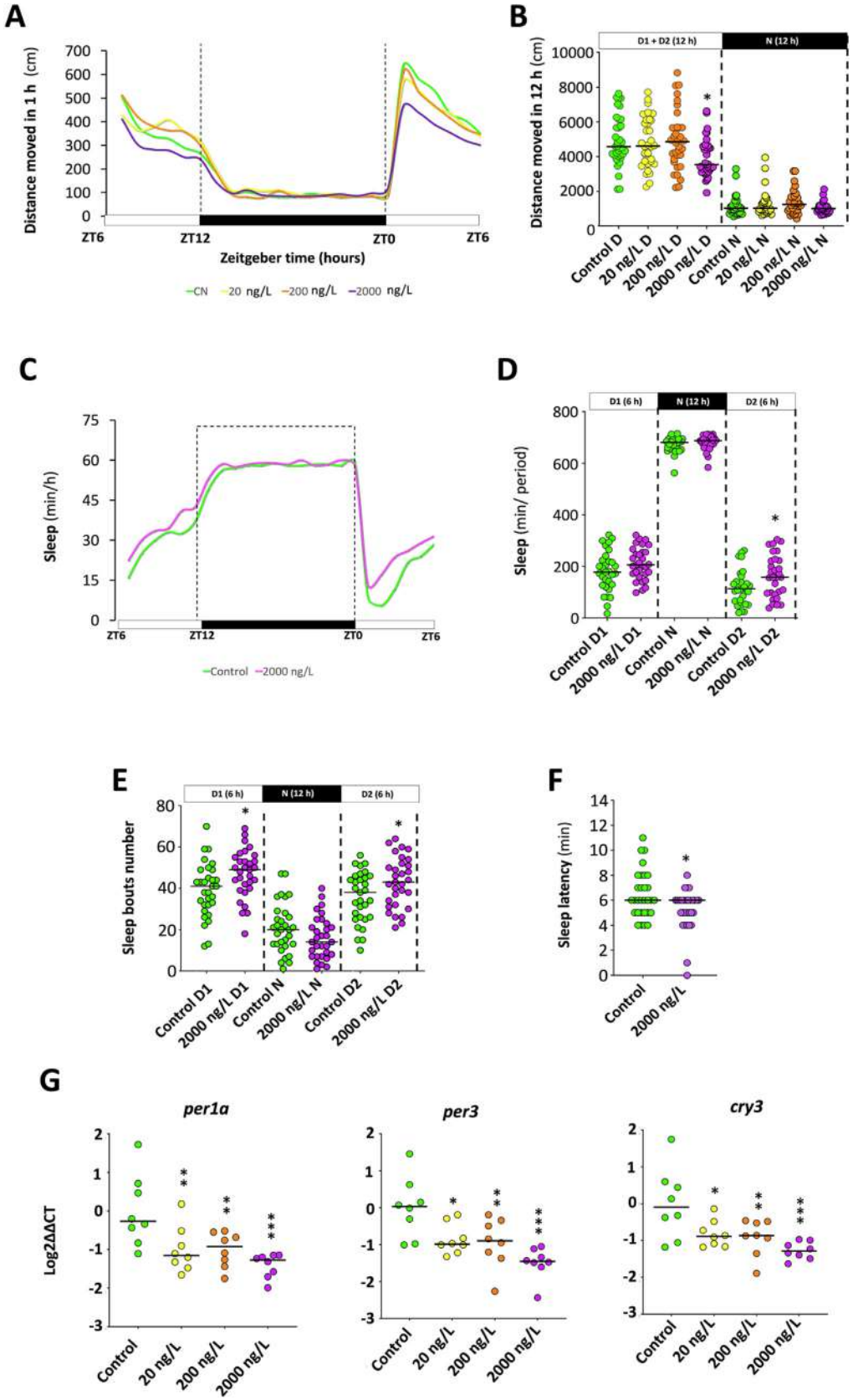



**Fig. 2.** Effects of 24 h of exposure to environmental concentrations of 6PPD-quinone on the behavior and neurotransmitter profile of zebrafish larvae. Behavioral endpoints analyzed include the basal locomotor activity (A), visual motor response (B), vibrational startle response (C) and habituation time to a series of vibrational stimuli (D). The neurotransmitters analyzed in the larval heads (E) include catecholamines (dopamine, norepinephrine, and epinephrine), serotonin, acetylcholine, GABA and glycine. Behavioral data are shown as scatter plots with the median ($n$ = 55–181 for basal locomotor activity, $n$ = 133–188 for visual motor response, $n$ = 60–210 for vibrational startle response, and n = 133–295 for habituation; Kruskal–Wallis test with Bonferroni correction). Neurotransmitter results are presented as the mean ± SE ($n$ = 4–6 pools, 15 heads each; one-way ANOVA with Dunnett's multiple comparison test) $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; data are from 2 to 3 independent experiments.

