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# Antibiotic drugs alter zebrafish behavior

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ABSTRACT

Antibiotics are widely used drugs in human and veterinary health as well as in the food industry. The majority of these compounds are, however, excreted unchanged and found as contaminants in water bodies. Although the toxicity of these drugs was previously studied in aquatic organisms, the behavioral effects of these pollutants have not been fully explored. Here we exposed adult zebrafish to environmentally relevant concentrations of different classes of antibiotics (Chlortetracycline, Ciprofloxacin, and Ceftazidime) and assessed zebrafish exploratory, cognitive, aggressive, and social behaviors. Ciprofloxacin, Chlortetracycline, and Ceftazidime exposure induced hyperlocomotion, which was characterized by an increase in the distance traveled in zebrafish. These antibiotics promoted cognitive decline and exacerbated aggressive behavior. In summary, this study shows that antibiotic contamination may impact zebrafish behavior in a short-time manner.

## 1. Introduction

Antibiotics are widely used drugs to treat bacterial infections, acting either by the death of microorganisms or by halting their growth (Blair et al., 2011; Genilloud, 2014). The estimated worldwide use of these drugs is ~100.000–200.000 tons annually, comprising medicinal, veterinary, and as a growth factor in food industry usage (Miranda et al., 2018; Wang et al., 2016). The indiscreet use of these substances, especially by overuse and misuse, induces problems at different levels. Accumulation in soil and water bodies poses an environmental hazard, resistance is a public health concern, and, at the individual level, health issues have been extensively reported (see Gothwal and Shashidhar, 2015; Haller, 2018; Thiemann et al., 2016 for reviews).

Contamination by antibiotics is mainly caused by discharge *via* feces or urine, being 25–75% of the intake by humans and animals excreted unchanged or only slightly modified (Karthikeyan and Meyer, 2006; Zhou et al., 2018). Residuals are present in the soil, sediment, sludge, groundwater, wastewater, tap water, surface water (lakes, streams, rivers, sea), plants, and aquatic animals (Gothwal and Shashidhar, 2015). Of these, aquatic ecosystems are of great importance. They are impacted by anthropogenic action, serve as reservoirs for resistance factors (Marti et al., 2014), and reports of adverse effects on aquatic organisms are plenty. Algae (Lanzky and Halting-Sørensen, 1997), ciliates (Láng and Kőhidai, 2012), amphibians (Peltzer et al., 2017), and fish (Carlson et al., 2015; Liu et al., 2012; Yin et al., 2014) are some examples where the overall health, survival, reproductive status, and/or biochemical markers have been altered by these drugs, demonstrating their influence in the entire food chain of aquatic organisms. However, in this context, the effects of antibiotic exposure on the behavior of aquatic animals were rarely explored (Liu et al., 2017; Pan et al., 2017; Wang et al., 2016). Considering that behavior in natural environments dictates individual fitness, reproduction, and even the species survival, it is relevant to evaluate the effect of antibiotics on behavioral responses in aquatic animals, especially in fish.

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For this purpose, zebrafish (*Danio rerio*) has been widely used for behavioral and toxicological studies. Several behavioral paradigms are available for this species at different developmental stages (Blank et al., 2014; Cognato et al., 2012; Howe et al., 2014). About 190 behavioral phenotypes have been described in zebrafish (Kalueff et al., 2013), both in the wild and laboratory conditions. This species presents as highly sociable, forming shoals and dominance hierarchies, and having preference for its conspecifics. They have neophiliac responses to new environments/situations, depth, color, and darkness preferences. Aggressive, fear/anxiety-like, and specific breeding ("courting") behaviors have been shown for both sexes (Graham et al., 2018; Kalueff et al., 2013; Suriyampola et al., 2015).

