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Research report

Fluoxetine and diazepam acutely modulate stress induced-behavior

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HIGHLIGHTS

- Stress increased the locomotor activity and the time spent at bottom of the tank.
- Fluoxetine and diazepam prevented these changes.
- Stress, fluoxetine and diazepam decreased social interaction.
- Stress increases aggressiveness, not reversed by fluoxetine and diazepam.
- The presence of fluoxetine and diazepam in environment may alters behavior of fish.

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ABSTRACT

Drug residue contamination in aquatic ecosystems has been studied extensively, but the behavioral effects exerted by the presence of these drugs are not well known. Here, we investigated the effects of acute stress on anxiety, memory, social interaction, and aggressiveness in zebrafish exposed to fluoxetine and diazepam at concentrations that disrupt the hypothalamic–pituitary–interrenal (HPI) axis. Stress increased the locomotor activity and time spent in the bottom area of the tank (novel tank). Fluoxetine and diazepam prevented these behaviors. We also observed that stress and fluoxetine and diazepam exposures decreased social interaction. Stress also increased aggressive behavior, which was not reversed by fluoxetine or diazepam. These data suggest that the presence of these drugs in aquatic ecosystems causes significant behavioral alterations in fish.

1. Introduction

The zebrafish is considered a good model to study anxiety, stress, and predator–prey relationships. Its hypothalamic–pituitary–interrenal (HPI) axis is well characterized [1,2], and its neuroendocrine system displays robust responses to stress [1,3–5]. The effects of acute and chronic stress have been studied in zebrafish. Acute stress modulates behavior and HPI axis, and induces an imbalance in the antioxidant status in zebrafish [6–8]. In a chronic model of unpredictable stress, the protocol increased anxiety-like behavior, impaired memory and induced neuroendocrine dysfunction [5]. Other protocols have extended the understanding of the effects of chronic stress in zebrafish [9–12]. However, the modulation of stress response by drugs is poorly understood.

Drug residue contamination in aquatic ecosystems has been studied extensively [13–19]. There are numerous reports of the presence of drugs and their metabolites in surface water and

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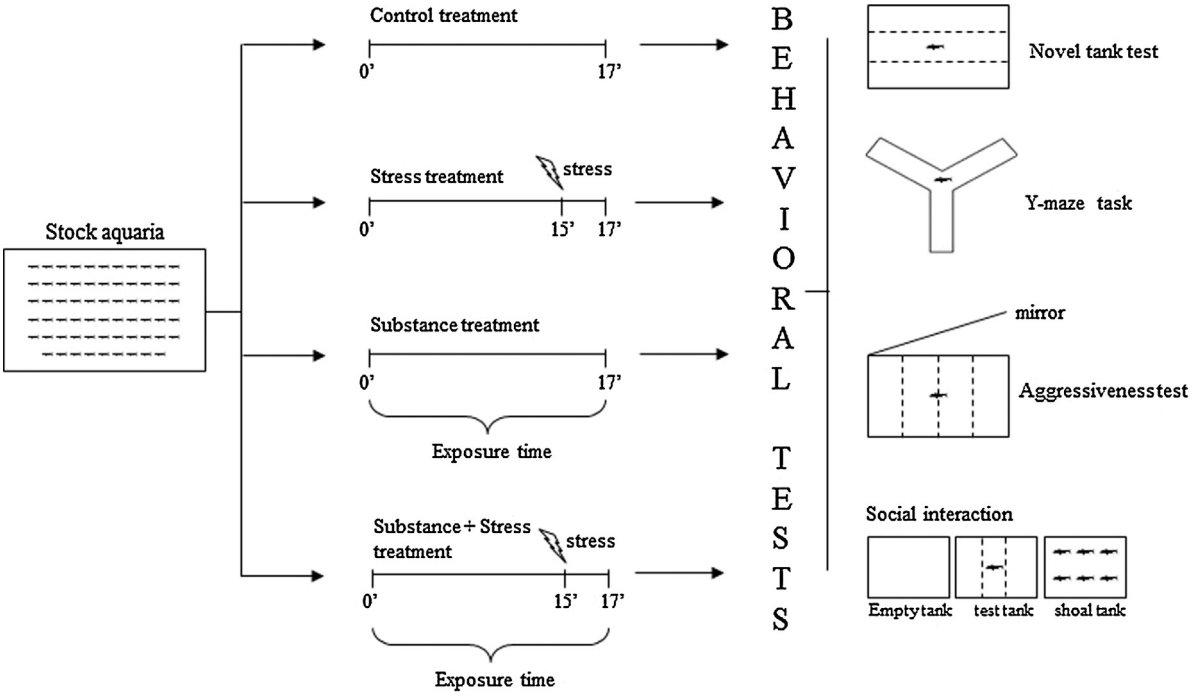

Fig. 1. Schematic view of experimental design.

wastewater in different countries [13,15–17,20–23]. These contaminants may negatively affect the human population as well as aquatic organisms [13–18,20,22–24]. Contaminants such as fluoxetine (FLU) and diazepam (DZP) can induce a number of neuroendocrine [25–27] and behavioral [26,28,29] alterations in exposed fish.

Here, we evaluated the effects of acute stress on behavioral parameters in zebrafish exposed to FLU and DZP. Specifically, we assessed anxiety, memory, social interaction, and aggressiveness.

2. Materials and methods

2.1. Ethical note

This study was approved by the Ethics Commission for Animal Use (CEUA) of the Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #10/2014-CEUA) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Experimental animals

A stock population of 400 mixed-sex (50/50) 180 days-adult wild-type zebrafish (*Danio rerio*), short-fin (SF) strain, were held in two tanks equipped with biological filters, under constant aeration, and with a natural photoperiod (approximately 14 h light:10 h dark). Water temperature was maintained at 26 ± 2 °C; pH 7.0 ± 0.25; dissolved oxygen at 6.5 ± 0.4 mg/L; total ammonia at <0.01 mg/L; total hardness at 6 mg/L; and alkalinity at 22 mg/L $CaCO_3$.

