Toxicology and Applied Pharmacology 426 (2021) 115616

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Oxytetracycline induces anxiety-like behavior in adult zebrafish

Darlan Gusso [a], Stefani Altenhofen [b], Pâmella Moreira Fritsch [a], Gabriel Rübensam [c], Carla Denise Bonan [a,b,*]

[a] Programa de Pós-Graduação em Biologia Celular e Molecular, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Programa de Pós-Graduação em Medicina e Ciências da Saúde, Escola de Medicina, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Centro de Pesquisa em Toxicologia e Farmacologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

ARTICLE INFO

Editor: Dr. Lawrence Lash

Keywords:
Anxiety
Behavior
Cortisol
Oxytetracycline
Zebrafish

ABSTRACT

Oxytetracycline (OTC) is one of the broad-spectrum antibiotics widely used for the treatment of fish-farm infection. Considering that behavior is directly related to reproduction, individual fitness, and survival, it is important to evaluate the impact of antibiotics on the behavioral repertoire in fish. Zebrafish (*Danio rerio*) presents a well-described behavioral repertoire to reliably demonstrate complex responses to chemical compound exposure. This work aims to identify the role of OTC in comprehensive behavioral parameters and whole-body cortisol levels in adult zebrafish. Here we report that OTC exposure (10, 20, and 100 mg/L) induces an anxiogenic-like phenotype in the novel tank test. OTC exposure also changes the behavior of social interaction with a shoal of unknown zebrafish - characterized as a stimulus group. Zebrafish exposed to OTC (10 mg/L) remains a longer period in the stimulus zone when compared to the control group. Clonazepam (0.006 mg/L) was able to reverse anxiogenic-like behavior and the changes in social behavior induced by OTC. We also demonstrated that cortisol levels were significantly decreased after exposure to OTC (10, 20, and 100 mg/L), which were not reversed by clonazepam. These findings highlight the growing utility of zebrafish as a model to understand the impact of antibiotics on behavior and their underlying mechanisms.

1. Introduction

Since their discovery, antibiotics had been used for human health, but also in the most diverse areas of animal production ranging from bees and fish (Ignasiak and Maxwell, 2018; Leal et al., 2017) to poultry, pigs, and cattle (Gajda et al., 2017; Omija et al., 1994). Currently, fish production in intensive systems increases the biomass of the environment and can result in increased susceptibility to diseases that cause financial loss. Therefore, it is essential to introduce constant prophylactic methods, such as treatments with antibiotics, which are a common practice among fish farms (Miranda et al., 2018). The dependence-relationship on the use of antibiotics in aquatic systems presents aspects of resistance (Fauci and Morens, 2012; Monteiro et al., 2016b), which is a global concern for animal and human health (Roth et al., 2019).

Oxytetracycline (OTC), a broad-spectrum antibiotic, is essential for veterinary use and is also used for treating infections in humans (Rok et al., 2017). Despite the benefits, OTC, as well as other antibiotics, has been shown to cause severe dysbiosis by reducing the microbial community and making room for opportunistic bacteria in the fish gut due to their antibacterial activity (Leal et al., 2017; Lu et al., 2019; Omija et al., 1994). In addition, in recent years, *in vivo* trials have proposed a relationship between the use of antibiotics and anxiety-like behavior (Li et al., 2019; Lurie et al., 2015; Schmidtner et al., 2019) through intestinal dysbiosis (Cryan et al., 2019; Han and Kim, 2019). Physiological functions can be impaired during stress situations and compromise fish welfare (Aerts et al., 2015), with environmental and financial impacts in aquaculture.

Studies have demonstrated that OTC is more than 90% excreted *via* the feces without significant biotransformation into the surrounding water (Cravédi et al., 1987). OTC has been detected in several aquatic environments around the world due to its continuous release and persistence at concentrations ranging from 10 to 7993 ng/L (120–152 ng/L - Drweca river, Poland, Harnisz et al., 2015; 14–7993 ng/L - Ilha

* Corresponding author at: Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Prédio 12D, Sala 301, 90619-900, Porto Alegre, RS, Brazil.
E-mail address: cbonan@pucrs.br (C.D. Bonan).

https://doi.org/10.1016/j.taap.2021.115616
Received 9 March 2021; Received in revised form 24 May 2021; Accepted 4 June 2021
Available online 6 June 2021

Solteira Reservoir, Brazil, Monteiro et al., 2016a; 10 ng/L - Luxembourg, Pailler et al., 2009; 287 ng/L - Bohai Bay, China; Zou et al., 2011). Despite the known antimicrobial action, the effects of OTC in behavioral and biochemical parameters on aquatic systems and non-target species remain to be better investigated. Previous studies have shown that long-term exposure to OTC induced changes in fish behavior (increased hyperactivity) as well as also decreased cellular energy (Cellular Energy Allocation, classified as energy available and energy consumption of an organism) and antioxidant enzymes (Glutathione as an antioxidant; Almeida et al., 2019b). Effects in alpha and beta diversity were also described in the fish gut and water bacterial communities (Almeida et al., 2019a). After the OTC exposure ceases, a recovery in energy allocation and water and gut microbiomes was observed (Almeida et al., 2021). Moreover, long-term exposure to low concentrations of OTC may alter the endocrine responses through changes in T3 and TSH contents as well as in genes involved in the hypothalamus–pituitary–thyroid (HPT) axis, disrupting the thyroid system and the development of zebrafish (Yu et al., 2020).

Considering that behavior is directly related to reproduction, individual fitness, and survival, it is important to evaluate the impact of antibiotics on the behavioral repertoire in fish (Juntti and Fernald, 2016). In this sense, zebrafish is a useful animal model for toxicological and neurobehavioral studies, since several behavioral tasks were already developed in larval and adult stages (Basnet et al., 2019; Cognato et al., 2012; Facciol and Gerlai, 2020; Gaspary et al., 2018). This species forms shoals and dominance hierarchies and has neophiliac responses to new environments/conditions, anxiety-like and aggressive behaviors (De Abreu et al., 2019; Rambo et al., 2017; Reolon et al., 2018).

Since OTC is widely used for human and animal health and there is a relationship between the presence of antibiotics in aquatic environments and behavioral responses in fish, in this study we investigated the effects of OTC on behavioral parameters, such as locomotion, anxiety, and social interaction in adult zebrafish. Considering that clonazepam acts as a GABA-A receptor agonist and induces anxiolytic effects (Möhler, 2012; Savage et al., 2018; Smart and Stephenson, 2019), we tested clonazepam to evaluate if behavioral changes promoted by OTC are related to the anxiety-like behavior. Given the possible complexity of interactions between antibiotics and behavior, we also quantified cortisol levels to address the potential mechanisms underlying the effects of OTC exposure on the zebrafish behavior.

2. Methods

2.1. Animals and housing conditions

A total of 439 adult (7–8 months) zebrafish (strain AB) from our breeding colony were used in equal proportions (male/female). Fish were kept in automated recirculating systems (Zebtec, Tecniplast, Italy). The Zebtec contain reverse osmosis filtered water at the recommended temperature (28 °C ± 2 °C), pH (7.0–7.5), conductivity (300–700 μS), hardness (80–300 mg/L), ammonia, nitrite, nitrate, and chloride levels for this species (Westerfield, 2007, 2000). The animals were maintained on a light/dark cycle - 14/10 h. All fish are fed with commercial flakes (TetraMin Tropical Flake Fish®) three times a day. Fourteen days post-fertilization, we supplemented the diet with brine shrimp (Westerfield, 2000). The OTC-exposed fish were kept in the same parameters of water quality, light, and food as the control animals. However, to expose them to treatment, 10 fish were taken from at least four different tanks, and then they were randomly distributed in the experimental tanks. All procedures followed the guidelines of the Brazilian Council of Animal Experimentation for Use of Fish in Research (CONCEA) and the Brazilian legislation (Lei 11.794/08). The sample number to behavioral and biochemical analyzes were based on previous studies performed for our group (Altenhofen et al., 2019; Gaspary et al., 2018) and other research groups that used zebrafish as an animal model (Kalichak et al., 2016; Menezes and Da Silva, 2017). The protocols were approved by the Institutional Animal Care Committee from Pontificia Universidade Catolica do Rio Grande do Sul (CEUA-PUCRS, protocol number 8950/2018). This study was registered in the Sistema Nacional de Gestão do Patrimonio Genetico e Conhecimento Tradicional Associado-SISGEN (Protocol No. A3B073D).

