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RESEARCH ARTICLE

Environmental enrichment modulates the response to chronic stress in zebrafish

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ABSTRACT

Several studies have shown that manipulations to the housing environment modulate susceptibility to stress in laboratory animals, mainly in rodents. Environmental enrichment (EE) is one such manipulation that promotes neuroprotection and neurogenesis, besides affecting behaviors such as drug self-administration. Zebrafish are a popular and useful animal model for behavioral neuroscience studies; however, studies evaluating the impact of housing conditions in this species are scarce. In this study, we verified the effects of EE on behavioral (novel tank test) and biochemical [cortisol and reactive oxygen species (ROS)] parameters in zebrafish submitted to unpredictable chronic stress (UCS). Consistent with our previous findings, UCS increased anxiety-like behavior, cortisol and ROS levels in zebrafish. EE for 21 or 28 days attenuated the effects induced by UCS on behavior and cortisol, and prevented the effects on ROS levels. Our findings reinforce the idea that EE exerts neuromodulatory effects across species, reducing vulnerability to stress and its biochemical impact. Also, these results indicate that zebrafish is a suitable model animal to study the behavioral effects and neurobiological mechanisms related to EE.

KEY WORDS: Neuromodulation, Behavior, Oxidative stress, Cortisol

INTRODUCTION

Recently, much effort has been made to clarify the effects of environmental enrichment (EE) and to investigate which mechanisms are involved (Young et al., 1999; Rampon et al., 2000; van Praag et al., 2000; Lazarov et al., 2005; Artola et al., 2006; Nithianantharajah and Hannan, 2006; Pang and Hannan, 2013). EE consists of interventions in the housing environment that contribute to improving the welfare of laboratory animals and attempts to resemble their family environment. It provides animals with greater social interaction and exposure to sensory stimuli (visual, motor, cognitive and somatosensory), stimulating several brain regions (Nithianantharajah and Hannan, 2006; Crofton et al., 2015). EE promotes neuroprotection and neurogenesis, and affects behaviors such as drug self-administration and response to stress (Bardo et al., 2001; Green et al., 2003, 2010; Chauvet et al., 2009; El Rawas et al., 2009; Solinas et al., 2009; Stairs and Bardo, 2009).

Evidence suggests that EE has neuromodulatory effects in brain reward circuits, mainly in the mesolimbic dopamine system (Bezard et al., 2003; Zhu et al., 2005; Solinas et al., 2008). Several studies in rodents demonstrated that EE improves hippocampus cytoskeleton, cell proliferation, survival of newborn cells, and increases the number and proportion of post-mitotic immature neurons and dendritic arborization (Bennett et al., 1964; Diamond et al., 1964; Tanti et al., 2013), along with increasing cell viability and glucocorticoid receptor expression (Veena et al., 2009; Sampedro-Piquero et al., 2014). EE promotes important changes in the expression of genes related to synaptic plasticity, neuronal signalling, learning and memory (Rampon et al., 2000). A recent study confirmed that EE induces neuroplasticity in the hippocampus and protection against the effects of unpredictable chronic stress (UCS) in mice (Vega-Rivera et al., 2016).

Regarding behavioral effects, some studies in rats submitted to chronic stress have shown that EE improved working memory performance in the T-maze task, and prevented anxiety-like behavior in the elevated plus maze test (Bhagya et al., 2017). EE also prevented depression-like behavior in the sucrose preference test, and partially prevented the anxiogenic-like effect of chronic stress, attenuating the spatial learning difficulty and memory impairment in the radial arm maze in rodents (Shilpa et al., 2017). Interestingly, zebrafish have been shown to prefer EE even when they are reared in barren conditions (Schroeder et al., 2014). In addition, studies with zebrafish revealed that the EE increased brain size (DePasquale et al., 2016) and proliferation of telencephalic cells (von Krogh et al., 2010), decreased anxiety-like behavior and increased exploration (Manuel et al., 2015), as well as blunting the cortisol response to acute stress in both isolated and group-housed zebrafish (Giacomini et al., 2016).