2.3. Drugs tested and exposure time

We used FLU (Daforin®, EMS, Brazil) and DZP (União Química, Brazil) at concentrations of 50 μg/L and 16 μg/L, respectively [27]. The animals were exposed to these drugs for 15 min, which is considered a sufficient period to elicit behavioral responses [28].

2.4. Stress protocol

After a 15 days period for habituation to laboratory conditions, fish were randomly distributed into the following groups: experimental fish exposed to FLU (50 μg/L) or DZP (16 μg/L) and untreated fish (control group). Experimental fish were then exposed to treatment for 15 min, and they underwent an acute stress challenge. This acute stress challenge consisted of harassing them with a pen net for 120 s in groups of three fish, except for Y-maze task where fish was stressed alone (Fig. 1). Different sets of fish then underwent the following behavioral tests: novel tank test, y-maze task, social interaction, and aggressiveness.

2.5. Evaluation of behavioral parameters

In all studies, fish behavior was recorded by a Logitech Quickcam PRO 9000 camera and the videos analyzed using ANY-maze® software (Stoelting CO, USA), which tracked animal behavior throughout testing.

2.5.1. Novel tank test

Fish were transferred individually to a test aquarium (24 × 8 × 20 cm; width × depth × height) and filmed for 6 min. The following parameters were analyzed: relative time in the bottom part of the tank (%), absolute turn angle, mean swimming speed (m/s), number of crossings, and total distance traveled (m).

2.5.2. Y-maze task

Fish were tested in a tank with three arms measuring 25 × 8 × 15 cm (length × width × height). Different geometric shapes (squares, circles, and triangles) were used as visual stimuli and placed on the outer wall of each arm, and the remaining area was covered with black plastic. The Y-maze arms were randomly assigned: start arm, in which the fish starts the test, new arm (locked during the initial test, but open during the second test), and the permanently open arm. The Y-maze center is a neutral area, and therefore, it was not counted in the analysis. The task consisted of two phases with a 1-h interval between them. In the first phase

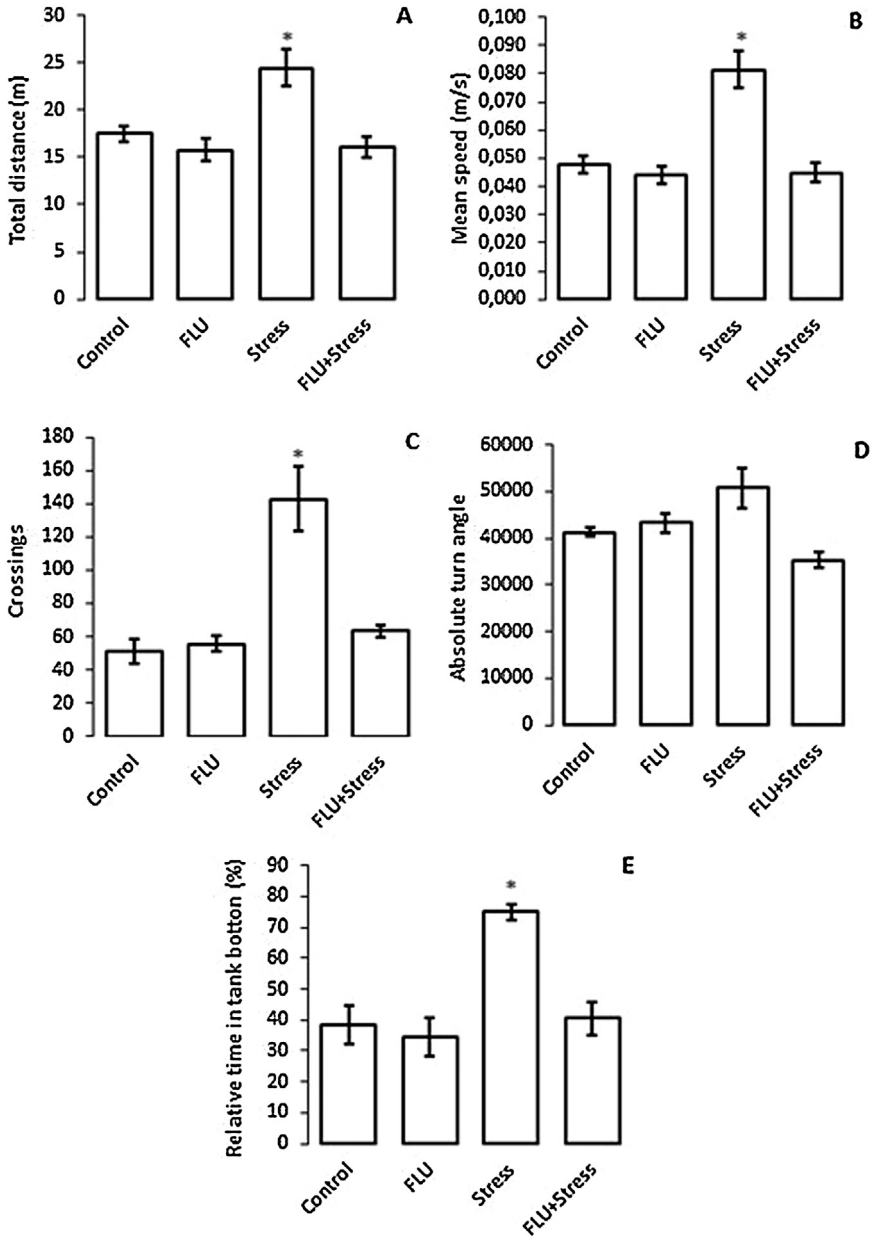

Fig. 2. Novel tank test in zebrafish exposed to fluoxetine and controls. (A) Total distance traveled; (B) mean speed; (C) number of crossings; (D) absolute turn angle, and (E) relative duration at the bottom of the tank. The * indicates statistical difference verified by two-way ANOVA followed by the Tukey test. Data are expressed as mean ± standard error of the mean of 9–12 animals per treatment.