reflected an effect on the circadian rhythm, the sleep state, including sleep bouts number, sleep bouts length and sleep latency at night, was analyzed (Fig. 3C-F). Exposure to 2000 ng/L 6PPD-quinone led to a significant increase in larval sleep time (min/h) during D2, the period corresponding to the last 6 h of exposure (U($N_{control}$ = 31, $N_{6PPD\text{-}quinone}$ = 31) = 636.000, z = 2.190, $P$ = 0.029; Fig. 3C-D). During D1, however, exposed



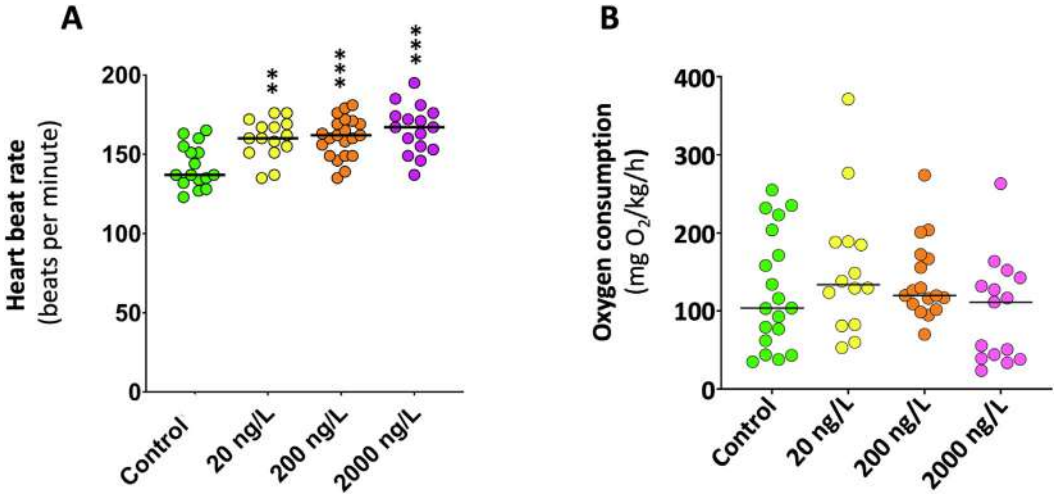



**Fig. 4.** Effects of 24 h of exposure to three environmental concentrations of 6PPD-quinone on the heart rate and oxygen consumption in zebrafish larvae. (A) Effect of 6PPD-quinone on heart beat frequency ($n$ = 15–21; one-way ANOVA with Dunnett's multiple comparison test). (B) Oxygen consumption rate in 8-day post fertilization zebrafish larvae, both control and those exposed for 24 h to 6PPD-quinone ($n$ = 14–19; one-way ANOVA with Dunnett's multiple comparison test). *p < 0.05, **p < 0.01, ***p < 0.001; data are from 2 independent experiments.

larvae exhibited a similar trend (Fig. 3C-D), although without reaching statistical significance ($t(60) = -1.712$, $P = 0.091$). No effect of 6PPD-quinone on sleep time was found during the nighttime period.