2.2. Oxytetracycline (OTC) exposure

The animals were chosen randomly and divided into the following treatments: Water (control group), nominal concentrations of 10 mg/L, 20 mg/L, and 100 mg/L OTC (CAS number 79–57-2), considering a density of two animals per liter. The concentrations chosen were based on indications for use in fish production systems (10, 20, and 100 mg/L; Noga, 2010), and studies using zebrafish as an animal model (10 mg/L; Almeida et al., 2019b). The test solutions were dissolving using water from the zebrafish recirculation system. For all groups, the exposure time was 96 h, with treatment exchanged daily. The essential parameters were monitored daily based on previous studies (Westerfield, 2000, 2007). After the novel tank test, more significant effects at 10 mg/L OTC were observed, which was the concentration tested in the subsequent assays.

2.3. Quantification of OTC by LC-UV

Oxytetracycline solution (10 mg/L) was quantified by liquid chromatography coupled to a UV detector (LC-UV). All collected samples were frozen and stored at −20 °C. Before LC-UV analysis, samples were thawed and diluted with formic acid for a final concentration of 0.1% of the acid. The chromatographic system was an Agilent LC 1100 equipped with a quaternary pump, autosampler, column oven, and a diode-array detector (Agilent Technologies, USA). The separation was performed on a Zorbax Extended-C18 RRHD (5 × 2.1 mm, 1.8 μm, Agilent Technologies, USA) column using a mobile phase consisted of 0.1% formic acid and 0.1% formic acid in acetonitrile, in isocratic mode (83:17 *v*/v) and flow of 0.25 mL/min. The total chromatographic run was 6 min, column temperature of 40 °C, and an injection volume of 10 μL. Quantification was performed by external standardization with calibration curve at concentrations of 0.0, 5.0, 10.0, 50.0, 100.0 and 150.0 mg/L. Standards were prepared individually in 0.1% formic acid at 1 mg/mL and diluted with mobile phase before LC-UV analysis.

2.4. Clonazepam exposure

To understand the mechanisms involved in the behavioral changes induced by OTC, we used clonazepam, a benzodiazepine drug widely used for generalized and social anxiety (Dokkedal-Silva et al., 2019; Perna et al., 2016). To validate the anxiety-like behavior, we tested a concentration of 0.006 mg/L clonazepam for 10 min of exposure as a positive control. The clonazepam concentration was chosen based on a study where it was demonstrated that 0.006 mg/L clonazepam for 10 min had an excellent anxiolytic response in the zebrafish (Magno et al., 2015). The fish were exposed to clonazepam in a 500 mL beaker before being recorded for behavioral analysis.

2.5. Novel tank test

In experimental tanks with water (30 cm long x 15 cm high x 10 cm wide), each fish was placed individually and recorded for 6 min (1280 × 720 pixels). After a 1 min habituation period, we analyzed the locomotion and exploratory patterns of the fish using the EthoVision XT® tracking software (version 11.5, Noldus, Wageningen, Netherlands) at a rate of 30 positions per second (Altenhofen et al., 2017; Gusso et al., 2020). The following behavioral parameters were evaluated: distance (m), erratic movement (deg/cm), turn angle (deg), time spent in the upper zone (s), latency to enter the upper zone (s), crossings to the upper zone, and freezing. Not moving time as a total absence of movement,

except for gills and eyes at less than 0.59 cm/s, is defined as freezing. The experiments were performed in experimental triplicate.

2.6. Social interaction

Immediately after the exposure period, each fish was placed in an experimental tank (30 cm length × 15 cm height × 10 cm width). On one side of the experimental tank, there was a tank with 10 fish classified as "stimulus" and on the other side a tank with only water. The dimensions from the stimulus and the non-stimulus tank were 10 cm length × 15 cm height × 10 cm width. After being introduced into the tank, the fish had 1 min to acclimatize followed by a 5 min video recording. To quantify social interaction and innate preference for conspecifics, the experimental tank was virtually divided into two halves: a "stimulus zone" closer to the "stimulus tank" and the other remaining half closer to the empty tank. The fish preference for the stimulus or non-stimulus side was assessed by dividing the aquarium during the analysis. The time spent by the fish in each zone, the number of times it crossed each zone, and latency to the first entry in the zone were measured using the EthoVision XT® tracking software (version 11.5, Noldus, Wageningen, Netherlands). The social interaction protocol was based on previous studies (Gerlai et al., 2000; Gusso et al., 2020; Meshalkina et al., 2018). The experiments were performed in experimental triplicate.

2.7. Whole-body cortisol determination

Immediately after treatment, the animals were removed using a net and euthanized by hypothermic shock. Fish were frozen immediately after euthanasia for further analysis. Each zebrafish was weighted, minced, and placed into a test tube. It was added 3 mL phosphate-buffered saline pH 7.4 (PBS) to each 0.5 g fish tissue to macerate using IKA® T10 basic – ULTRA-TURRAX®. The homogeneous content was transferred to a new test tube and ethyl ether (3 mL) was added 3 times. After each time that the ether was added, each tube was vortexed (60 s), frozen in liquid nitrogen (30 s), and the not frozen portion (ethyl ether containing cortisol) was transferred to a new tube to completely evaporate giving rise to a lipid extract containing cortisol. After complete ether evaporation (6 h), each sample was diluted with 0.2 mL of phosphate-buffered saline (PBS), pH 7.4, and transferred to 1.5 mL Eppendorf. We measured whole-body cortisol in duplicate samples of extracted tissue and determined the concentration by an enzyme immunoassay kit (ELISA; EIAgen CORTISOL test, Bio Chem Immuno Systems) of tissue extracts resuspended in PBS (Gaspary et al., 2018; Gusso et al., 2020). The linear regression test was performed between the curves, showing a high positive correlation (R2 = 0.9997). The inter-assay coefficient of variation was 1% to 7% and the intra-assay coefficient ranged from 1% to 5%. The experiments were performed in experimental quadruplicate.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). For all comparisons, a significance level of $p < 0.05$ was considered. The distribution of the data was evaluated for normality by the Shapiro-Wilk test. Data from novel tank test and anxiety were evaluated with one-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. Social interaction was analyzed by the Kruskal-Wallis test following Dunn's multiple comparisons test. The effects of OTC plus clonazepam on locomotion, anxiety, social interaction, and cortisol were analyzed by two-way analysis of variance (ANOVA), followed by Tukey test as a *post hoc*. The non-normal data were adjusted through the Log-transformation for each data and analyzed using two-way ANOVA. GraphPad Prism 8 (La Jolla, CA, USA) software was used for statistical analysis.