(5-min training), the fish could explore the start and the open arms with the new arm closed. In the second phase, fish were placed in the start arm and were allowed to freely access the three arms. The following parameters were analyzed: total distance, number of intersections, time, distance, and number of entries into the new arm [30].

2.5.3. Social interaction test

In this task, fish were transferred individually to the test aquarium measuring 30 × 15 × 10 cm (width × depth × height). The test tank was positioned between two equal-sized tanks, one without fish and the other containing a group of 15 conspecifics [31]. After transfer, fish were acclimated to the test aquarium for 30 s, and then behavior was recorded for 10 s. Image analysis was done by virtually dividing the test tank into three vertical segments. The first segment is nearest to conspecifics, while the third segment was next to the empty tank. The relative time zebrafish spent in the first segment was calculated as response to social stimuli.

2.5.4. Aggressiveness

Fish behavior when viewing their image in a mirror was used to indirectly quantify aggressiveness [31]. We used this test to verify if stress alters natural aggressive behavior and if FLU and DZP modulate this behavior. A test tank measuring 30 × 15 × 10 cm (length × width × height) was filled with 6 L water, and a mirror (45 × 38 cm) was placed on one side of the tank at an angle of 22.5°. Thus, the left side of the mirror was near the tank and the right side remote from the tank to reflect a closer or more remote image of the fish as it swims by. The interaction of the fish with its own image was recorded for 60 s after two acclimatization periods (30 s and 10 min). For the analysis of the recorded images, the tank was virtually divided into four equal segments to enable quantification of the number of entries into these areas. The entrance and the dura-

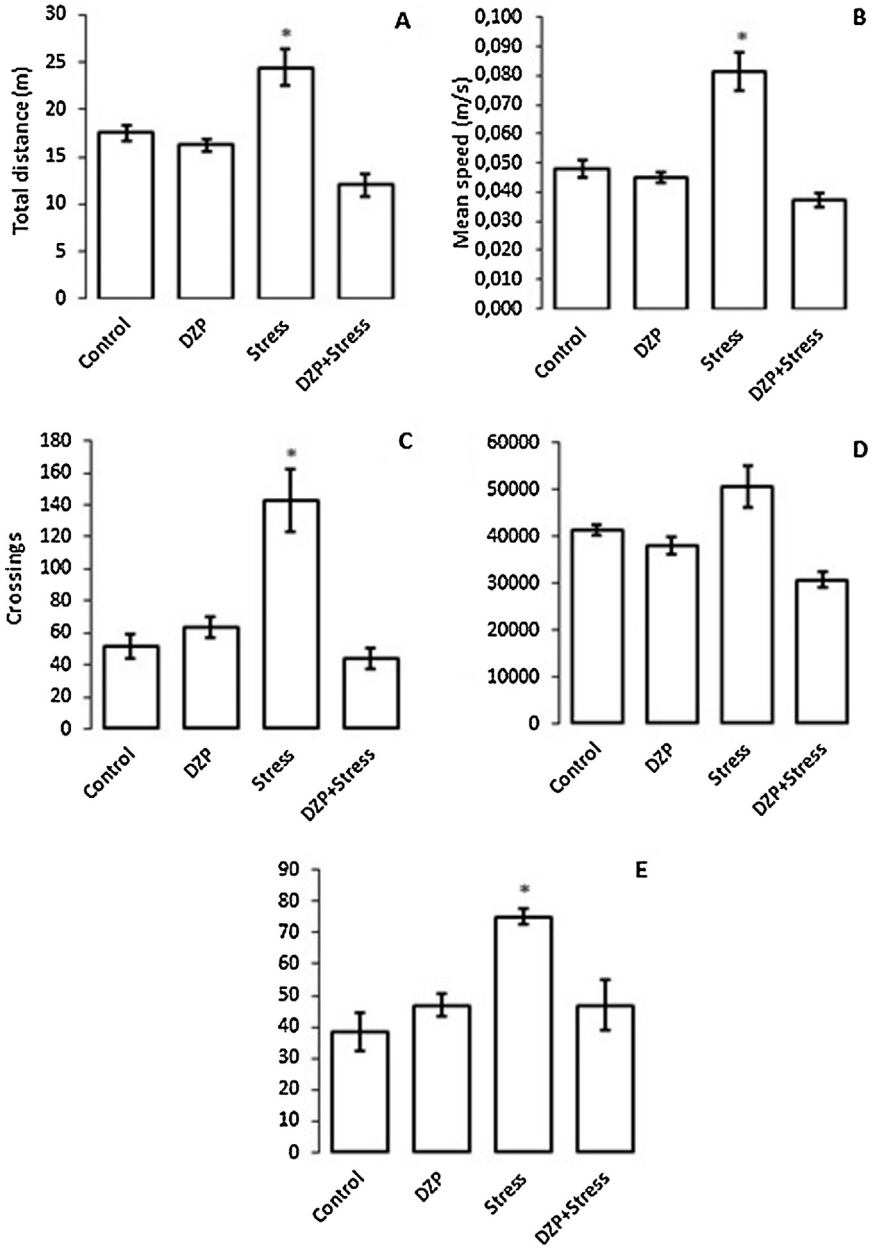

Fig. 3. Novel tank test in zebrafish exposed to diazepam and controls. (A) Total distance traveled; (B) mean speed; (C) number of crossings; (D) absolute angle turn, and (E) induration on the bottom of the tank. The * indicates statistical difference verified by two-way ANOVA followed by the Tukey test. Data are expressed as mean ± standard error of the mean of 9–12 animals per treatment.

tion of staying in segment 1 indicated the preference for proximity to the opponent and therefore aggressiveness.