In this study, we used adult, wild-type, mix-gendered zebrafish to test the behavioral effects of acute exposure to environmentally relevant concentrations of three antibiotics, chosen as representatives of widely-used classes of antibiotic drugs: Chlortetracycline, Ciprofloxacin, and Ceftazidime. Chlortetracycline is a tetracycline antibiotic that acts by linking itself to the 30S ribosomal subunit and inhibiting the connections of necessary components for bacterial synthesis and growth (F. Zhang et al., 2015). Ciprofloxacin is a second-generation quinolone that inhibits bacterial DNA replication by inactivating DNA topoisomerase I and IV (Plhalova et al., 2012). Ceftazidime is a β-lactam antibiotic that acts by impeding peptidoglycan synthesis in the bacterial cell wall (Q. Zhang et al., 2015). Thus, this study aimed to test different classes of antibiotics on zebrafish behavior, to evaluate if distinct mechanisms associated with the bacterial mortality would induce changes in the behavioral patterns of this species.

## 2. Methods

### 2.1. Animals

Adult wild-type zebrafish (*D. rerio*), 6–9 months old, were obtained from our breeding colony. The animals were kept (until antibiotic exposure) in an automated re-circulating system (Zebtec, Tecniplast, Italy) with reverse-osmosis-filtered water and conditions recommended for the species (temperature 28 °C ± 2 °C, pH 7.0–7.5, conductivity 300–700 μS, ammonia <0.02 mg/L, hardness 80–300 mg/L, nitrite <1 mg/L, nitrate <50 mg/L, and chloride 0 mg/L) until the start of the treatments. The light/dark cycle was 14/10 h, and the animals were fed commercial flakes (TetraMin Tropical Flake Fish®) three times a day; this feed was supplemented with brine shrimp until exposure when supplementation was ceased as to not interfere with the concentration of drugs in water. All protocols were approved by the Institutional Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS, permit number 7143), and the study followed the guidelines of the National Animal Experimentation Control Council (CONCEA). This study was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado - SISGEN (Protocol No. A3B073D).

### 2.2. Treatments

The concentrations of antibiotic drugs used in this study were chosen based on maximum reported concentrations in water bodies (Fick et al., 2009; Kümmerer and Henninger, 2003; Larsson et al., 2007; Zhu et al., 2001), toxicity, and behavioral studies in zebrafish larvae (Wang et al., 2016; F. Zhang et al., 2015; Q. Zhang et al., 2015). Animals were exposed to three concentrations (6.25, 12.5, or 25 mg/L) of Chlortetracycline, Ciprofloxacin, or Ceftazidime (Sigma-Aldrich, St. Louis, MO), dissolved in the tank water for 96 h. The control group was exposed only to tank water for 96 h.

### 2.3. Behavioral analysis

All behavioral experiments were conducted immediately after treatment in tanks with water. The water conditions were adjusted as described above. Analyses were conducted using sample rates of 15 samples per second. Subject detection was performed, resulting in a series of x, y coordinates from the subject position and size of the subjects in square distance units. The results presented in the following sections were calculated by the software according to these values. Immobility/mobility threshold was set up to 0.59 cm/s and 0.6 cm/s respectively. Sample sizes may vary in the groups due to a lack of subject detection and/or missed samples in the software.

#### 2.3.1. Exploratory behavior

Animals (n = 8–14) were placed individually in the experimental tank (30 cm long x 15 cm high x 10 cm wide) and recorded with a Microsoft LifeCam Cinema for 5 min, after a 60 sec habituation period. The recordings were analyzed with Any-Maze Software (Stoelting Instruments, Wood Dale, IL) for the following behavioral parameters: distance traveled (m), time mobile (s), time immobile (s), mean speed calculated from time mobile (m/s) and time spent in the upper or bottom zone (s). This last parameter is considered an indicator of anxiety since zebrafish tend to spend more time at the bottom zone of the tank when introduced to a new environment and then move to the upper zone after a few minutes (Levin et al., 2007).