In addition, studies have demonstrated the involvement of EE with changes in oxidative status. EE prevented the increase in reactive oxygen species (ROS) levels, thiobarbituric acid reactive substances (TBARS) and the superoxide dismutase (SOD) activity induced by chronic cerebral hypoperfusion in rats (Cechetti et al., 2012). Another study that investigated free radical levels through 8-hydroxy-2-deoxyguanosine (8-OH-dG), a marker of oxidative stress associated with DNA, reported that EE attenuated the upregulation induced by inescapable foot shocks in the hippocampus and prefrontal cortex of rats (Sun et al., 2016). Zebrafish exposure to chronic stress alters behavior and increases cortisol levels. Cortisol mobilizes energy reserves to deal with

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stressors (Kirschbaum et al., 1993), thus increasing mitochondrial respiration and the production of ROS as a by-product (Gutierrez et al., 2006).

Finally, despite the knowledge regarding the effects of EE in rodents, there are no studies investigating if EE could improve chronic stress-related phenomena in zebrafish. Therefore, considering the effects of EE as a neuromodulatory intervention, we hypothesized that EE-housed zebrafish would be less vulnerable to the effects of UCS. This study verified the effects of EE in zebrafish submitted to UCS on behavioral (novel tank test) and biochemical (trunk cortisol and ROS levels) parameters.

MATERIALS AND METHODS

Animals

A total of 96 adult zebrafish, *Danio rerio* (F. Hamilton 1822), wild-type short fin strain (6 months old, 3–4 cm long; 50:50 male: female ratio) were purchased from Delphis aquariums (Porto Alegre, Brazil). The fish were kept for 15 days in a closed 16 liter acclimation tank system (40×20×24 cm) identical to the experimental tanks (standard condition), with non-chlorinated tap water, well-aerated and a light:dark cycle of 14 h:10 h (lights on at 06:00 h). The tank water was kept at appropriate conditions (pH 7.0±0.3; temperature 26±1°C; total ammonia <0.01 mg l^{-1}; nitrite <0.01 mg l^{-1}; dissolved oxygen 7.0±0.4 mg l^{-1}; alkalinity 22 mg l^{-1} $CaCO_3$ and total hardness 5.8 mg l^{-1}). The fish were fed twice a day with a commercial flake fish food (Alcon Basic, Alcon, Brazil) and the amount of food was calculated based on the number of fish per tank and followed the manufacturer's instructions as well as zebrafish literature (4% of body weight in food per fish per day). All experiments were approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (reference number 30992/2015).

Reagents

2′,7′-Dichlorodihydrofluorescein-diacetate (H_2DCF-DA) and phosphate-buffered saline powder (PBS; pH 7.4) were purchased from Sigma-Aldrich (São Paulo, Brazil), and ethyl alcohol (absolute ethanol) from Merck (Brazil). H_2DCF-DA was dissolved in 1 mmol l^{-1} absolute ethanol and maintained at 4°C. A sachet of powdered PBS was diluted in 1 liter of Milli-Q water and maintained at 4°C, pH 7.4.

Housing conditions

After 15 days of acclimation, fish were randomly assigned to an experimental housing condition: standard condition (ST) or environmental enrichment (EE). The ST housing condition consisted only of a tank with water, heater, filter and aeration system (Fig. 1A). The EE housing condition consisted of a tank with gravel in the bottom (English sea stones, 4–9 mm, 3 cm high from the bottom of the tank), a ruin-like plastic object, and three submerged plastic plants (two 10 cm tall and one 20 cm tall), besides all equipment of the standard housing condition (Fig. 1B). Both types of tank were the same size (16-liter volume; 40×20×24 cm), and a piece of white frosted cardboard (30×60 cm) was placed between the tanks to prevent visual contact of fish from different tanks in the same horizontal plane. Tanks were not covered at the front. For every experimental group there were two identical tanks that were kept in the same room, and all the tanks were run in parallel. To confirm that the variation derived from the individual rather than from the tank, we tested for tank effects by including 'tank' as a factor in our initial statistical analysis. As we observed no main effects or interactions for the factor 'tank' in the ANOVAs, we excluded it as a variable in further analyses, and data from tanks of the same experimental group were pooled together.

Experimental design

The experimental design is shown in Fig. 2. The animals were kept under standard conditions (ST) or environmental enrichment for 21 days (EE21) or 28 days (EE28). In the last 7 days, animals were divided into two sub-cohorts (non-stressed or stressed, respectively: S− and S+). For S+ groups, 24 h after the last stressor animals were individually submitted to the behavioral test and immediately after that were anesthetized by rapid cooling (immersion in water at 2–4°C until unable to swim and cessation of opercular movement). After cessation of opercular movements for 2 min, zebrafish were removed and decapitated. The bodies and head were flash-frozen for whole-body (trunk) cortisol measurement and brain ROS analyses. S− groups were subjected to the same procedures on the 29th day of the experimental protocol.