2.6. Statistical analysis

Data were analyzed by two-way ANOVA followed by the Tukey test. The homogeneity of variance was determined using Hartley's test, and normality was assessed using the Kolmogorov–Smirnov test.

3. Results

All statistical data from behavior tests are shown in Table 1.

3.1. Novel tank test

Stress increased locomotor activity, as analyzed by total distance, average speed, and number of crossings, whereas it did not change the absolute turn angle. In addition, stressed fish spent more time in the bottom area of the tank. FLU and DZP did not affect locomotion *per se*, but prevented all of the stress-induced behavioral changes (Figs. 2 and 3).

3.2. Y-maze task

The acute stress protocol, FLU, or DZP, did not alter memory acquisition in this task (Figs. 4 and 5).

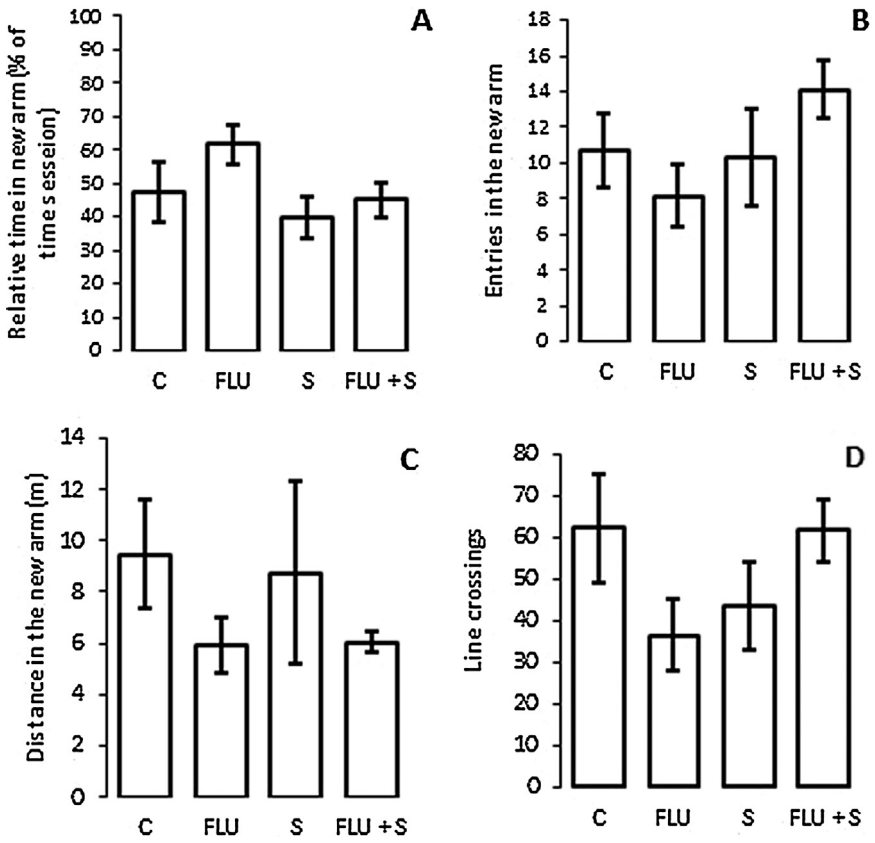

Fig. 4. Zebrafish response to the Y-maze task after training and locomotion parameters during the session and exposure to fluoxetine and stress. (A) Relative time in the new arm (% of shooting session length); (B) entries into the new arm; (C) distance traveled in the new arm and (D) crossings between lines. Data are expressed as mean ± standard error of the mean of 7–10 animals per treatment. Different symbols indicate statistical difference verified by two-way ANOVA followed by the Tukey test.

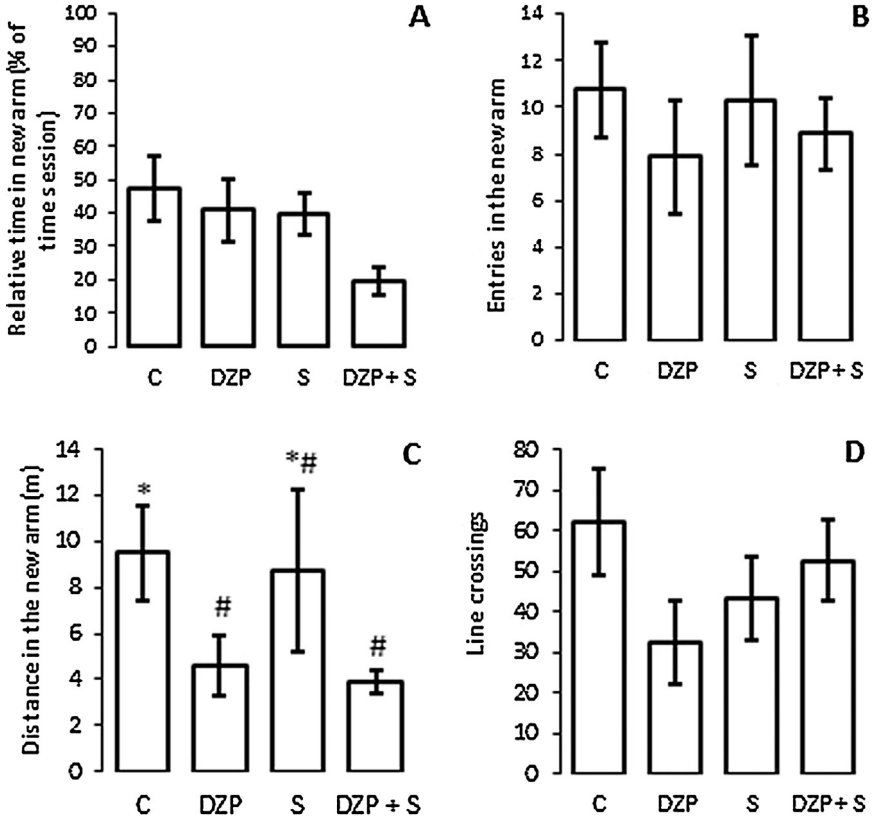

Fig. 5. Zebrafish response to the Y-maze task after training and locomotion parameters during the session and exposure to stress and diazepam. (A) Relative time in the new arm; (B) entries in the new arm; (C) distance traveled in the new arm and (D) crossings between lines. Data expressed as mean ± standard error of the mean of 7–10 animals per treatment.