The above data show that 18–24 h of exposure to 2000 ng/L 6PPD-quinone leads to a significant decrease in locomotor activity during the daylight period and that this effect parallels an increase in the time larvae spend in the sleep state.

The next step was to determine whether this effect on sleep was related to an increase in the number or length of sleep bouts. As shown in Fig. 3E, a significant increase in the number of sleep bouts was found during both daylight periods (D1: $t(60) = -2.502$, $P = 0.015$; D2: $t(60) = -2.321$, $P = 0.024$). In contrast, no effect on the number of sleep bouts was found during the nighttime period. Interestingly, the time elapsed between lights off and the first sleep bout during the nighttime period (sleep latency, Fig. 3F) was slightly but significantly reduced in exposed larvae (U ($N_{control} = 30$, $N_{6PPD\text{-}quinone} = 30$) = 311.000, z = −2.111, $P = 0.035$). Finally, no changes in sleep bout length were found between control and treated larvae (Supplementary Fig. S2).

The next step was to determine if the observed effects of 6PPD-quinone on locomotor activity and sleep time and number of sleep bouts were related to changes in the circadian clock genes. With this aim, larvae were collected during the D2 period, 3 h after lights on (D2, ZT3), since at this point, the differences in locomotion and sleep status were very consistent. Fig. 3G shows that at ZT3, the expression of *per1a*, *per3* and *cry3a* in 6PPD-quinone-exposed larvae was significantly downregulated (*per1a*: $F_{3,28} = 7.726$, $P = 0.0006$; per3: $F_{3,28} = 8.420$, $P = 0.0004$; *cry3a*: $F_{3,28} = 6.924$, $P = 0.0012$) compared with the control values. This effect of 6PPD-quinone on gene expression was concentration dependent, with the strongest effects found in larvae exposed to 2000 ng/L 6PPD-quinone (Fig. 3G). In contrast, 24 h of exposure to 6PPD-quinone did not lead to any significant effect on the expression of *per1b, per2, cry1a, arntl1b, per3, clock a, and clock b*, although for *clock b*, a clear downregulation trend was observed at the 2000 ng/L concentration (*clock b*: $F_{3,28} = 2.699$, $P = 0.065$).

### 3.5. Heart rate effects

Twenty-four hours of exposure to environmental concentrations of 6PPD-quinone induced a positive chronotropic effect in the heart of the exposed embryos ($F_{3,64} = 9.152$, $P = 4.0 \times 10^{-5}$). As shown in Fig. 4A, this effect was observed in larvae after 24 h of exposure to all of the three selected concentrations.

### 3.6. Oxygen consumption rate

In a recent report, tissue-specific disruption of mitochondrial respiration was proposed as a potential mode of action of 6PPD-quinone, and a significant increase in oxygen consumption was reported in zebrafish embryos exposed to 10–25 μg/L 6PPD-quinone for 96 h (Varshney et al., 2022). Therefore, we determined the oxygen consumption rate (OCR) in control and treated larvae to explore the possibility that the observed neurobehavioral effects were mediated by a previous alteration in mitochondrial respiration. However, as shown in Fig. 4B, no differences in oxygen consumption were found between control larvae and those exposed for 24 h to 20–2000 ng/L 6PPD-quinone.