Unpredictable chronic stress protocol

The UCS protocol followed our previous studies with slight adaptations (Piato et al., 2011; Marcon et al., 2016; Rambo et al., 2017). In this study, we replaced the social isolation stressor by the stress of exposure to a predator fish (*Archocentrus nigrofasciatus*). Stressors were presented randomly twice a day to ensure unpredictability and to avoid habituation over 7 days (Table 1). The stressors used were: (i) heating tank water up to 33°C (30 min); (ii) exposure to the predator (50 min); (iii) cooling tank water to

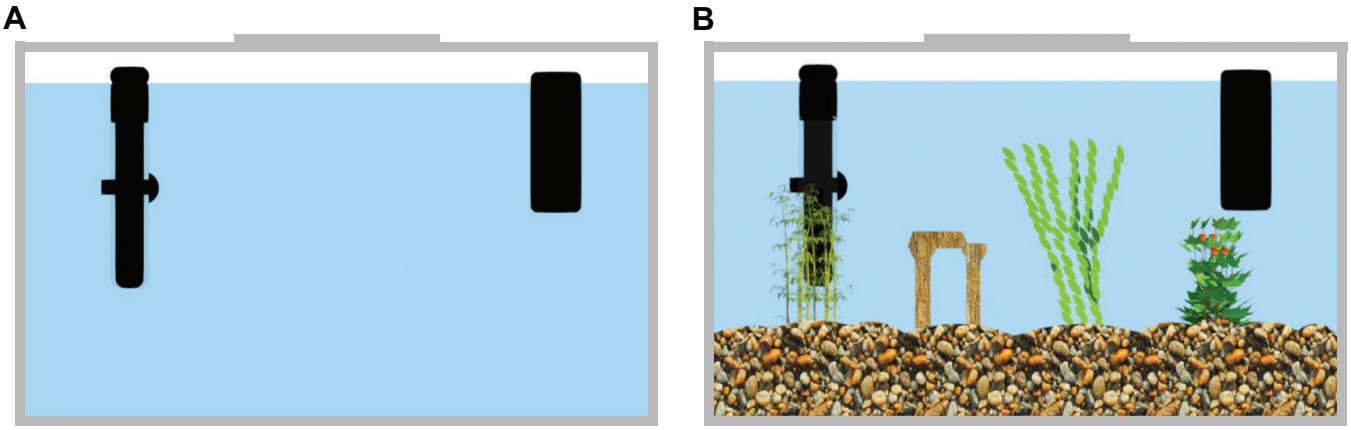

Fig. 1. Housing conditions for zebrafish (*Danio rerio*). (A) Standard condition (ST); (B) environmental enrichment (EE).

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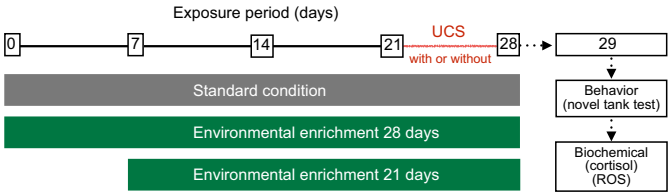

Fig. 2. Experimental design. The fish were exposed to environmental enrichment (EE) for 21 or 28 days or remained unchanged (standard condition). In the last 7 days of the experimental protocol, they were submitted to the unpredictable chronic stress (UCS) protocol or remained unchanged (control). The day after the last stressor, between 08:00 and 11:00 h, the fish were submitted to the novel tank test and euthanized for biochemical analysis (brain and trunk for the dosage of ROS levels and cortisol, respectively).

23°C (30 min); (iv) crowding of 12 animals in a 300 ml beaker (50 min); (v) transferring the animals to another tank with low water level, exposing the dorsal body wall (2 min); (vi) tank change, three consecutive times at 30 min intervals; and (vii) chasing with a net (8 min). The non-stressed (S–) animals were left undisturbed throughout the experiments.