3. Results

3.1. Novel tank test

In all OTC concentrations, there were no changes in the locomotor parameters tested, such as distance ($F_{(3,\ 88)} = 1.511$, $p = 0.2173$; Fig. 1A), freezing ($F_{(3,\ 82)} = 0.9251$, $p = 0.4325$; Fig. 1B), and in erratic movements ($F_{(3,\ 84)} = 0.5442$, $p = 0.6534$; Fig. 1C) compared to control. Following the anxiety parameters, we observed that animals exposed to 10 mg/L OTC took longer to entry the top of the tank compared to the control group ($F_{(3,\ 85)} = 10.97$, $p < 0.0001$; Fig. 2A). For all OTC concentrations tested, we observed that fish entered the top less often than the control group ($F_{(3,\ 96)} = 9.550$, $p < 0.0001$; Fig. 2B). In addition, at the concentrations of 10, 20, and 100 mg/L OTC, the fish spent less time at the top than the control group ($F_{(3,\ 88)} = 10.82$, $p < 0.0001$; Fig. 2C).

3.2. Social behavior

Fig. 3 demonstrated the effects of OTC on social interaction in zebrafish. We observed a decrease in the latency to enter the stimulus zone at 20 mg/L ($H = 10.19_{)}$, $p = 0.0170$; Fig. 3A). The group exposed to 10 mg/L OTC spent a longer time in the stimulus zone compared to the control group ($H = 13.43$, $p = 0.0195$; Fig. 3B). In addition, zebrafish exposed to 10 mg/L OTC has a lower frequency of entrances in the stimulus zone ($H = 12.48$, $p = 0.0042$; Fig. 3C) when compared to the control.

3.3. Quantification of OTC by LC-UV

Considering that we observed more significant effects with 10 mg/L OTC in the behavioral analysis, this concentration was chosen for the subsequent tests. OTC (10 mg/L) was quantified in tank solutions by the LC-UV method and resulted in a concentration of 9.44 ± 1.06 mg/L, which are values very close to the nominal concentration. Therefore, we maintained this concentration expressed as the nominal concentration.

3.4. Effects of clonazepam after OTC exposure on behavioral parameters

To evaluate the mechanisms involved in the anxiogenic-like responses induced by OTC, animals were exposed to 0.006 mg/L clonazepam for 10 min. The treatment with clonazepam after OTC exposure (10 mg/L) did not alter the distance covered (OTC; $F_{(1,\ 58)} = 1.813$, $p = 0.1833$); (clonazepam; $F_{(1,\ 58)} = 0.9313$, $p = 0.3385$); (Interaction; $F_{(1,\ 58)} = 1.392$, $p = 0.2428$; Fig. 4A), freezing (OTC $F_{(1,\ 58)} = 1.071$, $p = 0.3049$; clonazepam $F_{(1,\ 58)} = 2.544$, $p = 0.1161$; interaction $F_{(1,\ 58)} = 0.4871$, $p = 0.4880$; Fig. 4B), and erratic movements (OTC; $F_{(1,\ 57)} = 0.7029$, $p = 0.4053$; clonazepam; $F_{(1,\ 57)} = 2.619$, $p = 0.1111$; interaction; $F_{(1,\ 57)} = 0.06673$, $p = 0.7971$; Fig. 4C).

Fig. 5 demonstrates an increase of latency to first in the top (s) in zebrafish exposed to 10 mg/L compared to the non-exposed group ($p = 0.0004$). When added clonazepam, we did not see changes in time to get to the top (OTC; $F_{(1,\ 56)} = 30.85$, $p < 0.0001$; clonazepam; $F_{(1,\ 56)} = 4.195$, $p = 0.0452$; interaction; $F_{(1,\ 56)} = 0.1548$, $p = 0.6955$; Fig. 5A). We observed that there was a significant decrease in the top entries in the group exposed to 10 mg/L OTC in relation to the control non-exposed ($p = 0.0287$); (OTC; $F_{(1,\ 59)} = 2.396$, $p = 0.127$; clonazepam; $F_{(1,\ 59)} = 0.09941$, $p = 0.7537$; interaction; $F_{(1,\ 59)} = 5.417$, $p = 0.0234$; Fig. 5B). The results demonstrated a decrease in time spent in the top in zebrafish exposed to OTC compared to the non-exposed ($p = 0.0409$). We also found that OTC plus clonazepam spent more time at the top compared to the OTC alone ($p = 0.0107$), suggesting that clonazepam reversed the anxiogenic-like behavior induced by OTC (OTC; $F_{(1,\ 60)} = 12.45$, $p = 0.0008$; clonazepam; $F_{(1,\ 60)} = 18.22$, $p < 0.0001$; interaction; $F_{(1,\ 60)} = 0.01665$, $p = 0.8978$; Fig. 5C).

Regarding the social interaction parameters, there were no changes in the latency to enter in the stimulus zone after exposure to 10 mg/L

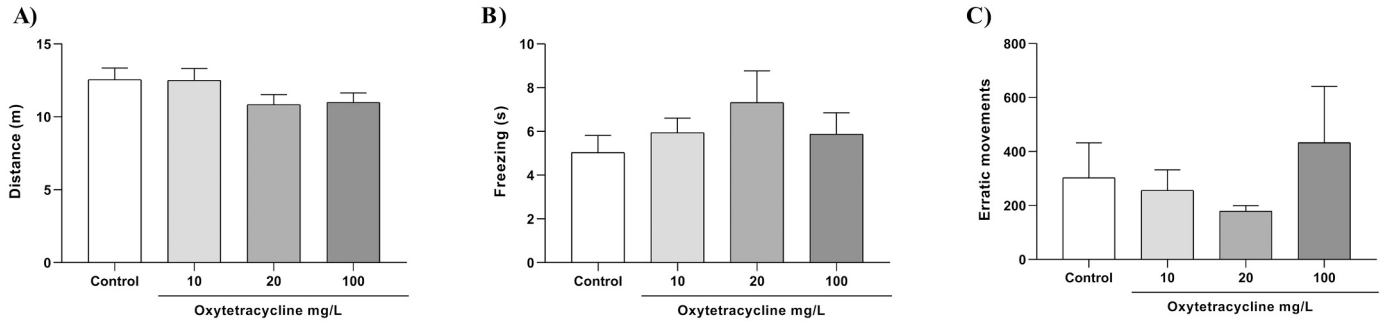

Fig. 1. Effects of OTC exposure on locomotor parameters: (A) distance, (B) freezing, and (C) erratic movements (Control $n = 26$; 10 mg/L $n = 23$; 20 mg/L $n = 18$; 100 mg/L $n = 25$). Data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used, followed by *post hoc* Tukey's test.

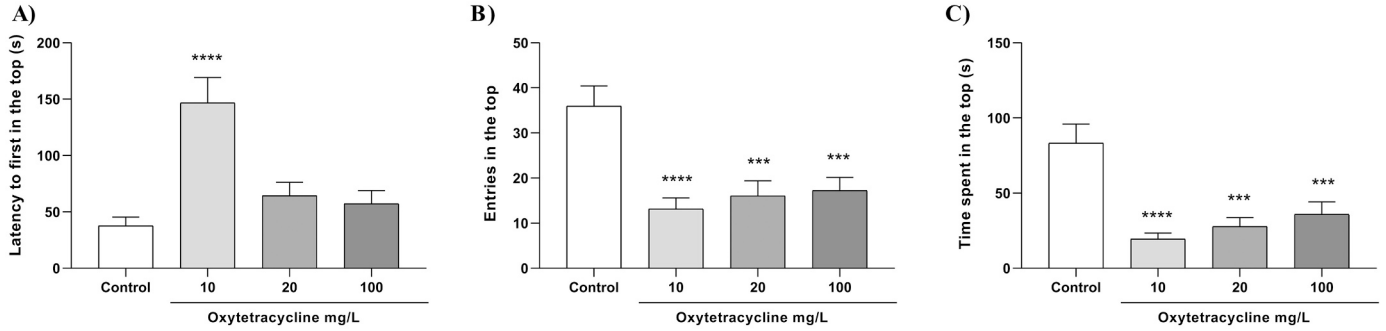

Fig. 2. Anxiogenic effects of OTC exposure on zebrafish behavior in the novel tank diving test: (A) latency to the first entry in the top, (B) entries in the top, and (C) time spent in the top movements (Control n = 26; 10 mg/L $n = 24$; 20 mg/L $n = 17$; 100 mg/L n = 25). Data are expressed as the mean ± SEM. One-way ANOVA was used, followed by *post hoc* Tukey's test. Symbols *** and **** indicate significant difference at $p < 0.001$ and $p < 0.0001$, respectively, when compared to control.