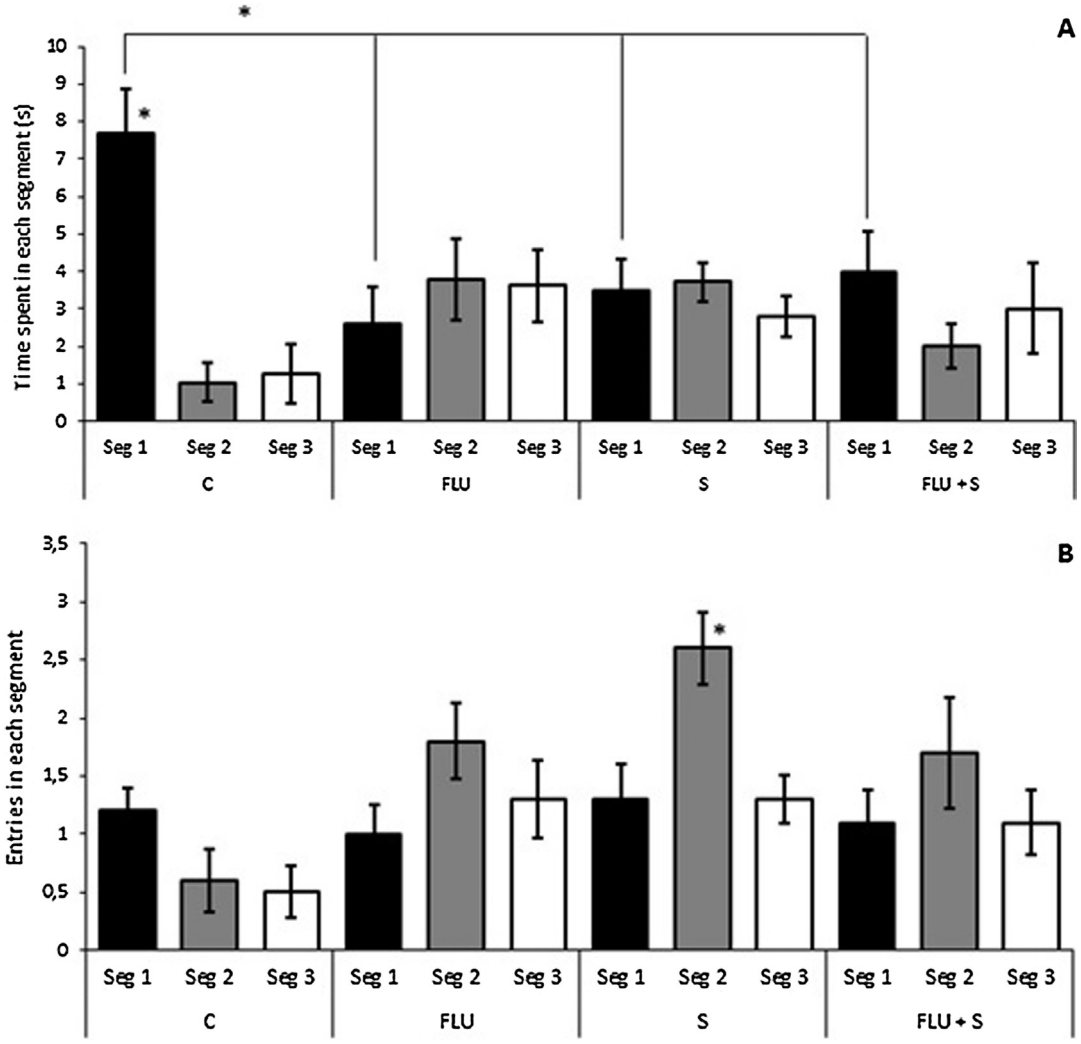

Fig. 6. Zebrafish response to the social interaction test after exposure to stress and fluoxetine. (A) Time in different time segments. The asterisk on the line indicates the time in segment 1 for the control fish was greater than all others that did not differ by Bonferroni test. The asterisk below the line indicates that only the control fish showed significant differences between the duration of time spent in those segments (two-way ANOVA followed by the Tukey test). (B) Entries in the different segments. The asterisk indicates that only in the stressed fish was difference between the time spent in the segments (two-way ANOVA followed by the Tukey test).

3.3. Social interaction test

The acute stress protocol and FLU and DZP exposure decreased social interaction, since fish in these treatments spent less time in the segment near to the shoal aquaria (Figs. 6 and 7). However, they showed no preference for any of the segments, as seen in the control group. Stress promoted an increase in the number of entries into segment 2. This effect was not observed in the control and drug-exposed groups.

3.4. Aggressiveness

After 30 s of habituation in the test tank, stressed fish showed more aggressive behavior as compared to control fish. FLU did not alter this behavior. After 10 min of habituation in the test tank, FLU reduced aggressiveness in non-stressed fish but not in stressed fish (Fig. 8). Moreover, DZP did not alter aggressiveness following either habituation time (Fig. 9).

4. Discussion

Here we show that acute stress increases anxiety-like behavior as evidenced by the increased time spent in the bottom area of the tank. We also showed that acute exposure to FLU or DZP exerted an anxiolytic-like effect, reversing the behavioral changes provoked by the acute stress protocol. The acute stress protocol induced locomotor changes that were reversed by FLU and DZP. These drugs, at the concentrations used, did not cause sedation or relevant motor side effects. Thus, the anxiolytic effects of FLU and DZP may be related to the blockade of cortisol responses to acute stress as verified previously in zebrafish [27].