## 4. Discussion

6PPD-quinone at concentrations commonly found in urban runoff and its receiving waters can lead to urban runoff mortality syndrome (URMS) in coho salmon and other sensitive fish species. However, information on the potential risk of environmental concentrations of 6PPD-quinone on "tolerant" fish species is still very scarce. In this study, 7 dpf zebrafish larvae, a "tolerant" species, were exposed for only 24 h to three concentrations of

**Fig. 3.** Effects of 24 h of exposure to environmental concentrations of 6PPD-quinone on the zebrafish larvae wake/sleep cycle. (A) Locomotor activity of zebrafish larvae, both control and those exposed to the three concentrations of 6PPD-quinone, entrained and tested under 12:12 h light–dark conditions. The period in which activity was recorded, from 7 to 8 dpf, corresponds to the 24 h exposure period. The bars at the bottom of the graph on the left indicate the light/dark/light periods (white bar on the left (D1): 2 pm to 8 pm/black bar in the middle (N): 8 pm to 8 am/white bar on the right (D2): 8 am to 2 pm). The results are presented as the mean ± SE. (B) Total distance moved during the daytime (D1 + D2) and N periods. Data are shown as scatter plots with the median ($n$ = 34–35; one-way ANOVA with Dunnett's multiple comparison test). (C) Time spent by the larvae in the sleep state per hour over 24 h. (D) Time spent by the larvae in the sleep state during D1 (6 h), N (12*h*), and D2 (6 h). Data are shown as scatter plots with the median (D1: $n$ = 31, Student's $t$-test; N and D2: n = 31, Mann–Whitney $U$ test). (E) Number of sleep bouts during the D1, N, and D2 periods (n = 31, Student's $t$-test). (F) Sleep latency at night ($n$ = 30, Mann–Whitney U test). (G) 6PPD-quinone leads to significant changes in the expression of the circadian clock genes *per1a, per3* and *cry3*. Log2 ΔΔCT values are shown as a scatter plot with the median ($n$ = 8 pools of 9 larvae; one-way ANOVA with Dunnett's multiple comparison test). *p < 0.05, **p < 0.01, ***p < 0.001; data are from 2 independent experiments.

6PPD-quinone in a range similar to those reported in urban runoff receiving waters, and significant effects on both locomotor activity and cognition were found. First, an NMCR relationship between 6PPD-quinone concentration and BLA was found, with larvae exposed to lower 6PPD-quinone concentrations (20–200 ng/L) showing a significant increase in BLA, a behavioral endpoint closely related to the exploratory behavior of larvae (Altenhofen et al., 2019). The NMCR relationship, also known as hormesis, is considered a highly conserved evolutionary adaptive strategy among living organisms for developing resilience (Agathokleous, 2022). Recently, we reported a hormetic effect between carbaryl and fenitrothion concentrations and BLA in zebrafish larvae (Bedrossiantz et al., 2023). Changes in larval locomotor activity, when unnecessary, can be ecologically relevant, as an increase in larval locomotor activity has a high energetic cost and increases the chances of attracting the attention of predators (Burgess and Granato, 2007). A previous study (Varshney et al., 2022) reported a decrease in BLA in zebrafish embryos exposed for 96 h to 10 and 25 μg/L 6PPD-quinone but no effect on those exposed to 1 μg/L. Despite the evident differences with our experimental design, the results presented by Varshney et al. (2022) are consistent with the NMCR relationship between the 6PPD-quinone concentration and BLA found in this study. In fact, if the results of both studies were analyzed together, the effect of 6PPD-quinone would show an increase in BLA at the lowest concentrations (20 and 200 ng/L, present study), no effect at the middle concentrations (1 μg/L, in (Varshney et al., 2022), and 2 μg/L, in the present study) and a decrease at the highest concentrations (10 and 25 μg/L, in (Varshney et al., 2022)). Although there is still a general misconception that hormetic effects are always beneficial, the current view is that these effects will be beneficial or detrimental depending on the biological context (Calabrese, 2010; Calabrese, 2011; Calabrese and Baldwin, 1999). The results presented in this manuscript support that the effect of 6PPD-Q on BLA can be detrimental for fish larvae.