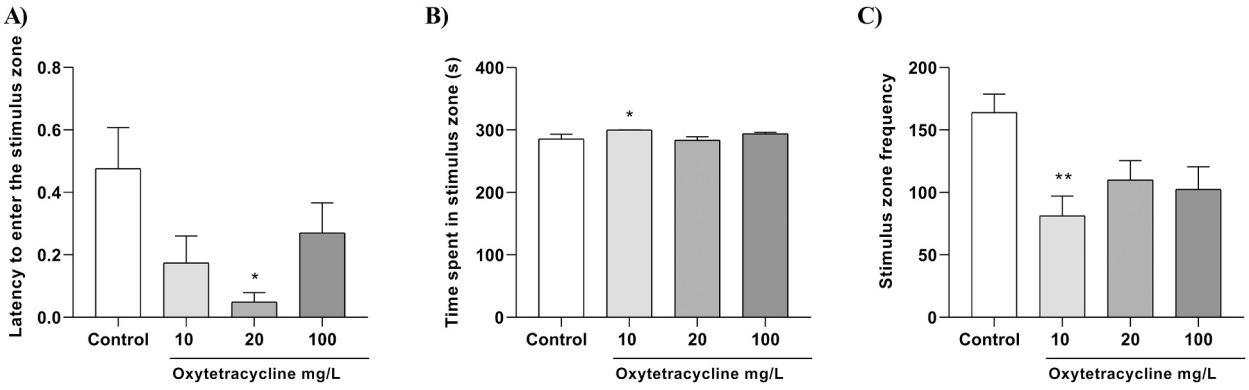

Fig. 3. Effect of OTC on social interaction: (A) latency to enter the stimulus zone, (B) time spent in the stimulus zone, and (C) stimulus zone frequency movements (Control $n = 22$; 10 mg/L n = 23; 20 mg/L $n = 24$; 100 mg/L $n = 24$). Data are presented as mean ± SEM and analyzed by the *Kruskal-Wallis* test following Dunn's multiple comparisons test.

OTC followed by clonazepam (OTC; $F_{(1,\ 64)} = 0.004671$, $p = 0.8590$; clonazepam; $F_{(1,\ 64)} = 0.8655$, $p = 0.0538$); interaction; $F_{(1,\ 64)} = 0.2639$, $p = 0.8818$; Fig. 6A). However, we observed that the group exposed to 10 mg/l OTC spent a longer time in the stimulus zone compared to the control group ($p = 0.0072$) and this effect was reversed by clonazepam ($p < 0.0001$); (OTC; $F_{(1,\ 87)} = 1.792$, $p = 0.1842$; clonazepam; $F_{(1,\ 87)} = 37.93$. $p < 0.0001$; interaction; $F_{(1,\ 87)} = 11.23$, $p = 0.0012$; Fig. 6B). In addition, we observed that zebrafish exposed to 10 mg/L OTC have a lower frequency of entrances in the stimulus zone ($p = 0.0091$). Clonazepam *per se* also induced a decrease in the frequency of entries in the stimulus zone ($p < 0.0001$) and it was unable to reverse the effects induced by OTC (OTC; $F_{(1,\ 86)} = 1.232$, $p = 0.2702$; clonazepam; $F_{(1,\ 86)} = 24.69$. $p < 0.0001$; interaction; $F_{(1,\ 86)} = 9.624$, $p = 0.0026$; Fig. 6C).

3.5. Whole-body cortisol levels

We observed that cortisol levels decreased in exposed fish to 10, 20, and 100 mg/L OTC when compared to the control group ($F_{(3.47)} = 5.382$; $p = 0.0029$; Fig. 7A). The exposure to 0.006 mg/L clonazepam after the fish had been exposed to 10 mg/L OTC did not show recovery of cortisol levels (OTC; $F_{(1,\ 46)} = 4.594$, $p = 0.0374$; clonazepam; $F_{(1,\ 46)} = 0.1011$. $p = 0.7520$; Interaction; $F_{(1,\ 46)} = 1.913$, $p = 0.1733$; Fig. 7B).

4. Discussion

The current study demonstrated that exposure to OTC caused anxiogenic-like behavior and altered the social behavior of adult

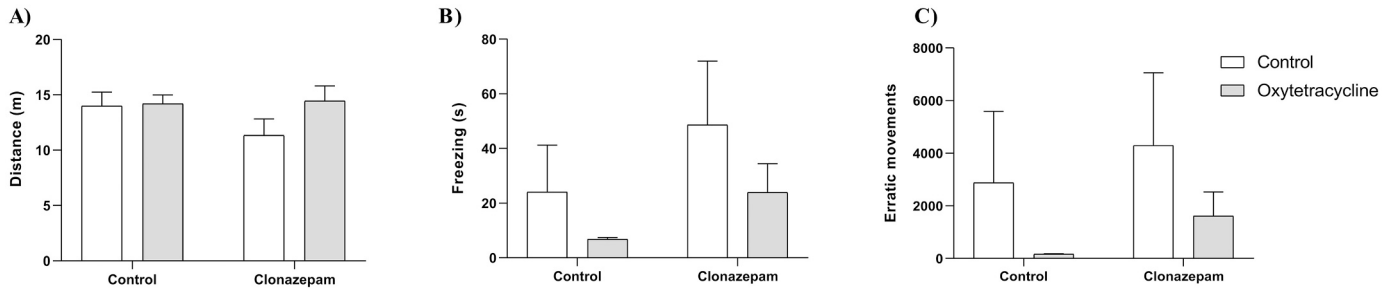

Fig. 4. Effects of OTC (10 mg/L) followed by clonazepam (0.006 mg/L) exposure on locomotor parameters: (A) distance, (B) freezing, and (C) erratic movements (Control $n = 17$; Oxytetracycline $n = 16$; Clonazepam = 13; Oxytetracycline plus clonazepam $n = 16$). Data are expressed as the mean ± SEM. Two-way ANOVA was used, followed by Tukey's *post hoc* test.

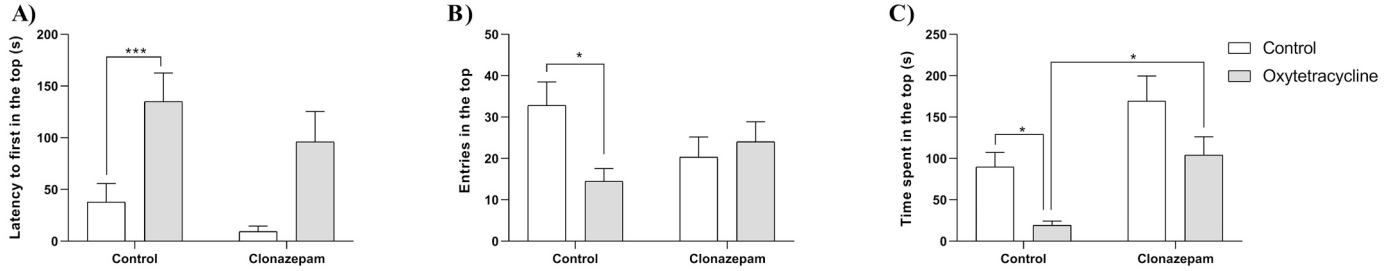

Fig. 5. Effects of clonazepam (0.006 mg/L) on OTC-induced changes on anxiety-like behavior in zebrafish: (A) latency to the first entry in the top, (B) entries in the top, and (C) time spent in the top (Control n = 17; Oxytetracycline n = 17; Clonazepam = 13; Oxytetracycline plus clonazepam $n = 16$). Data are expressed as the mean ± SEM. Two-way analysis of variance (ANOVA) was used followed by Tukey's *post hoc* test. *** indicate significant difference at $p < 0.001$.