Zebrafish show anxiety-like behavior when stressed [29,4,5,32–37]. This behavior was verified by the increased time spent in the bottom area of the tank. The anxiolytic effect of FLU has been reported in the literature following exposure daily or every 2 days at a concentration of 100 μg/L [4,35,36]. The influence of FLU on the stress neuroendocrine axis has been reported in some studies [2,38]. There are studies reporting that FLU influences the genetic expression of glucocorticoid [35–38] and mineralocorticoid receptors as well as the expression of GABA transporters in the brain, causing attenuation of the stress response [36,37].

The anxiolytic effect of FLU has been reported in rodents [39] and fish [4,40,41]. In addition to modulating serotonin, FLU exerts an anxiolytic effect that modulates neuropeptides and neurosteroids [36]. Although the anxiolytic effect exerted by FLU is well known,

Table 1
Results of two-way analysis of variance (ANOVA) of different behavioral tests.

Behavior test/parameter	Drug	Figs.	Comparison	DF	*F*-value	*P*-value	Partial Eta-squared
Novel tank/distance travelled	FLU	2 A	Interaction	1, 34	4.3	0.046	0.112
			Drug effect	1, 34	6.78	0.003	0.285
			Stress effect	1, 34	5.16	0.03	0.132
Novel tank/mean speed	FLU	2 B	Interaction	1, 34	10.86	0.002	0.242
			Drug effect	1, 34	10.69	<0.0001	0.386
			Stress effect	1, 34	12.12	0.001	0.263
Novel tank/crossing number	FLU	2 C	Interaction	1, 34	9.76	0.004	0.223
			Drug effect	1, 34	5.17	0.011	0.233
			Stress effect	1, 34	13.78	0.001	0.288
Novel tank/absolute turn angle	FLU	2 D	Interaction	1, 34	7.06	0.012	0.172
			Drug effect	1, 34	5.17	0.011	0.233
			Stress effect	1, 34	0	0.995	0
Novel tank/time in tank bottom	FLU	2 E	Interaction	1, 34	4.32	0.045	0.113
			Drug effect	1, 34	4.88	0.014	0.223
			Stress effect	1, 34	9.4	0.004	0.217
Novel tank/distance travelled	DZP	3 A	Interaction	1, 34	14.91	<0.0001	0.305
			Drug effect	1, 34	22.51	<0.0001	0.398
			Stress effect	1, 34	0.88	0.354	0.025
Novel tank/mean speed	DZP	3 B	Interaction	1, 34	20.4	<0.0001	0.375
			Drug effect	1, 34	26.52	<0.0001	0.438
			Stress effect	1, 34	8.24	0.007	0.195
Novel tank/crossing number	DZP	3 C	Interaction	1, 34	16.81	<0.0001	0.331
			Drug effect	1, 34	10.14	0.003	0.230
			Stress effect	1, 34	6.98	0.012	0.170
Novel tank/absolute turn angle	DZP	3 D	Interaction	1, 34	7.17	0.011	0.174
			Drug effect	1, 34	14.38	0.001	0.297
			Stress effect	1, 34	0.11	0,74	0.003
Novel tank/time in tank bottom	DZP	3 E	Interaction	1, 34	17.11	<0.0001	0.335
			Drug effect	1, 34	0.02	0.9	0
			Stress effect	1, 34	0.87	0.357	0.25
Y-maze task/relative time in the new arm	FLU	4 A	Interaction	1, 31	0.42	0.521	0.013
			Drug effect	1, 31	1.91	0.177	0.058
			Stress effect	1, 31	3.06	0.09	0.09
Y-maze task/entries in the new arm	FLU	4 B	Interaction	1, 31	2.39	0.132	0.072
			Drug effect	1, 31	0.03	0.865	0.001
			Stress effect	1, 31	3.62	0.066	0.105
Y-maze task/distance traveled in the new arm	FLU	4 C	Interaction	1, 31	0.02	0.875	0.001
			Drug effect	1, 31	2.79	0.105	0.083
			Stress effect	1, 31	0.04	0.845	0.001
Y-maze task/crossings between lines	FLU	4 D	Interaction	1, 31	4.95	0.033	0.138
			Drug effect	1, 31	0.14	0.711	0.004
			Stress effect	1, 31	0.1	0.749	0.003
Y-maze task/relative time in the new arm	DZP	5 A	Interaction	1, 28	0.83	0.369	0.029
			Drug effect	1, 28	3.19	0.085	0.102
			Stress effect	1, 28	3.68	0.065	0.116
Y-maze task/entries in the new arm	DZP	5 B	Interaction	1, 28	0.01	0.941	0
			Drug effect	1, 28	0.51	0.483	0.018
			Stress effect	1, 28	0.16	0.695	0.006
Y-maze task/distance traveled in the new arm	DZP	5 C	Interaction	1, 28	0	0.974	0
			Drug effect	1, 28	5.1	0.032	0.015
			Stress effect	1, 28	0.12	0.736	0.004
Y-maze task/crossings between lines	DZP	5 D	Interaction	1, 28	3.1	0.089	0.1
			Drug effect	1, 28	0.83	0.371	0.029
			Stress effect	1, 28	0.01	0.953	0
Social interaction/time in segment 1	FLU	6 A	Interaction	1, 36	7.36	0.01	0.170
			Drug effect	1, 36	5.03	0.031	0.123
			Stress effect	1, 36	1.89	0.178	0.05
Social interaction/entries in segment 1	FLU	6 B	Interaction	1, 36	0	1	0
			Drug effect	1, 36	0.58	0.449	0.016
			Stress effect	1, 36	0.15	0.704	0.004
Social interaction/time in segment 1	DZP	7 A	Interaction	1, 36	2.17	0.15	0.057
			Drug effect	1, 36	3.06	0.089	0.078
			Stress effect	1, 36	5.75	0.022	0.138
Social interaction/entries in segment 1	DZP	7 B	interaction	1, 36	0.16	0.69	0.004
			Drug effect	1, 36	2.59	0.16	0.067
			Stress effect	1, 36	0	1	0
Aggressiveness test (1′)	FLU	8	Interaction	1, 26	9.69	0.004	0.272
			Drug effect	1, 26	0.71	0.408	0.026
			Stress effect	1, 26	0.29	0.591	0.011
Aggressiveness test (10′)	FLU	8	Interaction	1, 26	0.47	0.5	0.018
			Drug effect	1, 26	0.13	0.720	0.005
			Stress effect	1, 26	2.65	0.116	0.093
Aggressiveness test (1′)	DZP	9	Interaction	1, 26	1.67	0.208	0.06
			Drug effect	1, 26	0.608	0.443	0.023
			Stress effect	1, 26	2.4	0.133	0.085
Aggressiveness test (10′)	DZP	9	Interaction	1, 26	1.41	0.245	0.052
			Drug effect	1, 26	2.94	0.098	0.102
			Stress effect	1, 26	4.62	0.041	0.151