6PPD-quinone also had a significant effect on habituation to a series of vibrational stimuli, with those larvae exposed to the two lower concentrations exhibiting faster habituation than the corresponding controls. Habituation is a primitive form of nonassociative learning in which when an animal is faced with a series of new stimuli, the animal responds to the first stimulus, and if this stimulus is not identified as beneficial or harmful, the animal learns to filter it out (Kandel, 1991). Vibrational stimuli in aquatic ecosystems are often associated with predator strikes (Bhattacharyya et al., 2020), so the significant decrease in the habituation time to vibrational stimuli found in the exposed larvae might result in an increased risk of predation, as the risk of filtering harmful stimuli should increase when the time taken to decide if the stimuli are harmful decreases.

Behavioral changes are usually associated with changes in neurotransmitter systems (Horzmann and Freeman, 2016). When the neurotransmitter profile in the heads of control and 6PPD-quinone-exposed larvae was analyzed, significant changes in the levels of acetylcholine, serotonin, norepinephrine and epinephrine were found. Specifically, 6PPD-quinone increased the levels of acetylcholine and epinephrine at only the lowest concentration, norepinephrine at the lowest and middle concentrations, and serotonin at all three concentrations. Different evidence suggests that serotonin is not directly involved in the observed effects on BLA, as and effect from 6PPD-quinone on BLA was not found at all three concentrations and increased serotonin levels have been linked to a decrease, and not an increase, in BLA (Faria et al., 2022b; Faria et al., 2021; Sallinen et al., 2009). Instead, the effect of 6PPD-quinone on BLA might be mediated by the observed increase in the levels of the nonselective adrenergic receptor agonists norepinephrine and epinephrine, since these neurotransmitters have been shown to increase locomotor activity in zebrafish larvae through the activation of the β-adrenergic receptors (Abbas et al., 2021; Basnet et al., 2019) and there is a good correlation between the 6PPD-quinone concentrations modulating behavior and those modulating these catecholamines. Moreover, the increased acetylcholine levels found in larvae exposed to the lowest 6PPD-quinone concentration could also be involved in the observed increase in BLA, as the nicotinic acetylcholine receptor (nAChR) activation has been reported to increase locomotor activity (Kalueff, 2017; Mora-Zamorano et al., 2016). The decrease in the habituation time in larvae exposed to 20 and 200 ng/L 6PPD, however, could be related to the concomitant increases in the norepinephrine and serotonin levels. A similar situation, with concomitant increases in serotonin and norepinephrine levels (Bellot et al., 2021a) and a decrease in habituation time, was previously reported in larvae exposed to deprenyl (Bellot et al., 2021b; Faria et al., 2021; Faria et al., 2019).

A highly relevant target for some neurotoxic pollutants present in aquatic ecosystems is the circadian rhythm. While different steroid hormones, pesticides, industrial chemicals, neuroactive drugs and metals have been recently linked to the disruption of circadian rhythms (Ding et al., 2022; Wei et al., 2022; Yang et al., 2021; Zhao et al., 2016; Zheng et al., 2021), research on the effect of environmental pollutants on circadian rhythms is still in its infancy. In this study, larvae exposed to 2000 ng/L 6PPD-quinone showed a decrease in locomotor activity during the daytime period, reflected as an increase in sleep time during that phase. A similar decrease in daytime activity has been reported in zebrafish larvae after 24 h of exposure to 50 ng/L fluoxetine (Wei et al., 2022) or 120 h of exposure to 386 μg/L tetrabromobisphenol S (Ding et al., 2022). Considering that control larvae spent almost all the nighttime period in a sleep state, it is not possible to assess whether the observed increase in sleep time is specific to the daytime period. The rapid effect of 6PPD-quinone on the sleep state parallels the fast decrease in 6PPD levels observed in the experimental solution during the same period when the larvae were exposed, strongly suggesting that this decrease is indirect evidence of 6PPD-quinone uptake by the larvae.

