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# Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol☆

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ABSTRACT

There is growing interest in zebrafish as a model organism in behavioral pharmacology research. Several anxiety behaviors have been characterized in zebrafish, but the effect of anxiolytic drugs on these parameters has been scarcely studied. The purpose of this work was to assess the predictive validity of acute treatment with anxiolytic drugs on behavioral parameters of anxiety. In the first task we simultaneously observed behavior of adult zebrafish on four parameters: height in the tank, locomotion, color, and shoal cohesion. The second task was the assessment of light/dark preference for 5 min. The benzodiazepines clonazepam, bromazepam, diazepam, and a moderate dose of ethanol significantly reduced shoal cohesion. Buspirone specifically increased zebrafish exploration of higher portions of the tank. In the light/dark task, all benzodiazepines, buspirone, and ethanol increased time spent in the light compartment. After treatment with anxiolytics, fish typically spent more than 60 s and rarely less than 40 s in the light compartment whereas controls ($n = 45$) spent $33.3 \pm 14.4$ s and always less than 60 s in the light compartment. Propranolol had no clear effects in these tasks. These results suggest that light/dark preference in zebrafish is a practical, low-cost, and sensitive screening task for anxiolytic drugs. Height in the tank and shoal cohesion seem to be useful behavioral parameters in discriminating different classes of these drugs.



## 1. Introduction

Zebrafish (*Danio rerio*) have many inherent advantages as a model organism, such as low cost, easy handling and maintenance as compared with other vertebrate models, and 70–80% genetic homology to humans (Barbazuk et al., 2000; Goldsmith, 2004; Egan et al., 2009). Although this degree of homology with humans is not as high compared to rodents, it is favorable compared to invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans*. Despite the increasing popularity of zebrafish in neuroscience, behavioral assessments in this model animal need further study.

In this species, behavior can be easily observed and quantified in a controlled environment (Beis and Stainier, 2006; Miklósi and Andrew, 2006; Levin et al., 2007). The behavioral repertoire of zebrafish is complex and allows the development of a range of behavioral parameters (Gerlai et al., 2000; Zon and Peterson, 2005). Furthermore, the characterization of zebrafish behavior is important for the generation of large-scale behavioral screenings and a system-level analysis of how chemicals affect behavior. Such behavioral screening tests may improve our understanding of neurobiology and drug action, and accelerate the pace of psychiatric drug discovery (Berghmans et al., 2007; Kokel and Peterson, 2008; Rihel et al., 2010). Recently, Rihel et al. (2010) evaluated the effect of 3968 compounds on locomotor activity and rest/wake regulation of larval zebrafish, finding specific behavioral fingerprints for many psychotropic classes. However, the characterization of more specific behavioral tasks is important for better use of zebrafish in the study of brain function.

Several measures of fear and anxiety have been proposed in zebrafish. Fear is defined as a response to imminent threat (Craske et al., 2009), which in zebrafish has been studied as reactions to predators and alarm pheromone, such as fleeing, erratic movements, freezing behavior, bottom-dwelling and crowding to form a dense shoal (Pfeiffer, 1977). Gerlai et al. (2009) has established automated measures of zebrafish escape from animated images of sympatric predators. Freezing behavior also increases with caffeine treatment, exposure to alarm substances (Egan et al., 2009) or to aversive environments (Blaser et al., 2010).

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In contrast, anxiety represents a response to future or possible threats, usually manifested as avoidance behavior (Craske et al, 2009). In zebrafish, Levin et al. (2007) have proposed that height in the tank may be a useful measure of anxiety that resembles thymogtaxis observed in rodents. Indeed, acute nicotine (Levin et al., 2007), buspirone (Bencan et al., 2009), and chronic fluoxetine (Egan et al., 2009) treatment reduce bottom-dwelling, but the results of the benzodiazepines diazepam and chlordiazepoxide were inconsistent in this task (Bencan et al., 2009). Moreover, preference of zebrafish for dark along with aversion to light environments has been put forward as a useful behavioral parameter (Serra et al., 1999; Maximino et al., 2010) and is reduced after treatment with fluoxetine, clonazepam, buspirone and ethanol (Maximino et al, 2011). Another behavior that may be related to anxiety and self-protection in zebrafish is their preference for being in groups, which is reduced by acute treatment with ethanol (Gerlai et al., 2000; Gerlai, 2003). However, these putative measures of anxiety have been poorly characterized in terms of face and predictive validity, especially regarding response specificity to different classes of anxiolytics.

Anxiolytics are generally divided into two groups of medications, benzodiazepines (BDZs) and non-benzodiazepines (barbiturates, propranolol, and buspirone) (Menard and Treit, 1999; Bianchi et al., 2009). Benzodiazepines potentiate $GABA_A$ receptor function by increasing channel opening frequency, producing hypnotic effects by acting on $\alpha_1$ (Mckernan et al., 2000) and anxiolytic activity by acting on $\alpha_2$ subunits (Löw et al., 2000). Buspirone exerts anxiolytic effects by acting as a partial agonist at serotonin 5-$HT_{1A}$ receptors (Ohlsen and Pilowsky, 2005). Ethanol also has acute anxiolytic effects, probably mediated by $GABA_A$ receptors (Radcliffe et al., 1999; Kumar et al., 2009), with depressant effects on the central nervous system at higher doses. Propranolol is a non-selective $\beta_1$- and $\beta_2$-adrenergic antagonist with anxiolytic effects only for performance and somatic anxiety (Granville-Grossman and Turner, 1966; Tyrer and Lader, 1974).