#### 2.3.2. Y-maze test

Animals (n = 11–17) were tested in a Y-maze glass aquarium with sides covered by black plastic self-adhesive film. There were three arms, each 25 cm long, 8 cm wide, and 15 cm high. The external floor of the apparatus was white to create a contrast between the fish and maze to facilitate video analysis. On the side of each arm, a geometric cue (square, triangle, or circle) allowed the fish to differentiate each arm. Three liters of water were used in the apparatus. The Y-maze arms were randomly designated as start arm, in which fish started exploration (always open); new arm, which was blocked during the first trial but opened during the second trial (test trial); and the other arm (always open). The center of the maze (neutral zone) was not included in the analysis. The Y-maze task consisted of two trials separated by an inter-trial interval (ITI) to assess response to novelty and spatial recognition memory (1 h ITI) (Cognato et al., 2012). During the first trial (training, 5 min), fish could explore only two arms (start and other), with the third arm (novel) closed. For the second trial (after the ITI), the fish were placed back in the same starting arm with free access to all three arms for 5 min. Training and test sessions were recorded using a Microsoft Life Cam Cinema and further analyzed using Any-Maze recording software (Stoelting Co., Wood Dale, IL, USA).

After conducting exploratory and cognition experiments, we continued the behavioral tests with the lowest concentration of each antibiotic (6.25 mg/L), as to minimize locomotor changes that could influence further testing.

#### 2.3.3. Social interaction

The social interaction test (n = 14–16), based on the natural social behavior of zebrafish, was conducted as described by Gerlai et al. (2000), with some modifications. In this protocol, each fish was placed in an experimental tank with water (30 cm long × 15 cm high × 10 cm wide). An empty fish tank was placed on one side of the experimental tank, while the other side contained an identically-sized tank with 15 zebrafish (designated as the "stimulus fish"). The fish undergoing evaluation could acclimate to the experimental tank for 1 min, after which the behavior was video recorded for 5 min for subsequent analysis using EthoVision XT software (Noldus, Wageningen, The Netherlands). The experimental tank was virtually divided into three zones, the "stimulus zone" that was closer to the "stimulus tank" that contained the conspecifics, the center zone, considered neutral, and the remaining zone which was closer to the empty tank. The time spent in each zone was measured to analyze fish social behavior and innate preference for conspecifics to the detriment of the empty tank. The results were expressed as a percentage of the time spent in each zone. The mean distance (m) to the stimulus zone was also assessed.

#### 2.3.4. Aggression

For the aggression test (n = 12–15), each fish was individually placed in an experimental tank (30 cm long × 15 cm high × 10 cm wide). A mirror (45 cm × 38 cm) was placed at the side of the tank at a 22.5° angle to the back wall so that the left vertical edge of the mirror touched the side of the tank and the right edge was further away. Thus, when a test fish swam to the left side of the tank, their mirror image appeared closer to them. After 1 min acclimation, the 5-min session was recorded for subsequent aggression quantification using EthoVision XT software. Virtual vertical lines divided the tank into four equal sections and allowed counting the time spent in each section. Time spent into the left-most segments (zones 1 and 2) indicated a preference for proximity to the "opponent" whereas entry into the right-most zones (zones 3 and 4) indicated less aggressive behavior. Episodes of bites against the mirror were also assessed. For this parameter, the video recordings were analyzed by three experimenters blinded for the treatment groups, resulting in three replicates for each fish (Gerlai et al., 2000; Rambo et al., 2017).

### 2.4. Statistical analysis

Behavioral data are expressed as mean ± standard error of the mean (S.E.M). The distribution of the data was evaluated for normality by the Shapiro-Wilk test. Depending on the distribution, a one-way ANOVA or Kruskal-Wallis test was used. Post-hoc tests were either Dunnett's, Dunn's, or Tukey's depending on the need to compare the treated groups to the control group or between zones. Nested one-way (ANOVA) was used in bite episodes assessment once this test included three replicate values for each animal. For all comparisons, $p \leq 0.05$ was considered significant.

## 3. Results

### 3.1. Exploratory behavior

The behavioral pattern of adult zebrafish was analyzed after 96-h antibiotic exposure. All antibiotic drugs influenced locomotor behavior (Fig. 1). Animals treated with 25 mg/L Ciprofloxacin, 12.5 or 25 mg/L Ceftazidime or 12.5 mg/L Chlortetracycline exhibited increased distance traveled when compared to the control group ($F_{(9,97)} = 3.654$; $p = 0.0006$) (Fig.1A). No other locomotor parameters such as