The table summarizes the main effects of and the interaction between drug and acute stress. DF = degrees of freedom.

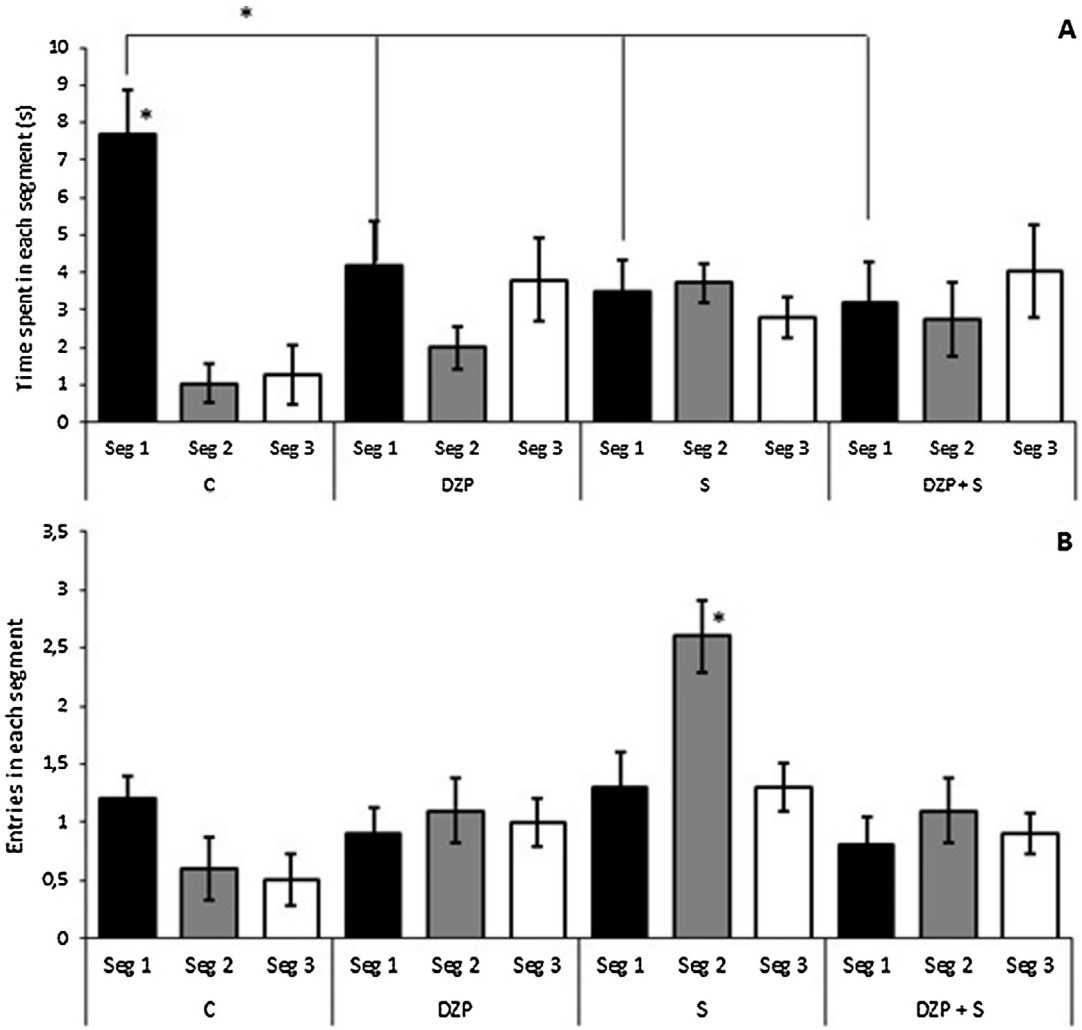

Fig. 7. Zebrafish response to social interaction test after exposure to stress and diazepam. (A) Time in different segments. The asterisk on the line indicates that the time spent by the control fish in the segment 1 was greater than that of to all others that did not differ by Bonferroni test. The asterisk below the line indicates that only the control fish showed differences between the time spent in those segments (two-way ANOVA followed by the Tukey test). (B) Entries into the different segments. The asterisk indicates that only in the stressed fish was there a significant difference between the duration of time spent in the segments (two-way ANOVA followed by the Tukey test).

the exact mechanisms by which this drug blocks the biological response of cortisol in response to stress are not yet clear. On the other hand, the anxiolytic effect of DZP in zebrafish is well established from studies using light/dark and novel tank tests [28,42].