Novel tank test

Twenty-four hours after UCS, the animals were individually submitted to the novel tank test: fish were placed for 6 min in 24×8×20 cm tanks with 15 cm water level. The water in the test apparatus was changed for each group and maintained at 26±1°C. The tanks were virtually divided into three equal horizontal sections (bottom, middle and upper zones). Behavioral tests were video recorded and analyzed with the ANY-Maze tracking software (Stoelting Co., Wood Dale, IL, USA). The following parameters were quantified: total distance traveled, time spent in the bottom and upper zones, and number of transitions to the upper zone (Marcon et al., 2016). Total distance moved was used as an indicator of overall locomotor activity. The time in the bottom zone is used as an indicator of anxiety, while time spent and entries in the upper zone are frequently used to illustrate the effects of anxiolytic interventions. After conducting the behavioral tests, video files were coded and the researcher responsible for behavioral analysis was unaware of the experimental group each animal belonged to.

Cortisol measurement

The extraction and quantification of trunk cortisol were carried out using a commercially available enzyme-linked immunosorbent assay kit (EIAgen Cortisol test, BioChem ImmunoSystems) based on our previous study (Marcon et al., 2016). Briefly, the zebrafish were euthanized and a pool of two zebrafish trunks was minced and 500 mg placed into a disposable stomacher bag with 2 ml of PBS (pH 7.4) for 6 min. Ethyl ether was added and samples were homogenated in a vortex and centrifuged for 10 min at 1000 ***g***. Immediately after centrifugation, samples were frozen in liquid nitrogen. The unfrozen portion (ethyl ether containing cortisol) was decanted and the ethyl ether was transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol. The extract was stored at −20°C until the ELISA was conducted on the samples suspended with 1 ml of PBS buffer.

ROS levels

Immediately after the behavioral evaluation, the fish brain was removed and gently homogenized in 150 µl of PBS. The samples were centrifuged at 10,000 ***g*** for 10 min at 4°C. The resulting pellets were discarded and the supernatants collected for the dosage of ROS levels. The fluorescent probe H_2DCF-DA was used to evaluate the free radical content (LeBel et al., 1990; Ali et al., 1992). A sample aliquot of 25 µl was incubated with 5 µl of 1 mmol l^{-1} H_2DCF-DA and 170 µl of PBS at 37°C for 30 min. ROS levels were detected with a fluorescence microplate reader at emission (520 nm) and excitation (480 nm) wavelengths using dichlorofluorescein (DCF) as standard. Results were expressed as relative fluorescence unit (RFU).

Statistical analysis

The normal distribution of the data and homogeneity of variance was confirmed by Kolmogorov–Smirnov and Levene tests, respectively. Results were analyzed by two-way ANOVA (stress and environmental enrichment as independent factors) followed by Tukey's *post hoc* test for comparisons within groups and between housing conditions. Correlation analysis between the time spent in the bottom of the tank and cortisol levels was assessed using Pearson's correlation analysis (we used the mean value from two fish to match the respective cortisol measure from the pooled animals). We conducted three-way ANOVAs with gender as the third independent variable; as no main effects or interactions were observed for gender, data from males and females were pooled together. Differences were considered significant at $P<0.05$. The data were expressed as means±s.e.m.

RESULTS

Fig. 3 shows the effects of EE on behavioral parameters in zebrafish submitted to UCS. The UCS protocol did not alter total distance traveled (Fig. 3A), but increased the time in the bottom zone, and decreased the time spent and the number of entries to the upper zone of the tank (Fig. 3B–D, respectively). EE did not induce general locomotor alterations as indicated by total distance (Fig. 3A). Although in non-stressed animals EE decreased time and entries to the upper zone, it attenuated the anxiogenic effect of UCS (Fig. 3B,C).

Fig. 4 shows the effects of EE on trunk cortisol in zebrafish submitted to UCS. As expected, the UCS protocol increased cortisol levels in animals housed in standard conditions. EE for 21 or 28 days attenuated this response. We also observed a positive correlation between cortisol levels and time spent in the bottom

Table 1. The procedure of the unpredictable chronic stress protocol

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
11:00 h Cooling	08:00 h Low water level	10:00 h Crowding	08:00 h Predator exposure	09:00 h Heating	11:00 h Chasing	10:00 h Tank change
05:00 h Heating	01:00 h Chasing	04:00 h Tank change	02:00 h Cooling	05:00 h Low water level	06:00 h Crowding	03:00 h Predator exposure

The UCS protocol included a total of seven stressors (cooling, low water level, crowding, predator exposure, heating, chasing, tank change; see Materials and methods for further details) that were applied at the indicated times to fish assigned to the stressed groups (environmental enrichment or standard condition).