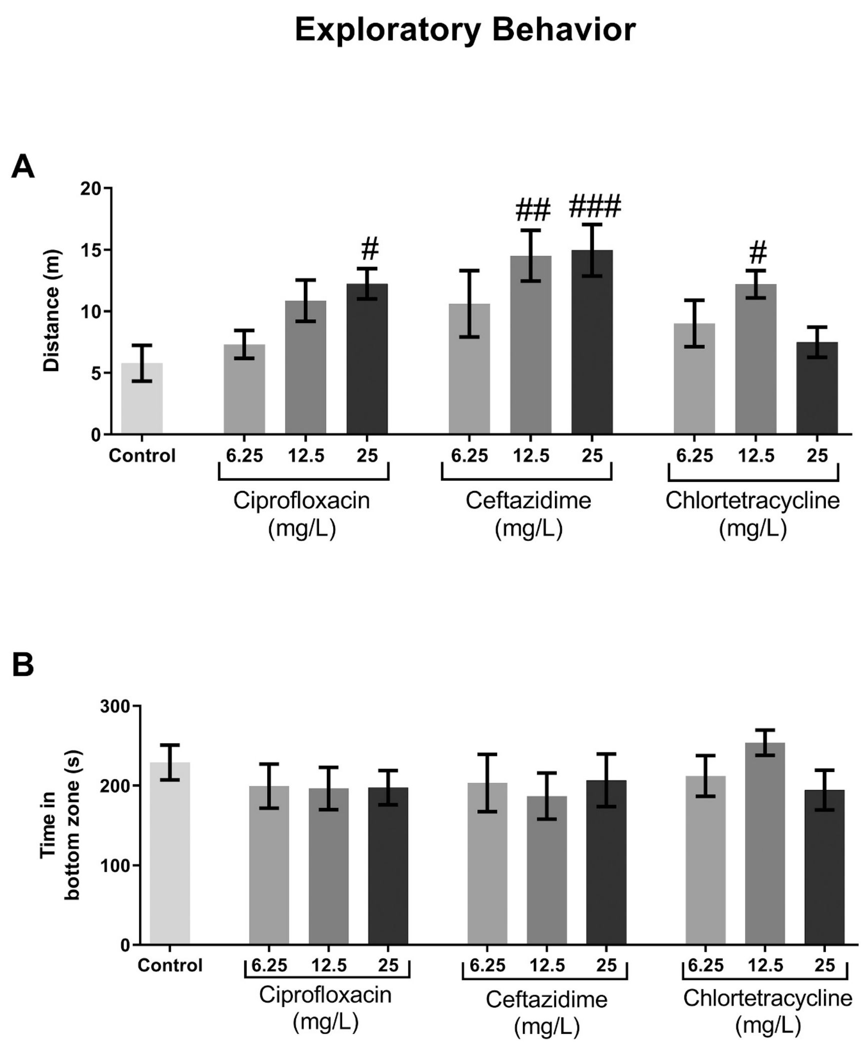


**Fig. 1.** Exploratory and locomotor patterns of control and antibiotic-treated zebrafish after 96-h treatment. (A) distance traveled (m); (B) Time in the bottom zone of the tank (s); (n = 8–14). Data are expressed as mean ± S.E.M. and were analyzed by one-way ANOVA followed by Dunnett's test. $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.005$, and $^{\#\#\#}p \leq 0.0005$ when compared to control group.