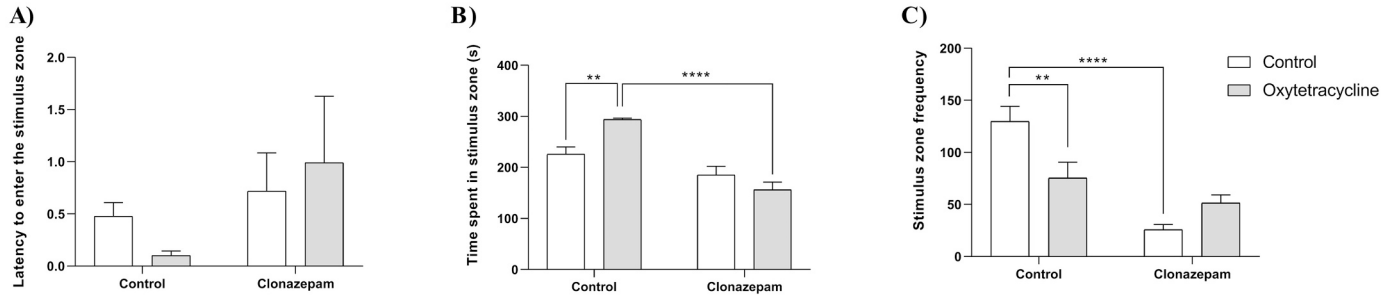

Fig. 6. Effects of clonazepam (0.006 mg/L) on OTC-induced changes on social interaction: A) latency to enter the stimulus zone, (B) time spent in stimulus zone, and (C) stimulus zone frequency (Control $n = 27$; Oxytetracycline n = 25; Clonazepam = 19; Oxytetracycline plus clonazepam $n = 19$). Data are presented as mean ± SEM. Two-way ANOVA was used, followed by Tukey's *post hoc* test. * indicate significant difference at $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

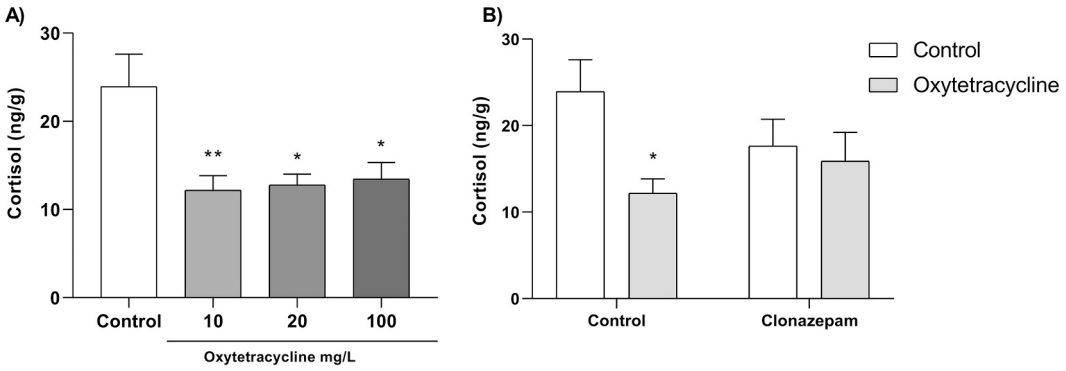

Fig. 7. Whole-body cortisol response of zebrafish exposed to (A) OTC. Data were analyzed by one-way ANOVA, followed by *post hoc* Tukey's test movements (Control $n = 15$; 10 mg/L $n = 13$; 20 mg/L $n = 12$; 100 mg/L $n = 11$). (B) Whole-body cortisol levels of zebrafish exposed to OTC followed by clonazepam. Data were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test. Data are expressed as mean ± SEM. (Control n = 15; Oxytetracycline n = 13; Clonazepam = 11; Oxytetracycline plus clonazepam n = 11). * indicate significant difference at $p < 0.05$ and ** $p < 0.01$.

zebrafish. In addition, a decrease in whole-body cortisol levels was observed after exposure to OTC. Clonazepam exposure reversed the changes observed in the novel tank test and social interaction, *i.e.*, in the time spent in the top zone and time spent in the stimulus zone, respectively.

In the novel tank test, the zebrafish preferred to remain at the bottom of the tank in all OTC concentrations tested. Our results showed a significant effect at the concentration of 10 mg/L OTC, where the zebrafish stay longer at the bottom of the tank and takes longer to enter the top zone when compared to the control group. The novel tank test stands out as an exploratory test that can be considered analogous to the open-field test in rodents (Mocelin et al., 2019). Zebrafish begins to explore the novel tank slowly and over time, usually during the total time, it explores all the tank (Kysil et al., 2017; Stewart et al., 2012). The reduction of exploration especially in the top part of the novel tank can be indicative of anxiogenic-like behavior (Stewart et al., 2012; Valcarce et al., 2020). However, zebrafish exposed to OTC did not present significant differences in the immobile time (*i.e.*, freezing). The zebrafish instinct is to look for safe places, especially when the environment is unknown (Cachat et al., 2010b; Egan et al., 2009). The search for a place to protect against predators in an unknown environment includes specific behavioral parameters such as reduced exploration and freezing in the first minute (Cachat et al., 2010a; Stewart et al., 2012). In addition, we did not observe changes in distance, suggesting that the anxiety-related effects induced by OTC were not linked with locomotor changes.

To investigate the anxiety-like behavior observed, clonazepam effects were evaluated on behavioral changes induced by OTC. Benzodiazepines such as clonazepam behave as a GABA-A receptor agonist and act by increasing the frequency of chloride channel opening. The GABA-A stimulated by clonazepam opens the chloride channel resulting in neuron hyperpolarization and decrease firing. As a result, clonazepam induces calming effects and reduces neurons excitability in the brain (Möhler, 2012; Savage et al., 2018; Smart and Stephenson, 2019). A study revealed that zebrafish presents a GABA-A subunit with receptor characteristics similarly conserved in vertebrates (Sadamitsu et al., 2021). In addition, it was previously demonstrated that the clonazepam at 0.006 mg/L prevents the anxiolytic effects in zebrafish without sedation effects (Magno et al., 2015). Our results demonstrated a similar distance, freezing, and erratic movements in the fish previously exposed to OTC plus clonazepam when compared to OTC or control groups, suggesting that clonazepam did not alter the locomotor behavior. However, clonazepam was able to reverse the effects induced by OTC in time spent in the top. Therefore, these findings demonstrated that OTC induces an anxiety-like behavior reverted by clonazepam.

Our findings also demonstrated that adult zebrafish exposed to OTC presented an altered social behavior. OTC, at the concentration of 10 mg/L, induced a more dependency of the animals with the co-specific group (*i.e.*, stimulus group) since they quickly move to the stimulus zone to stay in contact with the shoal. As prey, zebrafish naturally have mild anxiety-like behavior, which can be shaped by psychopharmaceutical compounds like clonazepam (Zenki et al., 2020). In our results, an anxiety-like behavior was observed in a novel tank test that coincides with social interaction. In addition, the animals exposed to OTC at a concentration of 10 mg/L remained in contact with the shoal for a longer time, which was reversed by clonazepam exposure.