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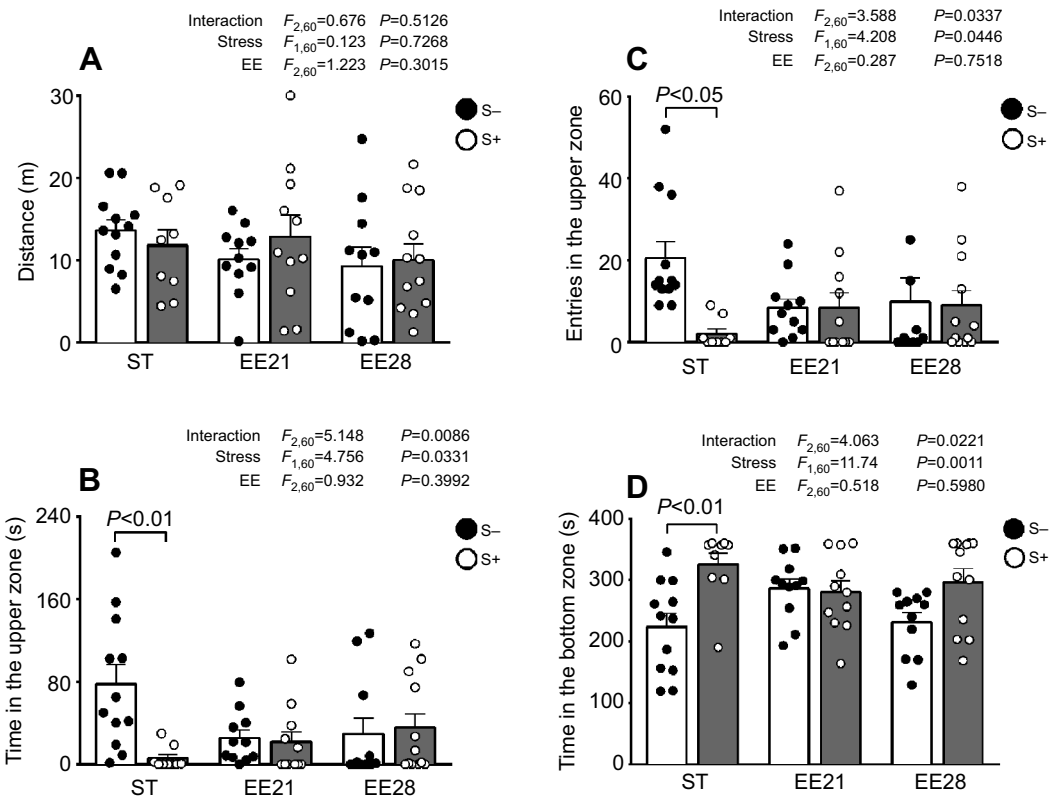

Fig. 3. Effects of environmental enrichment for 21 or 28 days on behavioral parameters (novel tank test) in zebrafish submitted to UCS or not. EE21, environmental enrichment for 21 days; EE28, environmental enrichment for 28 days; ST, standard condition; S+, subjected to UCS; S–, not subjected to UCS. Data are expressed as means±s.e.m.; *N*=9–12; two-way ANOVA/Tukey's *post hoc* test.

zone of the tank (r=0.4715, P=0.0056), showing the higher the cortisol levels, the more time fish spent in the bottom of the tank.

Fig. 5 shows the effects of EE on ROS levels in zebrafish submitted to UCS. The UCS protocol increased ROS levels but EE prevented this effect. There was no difference among non-stressed animals (ST×EE21×EE28).

DISCUSSION

Consistent with our previous findings, we show that UCS increased anxiety-like behavior and increased cortisol levels in zebrafish (Piato et al., 2011; Marcon et al., 2016). In this study, we characterize the effects of EE on behavior and biochemical parameter changes induced by UCS. We show that EE for 21 or