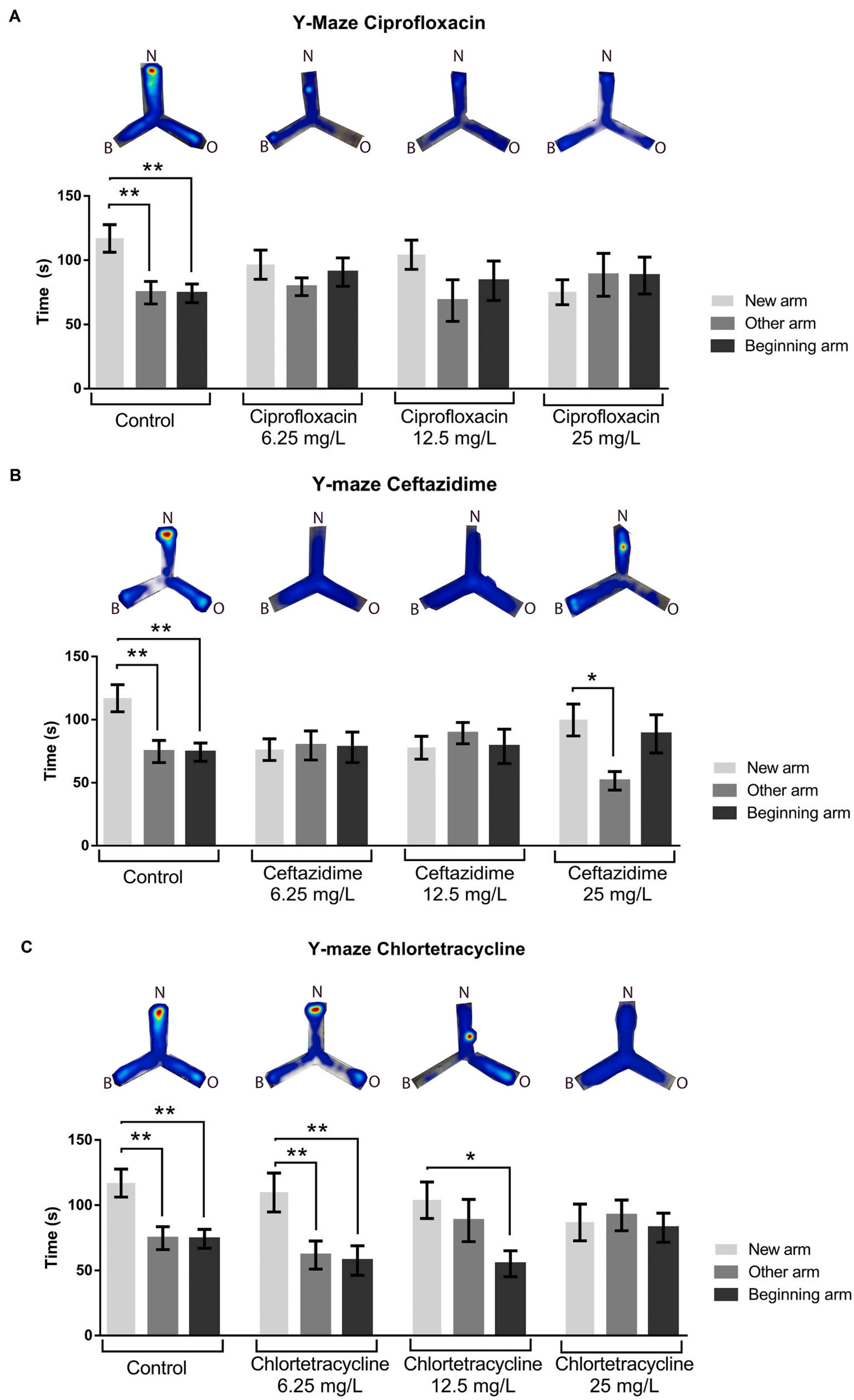


**Fig. 2.** Y-maze performance for control and antibiotic-treated zebrafish: (A) Ciprofloxacin, (B) Ceftazidime, and (C) Chlortetracycline treatments (n = 11–17). Data are presented as mean ± S.E.M. and were analyzed with one-way ANOVA followed by Tukey's test. *$p \leq 0.05$ and **$p \leq 0.005$ compared to control.

mean speed and line crossings were altered (data not shown). Time spent in the upper and bottom zones of the thank was recorded to evaluate anxiety, and there were no changes in these parameters (Fig. 1B).

### 3.2. Y-maze test

The Y-maze test was performed to investigate the effects of antibiotic drugs on cognition (Fig. 2). The control group behaved as expected, with more time spent in the novel arm (128.2 s ± 9.57) when compared to the start (67.57 s ± 6.28) and other arms (66.63 s ± 7.3; Fig. 2; $p < 0.005$). However, no discrimination between arms was observed at all concentrations of Ciprofloxacin ($F_{(11, 159)} = 2.729$; $p = 0.0030$; Fig. 2A), 6.25 and 12.5 mg/L Ceftazidime ($F_{(11, 171)} = 3.777$; $p < 0.0001$; Fig. 2B), and 12.5 mg/L and 25 mg/L Chlortetracycline ($F_{(11, 153)} = 6.145$; $p < 0.0001$; Fig. 2C), suggesting memory impairment.