To evaluate the involvement of the hypothalamic-pituitary-interrenal (HPI) axis in the anxiogenic-like behavior induced by OTC, we analyzed the whole-body cortisol levels in zebrafish exposed to 10, 20, and 100 mg/L OTC. Our results demonstrated a reduction in the cortisol levels in zebrafish exposed to OTC; however, cortisol was not recovered to the normal levels after clonazepam exposure. A study demonstrated that socially anxious individuals presented a reduced cortisol reactivity, which may be due to inadequate energy mobilization, leading to poor social performance (Cris, 2016). In addition, reduced cortisol response is a well-documented phenomenon that occurs in zebrafish after prolonged stress (Giacomini et al., 2015; Shams et al., 2017) and exposure to xenobiotics (Giacomini et al., 2016; Gusso et al., 2020). Our results demonstrated a reduction in cortisol levels in OTC-treated groups, which presented an anxiogenic-like phenotype. Despite the exact mechanism by which OTC reduced cortisol response is unknown, our results suggest that the fish decreased their capacity to display an adequate response to an adverse environment and maintain homeostasis. Therefore, further studies may focus on molecular consequences of OTC exposure on the HPI axis, especially on glucocorticoid receptor and corticotropin-releasing factor. Likewise, while exploratory and social behaviors were the focus here, analyses of other important behavioral paradigms also merit further investigation. For instance, while the novel tank test showed no effects after OTC exposure on locomotor parameters, evaluate other behaviors, such as aggression and cognition will be needed.

OTC plays a crucial role as a prophylactic and treatment measure in the production of fish as salmon and tilapia (Botelho et al., 2015; Miranda et al., 2018). OTC was found in the water in all sample seasons (Monteiro et al., 2016a), suggesting a continuous exposure to this compound for the fishes. The effects caused by OTC on zebrafish have a strong relationship with the microbiota since this compound is unable to cross the blood-brain barrier. OTC has a high molecular weight and it is hydrophilic, which causes a restriction in the passage to the brain (Chemello et al., 2016; Kalaiarasi et al., 2016). HPLC analysis in zebrafish exposed to OTC showed the highest proportion of the compound in the intestine both in fish exposed through water and iron oxide nanoparticles that carrying the compound (Chemello et al., 2016). As a cause of dysbiosis, OTC can eliminate some groups of intestinal bacteria and, at the same time, favor the growth of other groups (Navarrete et al., 2008). In our study, we found an anxiogenic-like phenotype after exposure to OTC, which could be related to changes in the fish microbiota, which was already investigated in previous studies (Almeida et al., 2021). The intestinal microbiota can regulate neurotransmitters such as serotonin (5-HT; 5-hydroxytryptamine) (Desbonnet et al., 2010). A recent study showed that exposure of zebrafish to 100 μg/L OTC reduced serotonin levels (Zenki et al., 2020). Hence, in future works, the analysis of serotoninergic and other neurotransmitter systems be helpful to better understand the impact of antibiotics on behavioral responses induced by OTC.

5. Conclusion

In summary, this study demonstrated behavioral responses induced by OTC characterized as an anxiety-like behavior and changes in social interaction in zebrafish, which were reverted by clonazepam. This study highlights the importance to investigate the effects of antibiotics on behavioral responses and the mechanisms underlying these responses since they are extensively used in veterinary medicine and food production with potential risks of long-term exposure to environmental antibiotics in human health.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - finance code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Proc. 420695/2018-4), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS; 17/2551-0000977-0) and Instituto Nacional de Ciências e Tecnologia para Doenças Cerebrais, Excitotoxicidade e Neuroproteção. C.D.B. (Proc. 304450/2019-7) was the recipients of a fellowship from CNPq.

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.

References

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G., De Saeger, S., 2015. Scales Tell a Story on the Stress History of 1–17. https://doi.org/10.1371/journal.pone.0123411.

Almeida, A.R., Alves, M., Domingues, I., Henriques, I., 2019a. The impact of antibiotic exposure in water and zebra fi sh gut microbiomes: a 16S rRNA gene-based metagenomic analysis. Ecotoxicol. Environ. Saf. 186, 109771. https://doi.org/10.1016/j.ecoenv.2019.109771.

Almeida, A.R., Tacão, M., Machado, A.L., Golovko, O., Zlabek, V., Domingues, I., Henriques, I., 2019b. Long-term effects of oxytetracycline exposure in zebrafish: a multi-level perspective. Chemosphere 222, 333–344. https://doi.org/10.1016/j.chemosphere.2019.01.147.

Almeida, A.R., Domingues, I., Henriques, I., 2021. Zebra fish and water microbiome recovery after oxytetracycline exposure. Environ. Pollut. 272, 116371. https://doi.org/10.1016/j.envpol.2020.116371.

Altenhofen, S., Wiprich, M.T., Nery, L.R., Leite, C.E., Vianna, M.R.M.R., Bonan, C.D., 2017. Manganese(II) chloride alters behavioral and neurochemical parameters in larvae and adult zebrafish. Aquat. Toxicol. 182, 172–183. https://doi.org/10.1016/j.aquatox.2016.11.013.

Altenhofen, S., Nabinger, D.D., Bitencourt, P.E.R., Bonan, C.D., 2019. Dichlorvos alters morphology and behavior in zebrafish (Danio rerio) larvae. Environ. Pollut. 245, 1117–1123. https://doi.org/10.1016/j.envpol.2018.11.095.

Basnet, R.M., Zizioli, D., Taweedet, S., Finazzi, D., Memo, M., 2019. Zebrafish Larvae as a Behavioral Model in Neuropharmacology, 7, pp. 7–23. https://doi.org/10.3390/biomedicines7010023.

Botelho, R.G., Christofoletti, C.A., Correia, J.E., Ansoar, Y., Olinda, R.A., Tornisielo, V.L., 2015. Genotoxic responses of juvenile tilapia (*Oreochromis niloticus*) exposed to florfenicol and oxytetracycline. Chemosphere 132, 206–212. https://doi.org/10.1016/j.chemosphere.2015.02.053.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., Dileo, J., Frank, K., Chang, K., Utterback, E., Hart, P., Kalueff, A.V., 2010a. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat. Protoc. 5, 1786–1799. https://doi.org/10.1038/nprot.2010.140.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., DiLeo, J., Frank, K., Chang, K., Utterback, E., Hart, P., Kalueff, A.V., 2010b. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat. Protoc. 5, 1786–1799. https://doi.org/10.1038/nprot.2010.140.

Chemello, G., Piccinetti, C., Randazzo, B., Carnevali, O., Maradonna, F., Magro, M., Bonaiuto, E., Vianello, F., Radaelli, G., Fifi, A.P., Gigliotti, F., Olivotto, I., 2016. Oxytetracycline delivery in adult female zebrafish by Iron oxide nanoparticles. Zebrafish 13, 495–503. https://doi.org/10.1089/zeb.2016.1302.

Cognato, G. de P., Bortolotto, J.W., Blazina, A.R., Christoff, R.R., Lara, D.R., Vianna, M. R., Bonan, C.D., 2012. Y-maze memory task in zebrafish (Danio rerio): the role of glutamatergic and cholinergic systems on the acquisition and consolidation periods. Neurobiol. Learn. Mem. 98, 321–328. https://doi.org/10.1016/j.nlm.2012.09.008.

Cravédi, J.P., Choubert, G., Delous, G., 1987. Digestibility of chloramphenicol, oxolinic acid and oxytetracycline in rainbow trout and influence of these antibiotics on lipid digestibility. Aquaculture 60, 133–141.

Cris, L.G., 2016. Reactivity to social stress in subclinical social anxiety: emotional experience. Cognit. Apprais. Behav. Physiol. 7, 1–10. https://doi.org/10.3389/fpsyt.2016.00005.