To be certain that the effect on larval motor activity is due to an altered circadian rhythm, larvae entrained with a light:dark cycle should be monitored in the absence of any circadian stimulus (Zhdanova, 2011). In this study, however, the locomotor activity of zebrafish larvae entrained on a 12 light:12 dark cycle was monitored using a similar light:dark cycle as the circadian cue. There is different evidence supporting that the decrease in locomotor activity found during the daytime period is directly related to an effect on circadian rhythms (Chiu and Prober, 2013). First, increases in sleep time and number of bouts were observed during the daytime period in larvae exposed to 6PPD-quinone. Moreover, the expression of the circadian clock genes *per1a*, *per3* and *cry3a* was altered in larvae exposed for 24 h to this chemical. Circadian rhythms are generated by a transcription-translation feedback loop with positive (Clock and Bmal) and negative (Per and Cry) regulators. The Clock/Bmal complex binds *E*-box elements in the promoters of the *per* and *cry* genes, inducing their expression. Once translated, the Per and Cry proteins heterodimerize and translocate to the nucleus to repress the transcriptional activation induced by this complex and repress their own transcription to close the loop (Sacksteder and Kimmey, 2022). Therefore, the observed effects of 6PPD-quinone on the expression of *per1a*, *per3* and *cry3a*, all three of which are key components of the negative regulators of the circadian transcription-translation feedback loop, strongly suggest a link between increased sleep time and the down-regulation of the expression of these circadian genes. Considering the relevance of circadian rhythms in controlling numerous molecular, physiological and neurobehavioral events (Yang et al., 2021; Zheng et al., 2021), additional efforts should be made to better characterize the effect of 6PPD-quinone on circadian rhythms.

Zebrafish heart rate seems to be controlled by adrenergic, cholinergic and serotonergic systems (Stoyek et al., 2017), the same neurotransmitter systems found altered in zebrafish larvae exposed to 6PPD-quinone. In fact, the positive chronotropic effect of 6PPD-quinone on the zebrafish heart is consistent with increases in the levels of norepinephrine, epinephrine, acetylcholine or serotonin (Stewart et al., 2013; Stoyek et al., 2017). However, considering that this positive chronotropic effect was found at all three tested concentrations, serotonin is the most suitable candidate to lead this effect, as its levels increase in the same range of concentrations. A negative chronotropic effect of 10 and 25 μg/L 6PPD-quinone has been reported on the hearts of 116 hpf zebrafish larvae after 96 h of exposure (Varshney et al., 2022). Differences in the experimental design (96 h of exposure to higher concentrations during early development vs. 24 h of exposure at the late larval stage), potential differences in the expression pattern of the receptors in the heart between 116 vs. 192 hpf larvae, or again, a nonmonotonic

concentration–response relationship (Agathokleous, 2022) could explain these differences in heart rate.

Whereas the mode of action of 6PPD-quinone is still unclear, mitochondrial function has been recently suggested as a potential target (Mahoney et al., 2022). The absence of effects on the oxygen consumption rate in larvae exposed to 6PPD-quinone in this study suggests that mitochondrial dysfunction is not involved in any of the neurobehavioral effects described here.

The results presented in this manuscript show that short-term exposure to levels of 6PPD-quinone commonly found in runoff or receiving waters leads to changes in essential behaviors, the neurotransmitter profile, the wake/sleep cycle, circadian rhythms, and heart rate in zebrafish larvae. Although these changes are not lethal under laboratory conditions, most of them could be lethal in nature. Therefore, the results provided here strongly suggest that 6PPD-quinone also endangers "tolerant" fish species and emphasize the need for further studies, mainly those that are mechanistic in nature, to identify the mode of action of this compound leading to its neurotoxic effects.

## CRediT authorship contribution statement

**Marina Ricarte:** Investigation, Data curation, Writing – review & editing. **Eva Prats:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Nicola Montemurro:** Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Juliette Bedrossiantz:** Data curation, Methodology, Writing – review & editing. **Marina Bellot:** Data curation, Formal analysis, Writing – review & editing. **Cristian Gómez-Canela:** Data curation, Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Demetrio Raldúa:** Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

## Data availability

Data will be made available on request.

## Declaration of competing interest

The authors declare no competing interests.

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## Appendix A. Supplementary data

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