### 3.3. Aggression and social interaction

For aggression and social interaction, only 6.25 mg/L for each antibiotic was evaluated, since this concentration did not induce locomotor changes that could influence these behavioral tests. None of the antibiotics induced social interaction deficits in zebrafish, and all treated animals presented the same preference for the stimulus area, as shown by the percent time in the stimuli zone (Fig. 3A) and mean distance in the stimuli zone (Fig. 3B).

Antibiotic exposure altered the aggressive behavior measured by the time spent in the segment nearest to the mirror image. Ciprofloxacin-treated animals spent more time in the segment nearest to the mirror than control zebrafish. Ceftazidime treatment produced a similar effect ($H_{(7)} = 51.06$; $p < 0.0001$; Fig. 3C). Moreover, the number of bites against the mirror was significantly increased in Ciprofloxacin- and Chlortetracycline-treated animals ($F_{(3, 57)} = 3.453$; $p = 0.0223$; Fig. 3D).

## 4. Discussion

This study demonstrated that acute exposure to antibiotic drugs affects the natural behaviors of adult zebrafish. Changes in the locomotor activity, cognition, and aggressive behavior were observed after antibiotics exposure, regardless of the mechanism of action of the drug tested.

Our results demonstrated that locomotor patterns were affected by at least one of the concentrations tested for each drug. Ciprofloxacin-treated animals traveled longer distances at the highest concentration (25 mg/L) tested, as did the animals treated with 12.5 or 25 mg/L Ceftazidime and 25 mg/L Chlortetracycline. Previous studies demonstrated that the long-term exposure to oxytetracycline (OTC) and a mixture of β-diketone antibiotics (DKAs) produced a similar effect in zebrafish (Almeida et al., 2019; Wang et al., 2016). Therefore, our findings agree with these studies, revealing that locomotor changes observed in aquatic organisms appear shortly after antibiotics exposure. However, the pattern of increased distance traveled was not observed in zebrafish after exposure to a high amoxicillin dose for 7 days (Gonçalves et al., 2020). In our study, we also evaluated the anxiety-like behavior by