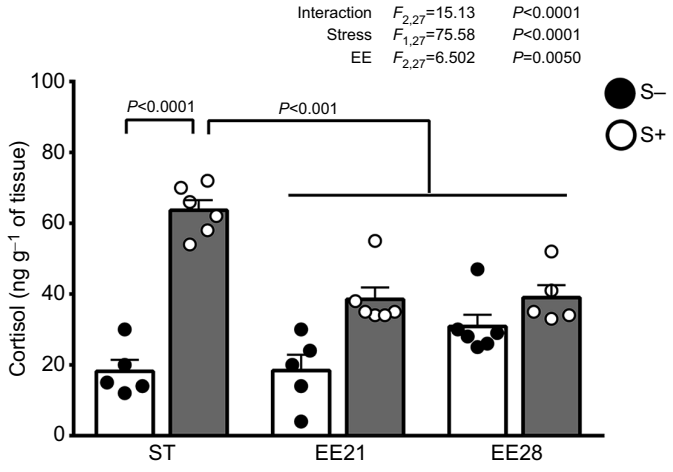

Fig. 4. Effects of environmental enrichment for 21 or 28 days on cortisol levels in zebrafish submitted to unpredictable chronic stress or not. EE21, environmental enrichment for 21 days; EE28, environmental enrichment for 28 days; ST, standard condition; S+, subjected to UCS; S–, not subjected to UCS. Data are expressed as means±s.e.m.; *N* =5–6; two-way ANOVA/Tukey's *post hoc* test.

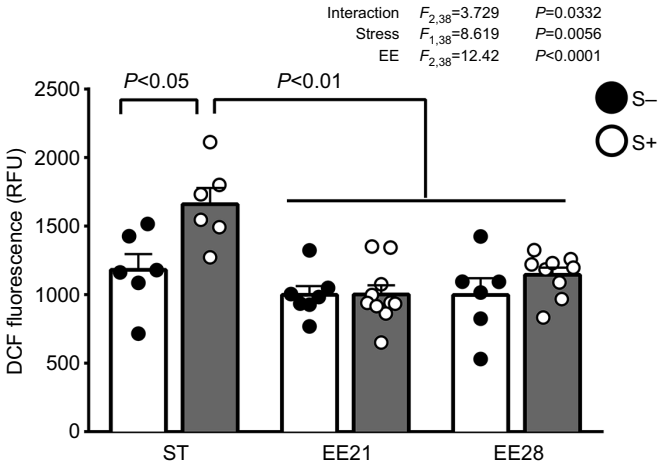

Fig. 5. Effects of environmental enrichment for 21 or 28 days on reactive oxygen species levels in zebrafish submitted to unpredictable chronic stress or not. EE21, environmental enrichment for 21 days; EE28, environmental enrichment for 28 days; ST, standard condition; S+, subjected to UCS; S–, not subjected to UCS. DCF, dichlorofluorescein (relative fluorescence units, RFU). Data are expressed as a means±s.e.m.; *N* =6–10; two-way ANOVA/Tukey's *post hoc* test.

28 days attenuated the effects induced by UCS on some behavioral parameters and trunk cortisol, whereas it prevented the effects of UCS on ROS levels.

UCS decreased the time and entries in the upper zone, and EE attenuated these anxiogenic behaviors. Interestingly, non-stressed animals kept in EE conditions seem more anxious in the novel tank test compared with non-stressed animals kept in ST conditions. We hypothesize that the transition from an enriched environment to the novel tank test, which is a barren tank akin to a standard housing condition, represents a novelty factor not present to ST-housed zebrafish, and is thus anxiogenic. Therefore, EE exerts differential effects depending on the stress status of the animals, i.e. is anxiogenic for non-stressed animals and anxiolytic for stressed animals in the novel tank test. This is in agreement with previous studies showing that group-housed zebrafish exhibit higher anxiety-related behavior in the novel tank test when compared with single-housed zebrafish (Parker et al., 2012; Shams et al., 2015). In this context, for fish already acclimated to the conditions of the test (barren tank, single individual) the novel environment will induce less anxiety behavior. It is important to note, however, that we accessed anxiety levels after removing the fish from their housing tanks. Therefore, no inferences can be made regarding the status of the animals while inside their maintenance tanks.

International guidelines recommend EE for animal maintenance and welfare. The feasibility of implementing EE in a zebrafish facility is certainly an important aspect to be considered. Furthermore, few studies have been carried out to address the behavioral consequences of modifying the conditions in which zebrafish are habitually kept in research facilities worldwide. The intention of our investigation was not to suggest the indispensable use of EE to avoid stress in all studies using zebrafish, but rather to elucidate how EE influences zebrafish behavior and biochemical parameters and whether it modifies the response of this species to stress. Our results show that studies previously published in the literature could have reached different conclusions (especially regarding behavior and stress response) if the housing conditions were enriched compared with what they usually are. Future studies in the field are necessary to further advance the discussion.