Cryan, J.F., O'Riordan, K.J., Cowan, C.S.M., Sandhu, K.V., Bastiaanssen, T.F.S., Boehme, M., Codagnone, M.G., Cussotto, S., Fulling, C., Golubeva, A.V., Guzzetta, K. E., Jaggar, M., Long-Smith, C.M., Lyte, J.M., Martin, J.A., Molinero-Perez, A., Moloney, G., Morelli, E., Morillas, E., O'Connor, R., Cruz-Pereira, J.S., Peterson, V.L., Rea, K., Ritz, N.L., Sherwin, E., Spichak, S., Teichman, E.M., van de Wouw, M., Ventura-Silva, A.P., Wallace-Fitzsimons, S.E., Hyland, N., Clarke, G., Dinan, T.G., 2019. The microbiota-gut-brain Axis. Physiol. Rev. 99, 1877–2013. https://doi.org/10.1152/physrev.00018.2018.

De Abreu, M.S., Anastácio, P.M., Kalueff, A.V., Maximino, C., Banha, F., Demin, K.A., Soares, M.C., 2019. Emotional behavior in aquatic organisms? Less. Crayfish Zebrafish 1–16. https://doi.org/10.1002/jnr.24550.

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J.F., Dinan, T.G., 2010. Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. Neuroscience 170, 1179–1188. https://doi.org/10.1016/j.neuroscience.2010.08.005.

Dokkedal-Silva, V., Berro, L.F., Galduróz, J.C.F., Tufik, S., Andersen, M.L., 2019. Clonazepam. Harv. Rev. Psychiatry 27, 279–289. https://doi.org/10.1097/HRP.0000000000000227.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205, 38–44. https://doi.org/10.1016/j.bbr.2009.06.022.

Facciol, A., Gerlai, R., 2020. Zebrafish Shoaling, Its Behavioral and Neurobiological Mechanisms, and Its Alteration by Embryonic Alcohol Exposure: A Review, 14, pp. 1–12. https://doi.org/10.3389/fnbeh.2020.572175.

Fauci, A.S., Morens, D.M., 2012. The perpetual challenge of infectious diseases. N. Engl. J. Med. 366, 454–461. https://doi.org/10.1056/NEJMra1108296.

Gajda, A., Jablonski, A., Gbylik-Sikorska, M., Posyniak, A., 2017. Correlation between oral fluid and plasma oxytetracycline concentrations after intramuscular administration in pigs. J. Vet. Pharmacol. Ther. 40, e39–e44. https://doi.org/10.1111/jvp.12413.

Gaspary, K.V., Reolon, G.K., Gusso, D., Bonan, C.D., 2018. Novel object recognition and object location tasks in zebrafish: influence of habituation and NMDA receptor antagonism. Neurobiol. Learn. Mem. 155, 249–260. https://doi.org/10.1016/j.nlm.2018.08.005.

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: Zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67, 773–782. https://doi.org/10.1016/S0091-3057(00)00422-6.

Giacomini, A.C.V.V., de Abreu, M.S., Koakoski, G., Idalêncio, R., Kalichak, F., Oliveira, T. A., da Rosa, J.G.S., Gusso, D., Piato, A.L., Barcellos, L.J.G., 2015. My stress, our stress: blunted cortisol response to stress in isolated housed zebrafish. Physiol. Behav. 139 https://doi.org/10.1016/j.physbeh.2014.11.035.

Giacomini, A.C.V.V., Abreu, M.S., Giacomini, L.V., Siebel, A.M., Zimerman, F.F., Rambo, C.L., Mocelin, R., Bonan, C.D., Piato, A.L., Barcellos, L.J.G., 2016. Fluoxetine and diazepam acutely modulate stress induced-behavior. Behav. Brain Res. 296, 301–310. https://doi.org/10.1016/j.bbr.2015.09.027.

Gusso, D., Reolon, G.K., Gonzalez, J.B., Altenhofen, S., Kist, L.W., Bogo, M.R., Bonan, C. D., 2020. Pyriproxyfen exposure impairs cognitive parameters and alters cortisol levels in zebrafish. Front. Behav. Neurosci. 14, 32–39. https://doi.org/10.3389/fnbeh.2020.00103.

Han, S.-K., Kim, D.H., 2019. Lactobacillus mucosae and Bifidobacterium longum synergistically alleviate immobilization stress-induced anxiety/depression in mice by suppressing gut Dysbiosis. J. Microbiol. Biotechnol. 29, 1369–1374. https://doi.org/10.4014/jmb.1907.07044.

Harnisz, M., Korzeniewska, E., Gołas, I., 2015. The impact of a freshwater fish farm on the community of tetracycline- resistant bacteria and the structure of tetracycline resistance genes in river water. Chemosphere 128, 134–141. https://doi.org/10.1016/j.chemosphere.2015.01.035.

Ignasiak, K., Maxwell, A., 2018. Oxytetracycline reduces the diversity of tetracycline-resistance genes in the galleria mellonella gut microbiome. BMC Microbiol. 18, 228. https://doi.org/10.1186/s12866-018-1377-3.

Juntti, S.A., Fernald, R.D., 2016. Timing reproduction in teleost fish: cues and mechanisms. Curr. Opin. Neurobiol. 38, 57–62. https://doi.org/10.1016/j.conb.2016.02.006.

Kalaiarasi, S., Arjun, P., Nandhagopal, S., Brijitta, J., Iniyan, A.M., Vincent, S.G.P., Kannan, R.R., 2016. Development of biocompatible nanogel for sustained drug release by overcoming the blood brain barrier in zebrafish model. J. Appl. Biomed. 14, 157–169. https://doi.org/10.1016/j.jab.2016.01.004.

Kalichak, F., Idalencio, R., Rosa, J.G.S., Oliveira, T.A.D., Koakoski, G., Gusso, D., Abreu, M.S.D., Giacomini, A.C.V., Barcellos, H.H.A., Fagundes, M., Piato, A.L., Barcellos, L.J.G., 2016. Waterborne psychoactive drugs impair the initial development of zebrafish. Environ. Toxicol. Pharmacol. 41 https://doi.org/10.1016/j.etap.2015.11.014.

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., Song, C., Kalueff, A.V., 2017. Comparative analyses of zebrafish anxiety-like behavior using conflict-based novelty tests. Zebrafish 14, 197–208. https://doi.org/10.1089/zeb.2016.1415.

Leal, J.F., Henriques, I.S., Correia, A., Santos, E.B.H., Esteves, V.I., 2017. Antibacterial activity of oxytetracycline photoproducts in marine aquaculture's water. Environ. Pollut. 220, 644–649. https://doi.org/10.1016/j.envpol.2016.10.021.

Li, N., Wang, Q., Wang, Y., Sun, A., Lin, Y., Jin, Y., Li, X., 2019. Fecal microbiota transplantation from chronic unpredictable mild stress mice donors affects anxiety-like and depression-like behavior in recipient mice via the gut microbiota-inflammation-brain axis. Stress 22, 592–602. https://doi.org/10.1080/10253890.2019.1617267.

Lu, Y., Zhang, Z., Liang, X., Chen, Y., Zhang, J., Yi, H., Liu, T., Yang, L., Shi, H., Zhang, L., 2019. Study of gastrointestinal tract viability and motility via modulation of serotonin in a zebrafish model by probiotics. Food Funct. 10, 7416–7425. https://doi.org/10.1039/C9FO02129A.

Lurie, I., Yang, Y.-X., Haynes, K., Mamtani, R., Boursi, B., 2015. Antibiotic exposure and the risk for depression, anxiety, or psychosis. J. Clin. Psychiatry 76, 1522–1528. https://doi.org/10.4088/JCP.15m09961.

Magno, L.D.P., Fontes, A., Gonçalves, B.M.N., Gouveia, A., 2015. Pharmacological study of the light/dark preference test in zebrafish (Danio rerio): waterborne administration. Pharmacol. Biochem. Behav. 135, 169–176. https://doi.org/10.1016/j.pbb.2015.05.014.