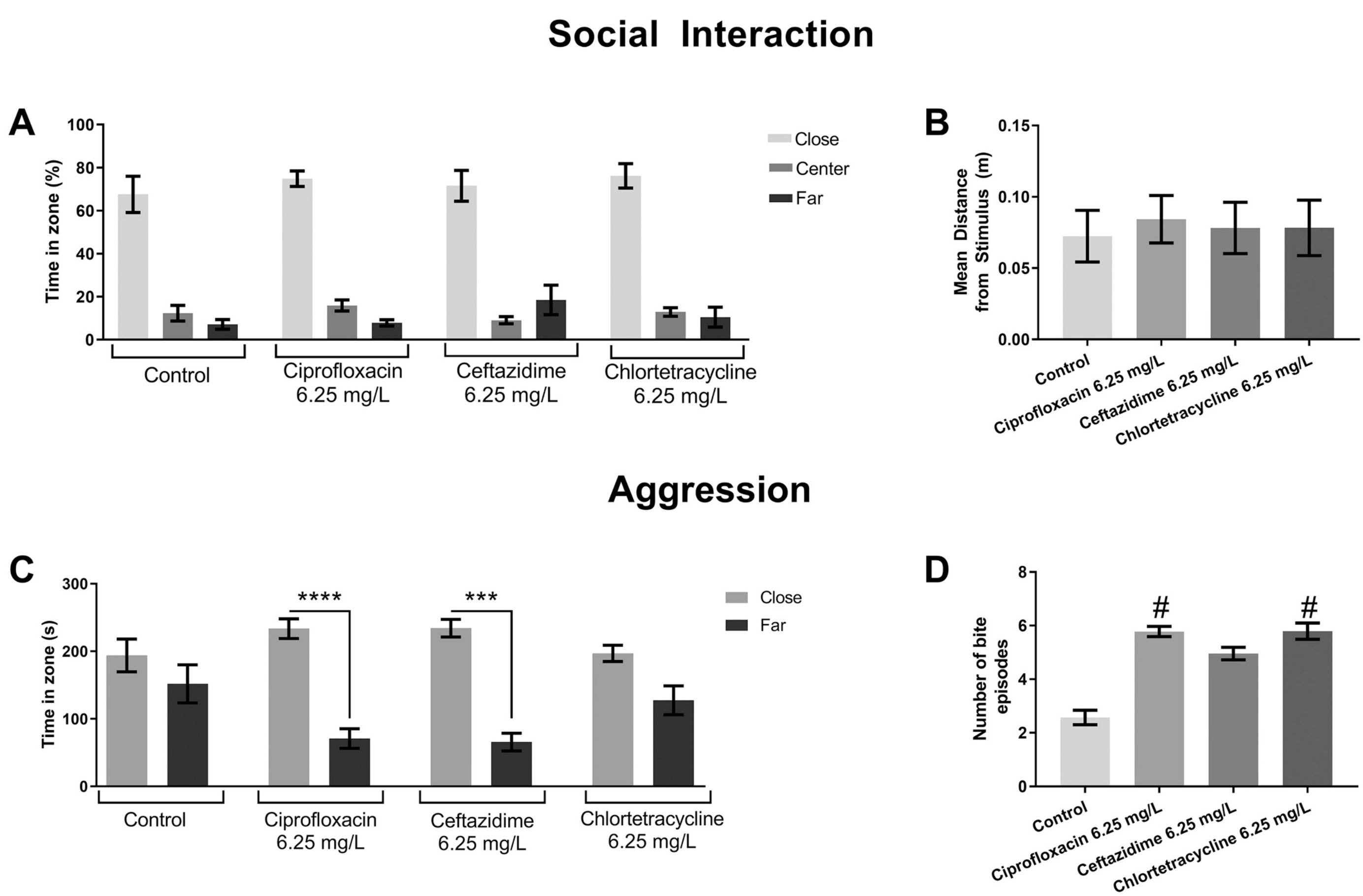


**Fig. 3.** Social interaction and aggression patterns observed after antibiotics exposure in zebrafish. Social interaction was measured by (A) percent time (%) in the zone nearest to the shoal and (B) by distance to the stimuli zone (m). n = 14–16. (C) Aggression measured by time in the zone nearest to the mirror and (D) by number of bite episodes against the mirror. n = 12–15. Data are presented as mean ± S.E.M. Time in zone data were analyzed by Kruskal-Wallis test followed by Dunn's post-hoc. *** represents $p \leq 0.0005$, **** represents $p \leq 0.00005$ compared to other zones. Distance to stimuli zone was analyzed by Kruskal-Wallis followed by Dunn's test. Bites against the mirror were analyzed by Nested ANOVA followed by Dunnett's test. $^{\#}p \leq 0.05$ compared to control.

the analysis of the time spent in the top/bottom zone of the tank. Our results demonstrated that there were no differences in these parameters after antibiotics exposure. Anxiety levels were reported to be altered by long-term exposure (3 months) to OTC or DKAs, but not after short-term exposure (7 days) to high doses of amoxicillin (Wang et al., 2016; Gonçalves et al., 2020). These findings suggest that antibiotic exposure alters parameters of exploratory parameters in zebrafish with different effects appearing over the time exposure.

In this study, we also investigated complex behaviors able to impact fitness and survival in natural environments. Learning and remembering the specificities of their habitat allows animals to forage, locate mating sites, and avoid predators and risky situations (Roy and Bhat, 2016). The Y-Maze test, which we use to measure cognition, is less emotional than avoidance or reward-based tests, once it is based on the natural neophilia of zebrafish (Cognato et al., 2012). In this test, animals use visual cues to orient themselves and distinguish old from the novel arm from the Y-shaped apparatus. Impairment in this ability was recorded for animals exposed to Ciprofloxacin at all concentrations tested, 6.25 and 12.5 mg/L Ceftazidime, and 25 mg/L Chlortetracycline. However, it is important to note that some of these results may be affected by altered locomotion. To our knowledge, this is the first study to measure cognition following antibiotics exposure in adult zebrafish, but the cognitive impairment observed here is in agreement with previous studies that used these drugs in murine species (Desbonnet et al., 2015; Fröhlich et al., 2016). With no other evidence of fear or anxiety in the exploratory tests that could impact this trait, our results seem to reflect the effects of antibiotic exposure on memory/learning processes.