Rodent studies have reported anxiogenic effects of EE in the sucrose neophobia test, cold-stress defecation (Green et al., 2010) and latency to ejaculation (Urakawa et al., 2014). Alternatively, another study assessed anxiety-related behavior in zebrafish raised in EE and observed decreased anxiety as indicated by increased time spent in the light compartment in the light/dark test (Manuel et al., 2015). This divergence of EE-induced behavior in anxiety tests (novel tank versus light/dark test) suggests that they are measuring distinct, although related, phenomena (Kysil et al., 2017). Furthermore, the differences in the EE protocols may underlie the contrasting observations of animal behavior. Aspects such as the degree of enrichment, types of objects used, length of exposure, reorganization or not of the environment, housing density and age are the main variables (Crofton et al., 2015).

Chronic stress is characterized by sustained activation of the neuroendocrine axis and production of cortisol (Egeland et al., 2015; McEwen et al., 2015). As demonstrated in rodents (Miklós and Kovács, 2012) and recently in zebrafish (Song et al., 2017), chronic stress reshapes the neural networks that regulate neuroendocrine function, providing an anatomical reorganization that renders cortisol responses more sensitive to stress. The sustained release of cortisol leads to mobilization of energy resources and accelerates cellular metabolic processes (Otte et al., 2016), increasing oxidative phosphorylation and ROS as products of this process (Thannickal and Fanburg, 2000; Murphy, 2009; Zorov et al., 2014). The increase in ROS levels can lead to tissue damage and oxidative stress, and may underlie some of the deleterious consequences of chronic stress (Polidori et al., 2000). Our EE protocol attenuated the increase in trunk cortisol induced by UCS in zebrafish. It is arguable that the EE provides a perception of shelter, protection and security for zebrafish, contributing to a decreased activation of the neuroendocrine axis. However, ST-housed zebrafish may feel unprotected and vulnerable in a barren environment, and thus stay alert and respond more intensely to stress. Similarly, a study has recently found that EE during adolescence increased the amount of glucocorticoid receptors in the hippocampus and reduced circulating levels of corticosterone, improving the negative feedback of the neuroendocrine axis in rats from stressed strains (McCreary et al., 2016). At the same time, EE attenuated the increase in corticosterone after alcohol ingestion (Lopez and Laber, 2015) and decreased corticosterone production as measured by fecal corticosterone metabolites in mice (Gurfein et al., 2017). In this context, it is notable that EE influences the neuroendocrine axis and affects the production of glucocorticoids induced by different factors, in agreement with our study. However, this phenomenon is not yet fully understood, and more studies are needed to investigate the underlying mechanism.

Limitations of our study include the fact that correlations between biochemical and behavioral data could only be carried out for cortisol levels. Also, we only performed biochemical analysis at a single time point, i.e. after behavioral tests. Future studies are necessary to assess cortisol and ROS levels before and during the novel tank test. It would also be relevant to evaluate the effects of anti-oxidants and drugs able to block cortisol synthesis in order to test for mechanisms. Despite these limitations, our study extended the characterization of the UCS protocol, revealing that UCS increases ROS levels in the zebrafish brain. Also, we demonstrated that EE prevented this effect. The increase in ROS levels may lead to tissue damage and oxidative stress. These findings reinforce that environmental enrichment is a non-pharmacological neuromodulation strategy to protect against oxidative stress-related phenomena induced by UCS (Nithianantharajah and Hannan, 2006).

We have demonstrated for the first time that EE for 21 or 28 days promotes positive effects in zebrafish submitted to an unpredictable chronic stress protocol. Our results indicate that cortisol levels were correlated with anxiety levels, but future studies are required to address mechanistic relationships. We suggest that EE can be used as an alternative neuromodulatory strategy for reducing vulnerability to stress. This study also provides a new protocol to study the behavioral effects and neurobiological mechanisms involved in environmental enrichment.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.M., A.P.H., D.L., A.P.; Methodology: M.M., T.C., A.P.H., L.B., A.P.; Validation: A.P.; Formal analysis: M.M., R.M., R.B., A.P.H., G.K., A.P.; Writing - original draft: M.M., R.M., R.B., A.P.H., L.B., A.P.; Writing - review & editing: M.M., R.M., R.B., A.P.H., D.L., L.B., A.P.; Supervision: A.P.

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