Menezes, F.P., Da Silva, R.S., 2017. The influence of temperature on adult zebrafish sensitivity to pentylenetetrazole. Epilepsy Res. https://doi.org/10.1016/j.eplepsyres.2017.05.009.

Meshalkina, D.A., Kysil, E.V., Antonova, K.A., Demin, K.A., Kolesnikova, T.O., Khatsko, S.L., Gainetdinov, R.R., Alekseeva, P.A., Kalueff, A.V., 2018. The effects of chronic amitriptyline on zebrafish behavior and monoamine neurochemistry. Neurochem. Res. 43, 1191–1199. https://doi.org/10.1007/s11064-018-2536-5.

Miranda, C.D., Godoy, F.A., Lee, M.R., 2018. Current status of the use of antibiotics and the antimicrobial resistance in the Chilean Salmon farms. Front. Microbiol. 9, 1–14. https://doi.org/10.3389/fmicb.2018.01284.

Mocelin, R., Marcon, M., Sander, A., Paula, A., 2019. Progress in Neuropsychopharmacology & Biological Psychiatry Withdrawal e Ff Ects Following Repeated Ethanol Exposure Are Prevented by N - Acetylcysteine in Zebra Fish, 93, pp. 161–170. https://doi.org/10.1016/j.pnpbp.2019.03.014.

Möhler, H., 2012. The GABA system in anxiety and depression and its therapeutic potential. Neuropharmacology 62, 42–53. https://doi.org/10.1016/j.neuropharm.2011.08.040.

Monteiro, S.H., Francisco, J.G., Andrade, G.C.R.M., Botelho, R.G., Figueiredo, L.A., Tornisielo, V.L., 2016a. Study of spatial and temporal distribution of antimicrobial in water and sediments from caging fish farms by on-line SPE-LC-MS/MS. J. Environ. Sci. Heal. Part B 51, 634–643. https://doi.org/10.1080/03601234.2016.1181917.

Monteiro, S.H., Garcia, F., Gozi, K.S., Romera, D.M., Francisco, J.G., Moura-Andrade, G. C.R., Tornisielo, V.L., 2016b. Relationship between antibiotic residues and occurrence of resistant bacteria in Nile tilapia (Oreochromisniloticus) cultured in cage-farm. J. Environ. Sci. Heal. Part B 51, 817–823. https://doi.org/10.1080/03601234.2016.1208457.

Navarrete, P., Mardones, P., Opazo, R., Espejo, R., Romero, J., 2008. Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic Salmon. J. Aquat. Anim. Health 20, 177–183. https://doi.org/10.1577/H07-043.1.

Noga, E.J., 2010. Fish Disease: Diagnosis and Treatment, Second ed. https://doi.org/10.1002/9781118786758 North.

Omija, B., Mitema, E.S., Maitho, T.E., 1994. Oxytetracycline residue levels in chicken eggs after oral administration of medicated drinking water to laying chickens. Food Addit. Contam. 11, 641–647. https://doi.org/10.1080/02652039409374265.

Pailler, J., Krein, A., Pfister, L., Hoffmann, L., Guignard, C., 2009. Science of the Total Environment Solid phase extraction coupled to liquid chromatography-tandem mass spectrometry analysis of sulfonamides, tetracyclines, analgesics and hormones in surface water and wastewater in Luxembourg. Sci. Total Environ. 407, 4736–4743. https://doi.org/10.1016/j.scitotenv.2009.04.042.

Perna, G., Alciati, A., Riva, A., Micieli, W., Caldirola, D., 2016. Long-term pharmacological treatments of anxiety disorders: an updated systematic review. Curr. Psychiatry Rep. 18, 23. https://doi.org/10.1007/s11920-016-0668-3.

Rambo, C.L., Mocelin, R., Marcon, M., Villanova, D., Koakoski, G., de Abreu, M.S., Oliveira, T.A., Barcellos, L.J.G., Piato, A.L., Bonan, C.D., 2017. Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress. Physiol. Behav. 171, 50–54. https://doi.org/10.1016/j.physbeh.2016.12.032.

Reolon, G.K., de Melo, G.M., da Rosa, J.G. dos S., Barcellos, L.J.G., Bonan, C.D., 2018. Sex and the housing: effects on behavior, cortisol levels and weight in zebrafish. Behav. Brain Res. 336, 85–92. https://doi.org/10.1016/j.bbr.2017.08.006.

Rok, J., Wrześniok, D., Beberok, A., Otręba, M., Delijewski, M., Buszman, E., 2017. Phototoxic effect of oxytetracycline on normal human melanocytes. Toxicol. in Vitro 48, 26–32. https://doi.org/10.1016/j.tiv.2017.12.008.

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., Domig, K.J., 2019. The application of antibiotics in broiler production and the resulting antibiotic resistance in Escherichia coli: a global overview. Poult. Sci. 98, 1791–1804. https://doi.org/10.3382/ps/pey539.

Sadamitsu, K., Shigemitsu, L., Suzuki, M., Ito, D., Kashima, M., Hirata, H., 2021. Characterization of zebrafish GABAA receptor subunits. Sci. Rep. 11, 1–11. https://doi.org/10.1038/s41598-021-84646-3.

Savage, K., Firth, J., Stough, C., Sarris, J., 2018. GABA-modulating phytomedicines for anxiety: a systematic review of preclinical and clinical evidence. Phyther. Res. 32, 3–18. https://doi.org/10.1002/ptr.5940.

Schmidtner, A.K., Slattery, D.A., Gläsner, J., Hiergeist, A., Gryksa, K., Malik, V.A., Hellmann-Regen, J., Heuser, I., Baghai, T.C., Gessner, A., Rupprecht, R., Di Benedetto, B., Neumann, I.D., 2019. Minocycline alters behavior, microglia and the gut microbiome in a trait-anxiety-dependent manner. Transl. Psychiatry 9, 223. https://doi.org/10.1038/s41398-019-0556-9.

Shams, S., Seguin, D., Facciol, A., Chatterjee, D., Gerlai, R., 2017. Effect of social isolation on anxiety-related behaviors, cortisol, and monoamines in adult zebrafish. Behav. Neurosci. 131, 492–504. https://doi.org/10.1037/bne0000220.

Smart, T.G., Stephenson, F.A., 2019. A half century of γ-aminobutyric acid. Brain Neurosci. Adv. 3 https://doi.org/10.1177/2398212819858249.

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., Kalueff, A.V., 2012. Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacology 62, 135–143. https://doi.org/10.1016/j.neuropharm.2011.07.037.

Valcarce, D.G., Martínez-v, J.M., Riesco, M.F., Robles, V., 2020. Heliyon Probiotics Reduce Anxiety-Related Behavior in Zebra Fish, 6. https://doi.org/10.1016/j.heliyon.2020.e03973, 4–7.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 4th ed. (Book).

Westerfield, M., 2007. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 5th Ed. Univ. Oregon Press, Eugene.

Yu, K., Li, X., Qiu, Y., Zeng, X., Yu, X., Wang, W., Yi, X., Huang, L., 2020. Low-dose effects on thyroid disruption in zebrafish by long-term exposure to oxytetracycline. Aquat. Toxicol. 227, 105608. https://doi.org/10.1016/j.aquatox.2020.105608.

Zenki, K.C., De Souza, L.S., Mendonc, A., 2020. *Coriandrum sativum* Extract Prevents Alarm, 17, pp. 1–11. https://doi.org/10.1089/zeb.2019.1805.

Zou, S., Xu, W., Zhang, R., Tang, J., Chen, Y., Zhang, G., 2011. Occurrence and distribution of antibiotics in coastal water of the Bohai Bay, China: impacts of river discharge and aquaculture activities. Environ. Pollut. 159, 2913–2920. https://doi.org/10.1016/j.envpol.2011.04.037.