We showed that acute stress and exposure to FLU and DZP immediately before training did not interfere with memory acquisition as evidenced by results of the Y-maze task. However, other studies have reported different influences of FLU on memory acquisition. For example, perinatal exposure to environmental concentrations of FLU modifies memory processes in the cuttlefish *Sepia officinalis*, altering learning and consolidation [43]. Subcutaneous administration of FLU increases consolidation recovery but not acquisition memory in mice [44]. Similarly, studies have reported amnesic effects of DZP that compromise acquisition memory [45,46], object recognition [46], and spatial memory [47] in humans [45] and rodents [46,47]. In young chicks, the effect of DZP on GABA receptors is dose-dependent; low doses increase memory and high doses inhibit memory [48]. The fact that we do not have evidence of memory changes in this study can be attributed to the time interval between exposure to stress and the memory test, which was greater than 60 min, during which time cortisol levels have a tendency to decline to pre-stress levels [27,49]. It was not possible to correlate behavior with cortisol levels in this study.

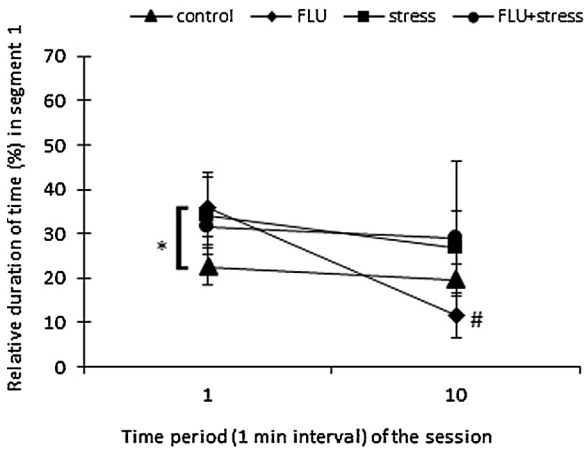

Fig. 8. Zebrafish response to the aggressiveness test after exposure to stress and fluoxetine. Stressed fish spent more time in the segment nearest to the mirror image than did controls in time interval 1 (two-way ANOVA followed by the Tukey test), but not in the 10-min time interval (two-way ANOVA followed by the Tukey test). The # symbol indicates that fish exposed to fluoxetine spent more time in the segment nearest to the mirror image at time interval 1 than they did in time interval 10.

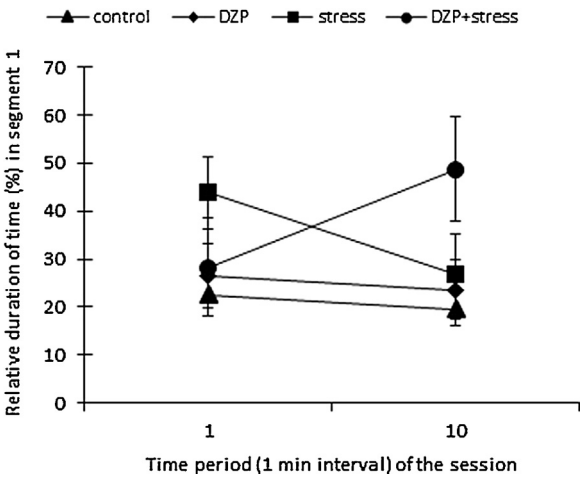

Fig. 9. Zebrafish response to aggressiveness test following exposure to stress and diazepam. The # symbol indicates that fish exposed to fluoxetine spent more time in the segment nearest to the mirror image at time interval 1 than at time interval 10. The time spent in the segment nearest to the mirror image did not differ between both time intervals (two-way ANOVA followed by the Tukey test).

Zebrafish live in shoal communities and exhibit social behavior from the beginning of life. This form of interaction is important to minimize the risk of predation. This species displays a natural tendency to swim close to conspecifics in preference to an empty tank [50]. Here, we showed that fish exposed to acute stress reduce their social interactions. Stress can alter the expression of corticotropin-releasing hormone (CRH) receptors in the hippocampus and amygdala, and thus, can promote behavioral changes related to socialization and aggression in mice [51] and fish [52]. In addition, neurotransmitters and neuropeptides are related to stress, social interaction, and aggressiveness in humans [53], rodents [54], and fish [55]. In fact, FLU increases the expression of the neuropeptides isotocin and vasotocin, promoting an increase in social interaction and decrease in anxiety and aggressiveness in zebrafish [55]. Similarly, chronic FLU treatment normalizes behavioral and biochemical changes in mice with social aversion after chronic stress [56]. Nevertheless, we showed that acute exposure to FLU and DZP eliminated the preference for proximity to conspecifics both in stressed and non-stressed fish. We attribute this effect to fish's altered perception of their relation to the shoal. Considering the importance of fish agglomeration near the shoal, this lack of interaction can lead to vulnerability that affects reproduction and survival.

Stressed fish showed more aggressive behavior compared to non-stressed fish. FLU reduced aggressive behavior in non-stressed fish, but it did not exert this effect on stressed ones. Moreover, DZP did not alter the aggressive behavior. Aggressiveness increased in *Rhesus* monkeys after stress [57]. In rainbow trout, CRH interfered with the levels of 5-HT and dopamine, thereby inhibiting aggressive behavior [52].

In aquatic ecosystems, response to acute stress is important for reproduction, osmoregulation, and predator avoidance [58–60]. On the other hand, the modulation of behavior by FLU and DZP may impair the balance of aquatic ecosystems, although the concentrations used in this study are higher than those measured in the environment. However, there are critical points at which the release of urban effluents may give rise to concentrations greater than those reported in the natural environment [61].

One limitation of this study is that we cannot directly extrapolate these results to the aquatic environment, where fish are chronically exposed to xenobiotics since early development, and in this study, fish were briefly exposed to the drugs. However, this does not lessen the importance of our results, since data about the effects of these drugs modulating acute stress-induced behavior, are very scarce.

Considering the evidence pointing to the presence and persistence of psychotropic drug residues or their active metabolites in water [15,23], as well their action on the CNS, it is essential to assess their effects on aquatic ecosystems and the human populations that may use contaminated water sources.

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