Another key trait in zebrafish's behavioral repertoire is its sociability, which is highly complex and conserved (Graham et al., 2018). Previous studies on antibiotics exposure have investigated social interaction with differing results. The exposure to OTC and DKAs induced an increase in social behavior (Almeida et al., 2019; Wang et al., 2016) whereas amoxicillin promoted an impairment in this behavioral trait in zebrafish (Gonçalves et al., 2020). We evaluated this parameter, using a preference-based test, where the animal was placed in a tank with one side facing its co-specifics and the other facing an empty tank. The percent time spent in and the distance to the zone close to other fishes were measured and neither of these parameters was altered. Considering these findings, we hypothesize that the changes in social behavior caused by antibiotics in zebrafish may be related to long-lasting time exposures or high concentrations of these drugs.

We also observed that antibiotics play a role in zebrafish aggression, a finding that, to the best of our knowledge, was not previously reported. Aggression traits are essential in many species to maintain groups, with effects on social hierarchy and mating, among others (Gerlai et al., 2000). The mirror-induced aggression test is based on the behaviors exhibited by zebrafish when posed against a clear opponent. In this test, fish tend to either have a submissive fleeing and freezing posture or aggressive chasing and biting posture (Oliveira et al., 2011). To measure these traits, we evaluated time in the portion of the tank close to the mirror and biting episodes. The time close to the mirror image was increased in 6.25 mg/L Ciprofloxacin and 6.25 mg/L Ceftazidime-exposed animals. The biting episodes were significantly increased in 6.25 mg/L Ciprofloxacin and 6.25 mg/L Chlortetracycline treatments. The control group had no preference for the zone near the mirror and had fewer biting episodes. Therefore, these findings support the idea that the patterns presented by treated animals were due to changes in aggressive behavior and not only the desire to interact with the image in the mirror.

In aquatic animals, the behavior is often used to identify an early disease sign and its application for conservation has been noted (Shumway, 1999). Regarding antibiotic pollution, behavioral effects have not been much studied, especially in adult zebrafish. However, these drugs may impact the F0 and F1 generation, lipid metabolism, cardiotoxicity, oxidative stress, genes associated with apoptosis in zebrafish embryos, and neurobehavioral development (Phelps et al., 2017; Sheng et al., 2018; Wang et al., 2018; Yan et al., 2019; Zheng et al., 2016).

Here we demonstrated a myriad of behavioral alterations in zebrafish caused by acute exposure to antibiotics in water, especially with impacts in learning/memory processes and aggression. Therefore, wild behaviors such as locomotion, exploration, learning, and aggression may be changed by antibiotic contamination, influencing foraging, predator escape, the formation of social hierarchies, reproduction, and the maintenance of populations.

Our results indicate that contamination by antibiotics in water bodies and their impacts on aquatic wildlife is an pressing issue and that these effects do not depend on the drug used and its specific mechanism of action but on the antibiotics *per se*. Along this line, the overuse of these substances in human, veterinary, and industrial scenarios should be looked at outside of the bacterial resistance phenomenon. The effects of the discharge of antibiotic drugs in the environment should be further studied in the population and ecosystem levels, once bioaccumulation across the food chain is a possibility. The mechanisms responsible for these behavioral alterations, possibly linked to the diversity of symbiotic bacteria, should also be further explored.

## 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.

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### *Contributions*

B.D.P.: conceptualization, methodology, investigation, writing – original draft. T.C.B.P, S.A and D.D.N.: investigation. M.R.B.: resources, writing – review & editing. P.M.A.F.: formal analysis. C.D.B: conceptualization, supervision, writing – review & editing, funding acquisition.

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