Our objective was to assess the effect of anxiolytic drugs on putative behavioral parameters of anxiety in zebrafish. With the goal to develop fast, simple and valid tasks and endpoints to assess anxiolytic action, we used a protocol (the Group Behavior Task, GBT) that allows evaluating shoal cohesion, height in the tank and locomotion simultaneously, which are parameters putatively related to anxiety in zebrafish. Thus, we investigated the behavioral responses of zebrafish acutely treated with different anxiolytics in the GBT and the light/dark task.

## 2. Material and methods

### 2.1. Animals and housing

A total of 391 adult male and female 'wild type' (short fin) zebrafish (*Danio rerio*) were obtained from a commercial supplier (Red Fish, Porto Alegre, Brazil). All fish were acclimated for at least two weeks in the laboratory environment and housed in groups of 30–50 fish in a 50 L thermostat tank (28 ± 2 °C) with water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines and heavy metals; pH 7.2; conductivity 501 μS, filtered with Tetra Whisper® PF10) and continuously aerated (7.2 mg $O_2$/L). Fish were kept on a 14–10 h day/night cycle and fed three times a day with commercial flakes and supplemented with live brine shrimp.

All protocols were reviewed and approved by an Institutional Review Committee for the use of Human or Animal Subjects (110/08-CEUA-PUCRS) and the procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

### 2.2. Chemicals

Clonazepam (Rivotril®), bromazepam (Lexotan®), diazepam (União Química, Brazil), and ethanol were purchased from common commercial suppliers. Buspirone and propranolol were from Sigma-Aldrich (USA).

### 2.3. Treatments

Three fish were placed into a beaker filled with 300 mL of the water from the respective test tanks (with the corresponding drug concentration) for a pretreatment of 10 min. Oxygen levels remained adequate during this treatment (8 ppm). In the GBT, drug treatment during the task was maintained as indicated. Pilot studies comparing 10, 30, and 50 min indicated that 10 min were sufficient to induce clear behavioral effects for all drugs tested. Drug concentrations (clonazepam 0.3 mg/L; bromazepam 1.5 mg/L; diazepam 0.16 mg/L; buspirone 1 and 3 mg/L; propranolol 3 mg/L) were determined based on pilot drug response curves and the relative potency of benzodiazepines. Ethanol concentrations (0.25 and 0.5%) were chosen based on previous data (Gerlai et al., 2000, 2006).

Raters (interrater reliability $r > 0.90$, Spearman test) were blind to treatments, which were administered by another researcher, and a control group was included in every experiment.

### 2.4. Behavioral apparatus

All procedures were performed in an isolated room. One day (24 h) prior to the experiments, both male and female fish (approximately 1:1 distribution) were moved to the experimental room with identical conditions to the fish housing apparatus to reduce environmental variance during behavioral assays.

The Group Behavior Task (GBT) was performed according to Piato et al. (2011). Test tanks (24 × 8 × 20 cm, length × width × height) with 2.7 L of water (15 cm high) were used for simultaneous evaluation of height in the tank, locomotion, color, and shoal cohesion. Water temperature was maintained with heaters. The lateral and back sides were visually blocked with white opaque self-adhesive plastic film to reduce the influence of the surrounding area and to facilitate observation.

For the light/dark task, a glass tank (18 × 9 × 7 cm, length × width × height, adapted from Rawashdeh et al., 2007) was divided by a sliding guillotine-type partition (9 × 7 cm) in two equally sized dark and white compartments using black or white self-adhesive film externally covering walls, floor and the corresponding sides of the partition. The tank water level was 3 cm and the partition was raised 1 cm above the tank floor to allow zebrafish to swim freely from one side of the tank to the other.

### 2.5. Behavioral scores in the GBT

#### 2.5.1. Height in the tank

The position (bottom × middle × upper levels) was considered an index of anxiety, similar to the position near the wall versus the center of an open field with rodents (Levin et al., 2007; Egan et al., 2009).

Fish position in the test tank experiment was noted according to the following scores during 1 min-observations: 1—only in the bottom third of the tank; 2—preference for the lower two thirds of the tank; 3—similar times exploring the three thirds; 4—preference for the upper two thirds; and 5—only in the upper third. This score has a 0.90 correlation (Spearman test) with an objective measure using stopwatch performed by separate and independent observers blinded to each others' results ($n = 65$).

#### 2.5.2. Locomotion

Locomotor activity was used as a general index of behavioral excitation/inhibition. Activity was evaluated by comparing to 'internal control' fish, using the following scores: 1—virtually immobile; 2—slower than normal; 3—normal; 4—increased locomotion; and 5—intense locomotion. This score has a 0.50 correlation (Spearman test)

with an objective measure using crossing counts (frontal area of the tank divided in 9 rectangles) performed by separate and independent observers blinded to each others' results (n = 63). Although this correlation level is only moderate, it is acceptable to detect gross changes in locomotion, as in our pilot experiments with higher doses of benzodiazepines.

#### 2.5.3. Color

Zebrafish change their color in response to certain stimuli. Fish that exhibit signs of fear (e.g., freezing or erratic movement) quickly become pale, especially when the background is light. When fish are more excited or aggressive, they become more chatoyant (Gerlai, 2003).

Fish color was rated visually by comparing to 'internal control' fish and scored as follows: 1—pale; 2—lighter than normal but not pale; 3—normal; 4—darker than normal but not chatoyant with dark-blue stripes; and 5—chatoyant with dark-blue stripes.

#### 2.5.4. Shoal cohesion

Zebrafish prefer swimming in groups and group aggregation is termed shoal cohesion (Engeszer et al., 2007; Miller and Gerlai, 2007; Saverino and Gerlai, 2008). This behavioral strategy is thought to be effective against predators in several fish species (Detrich et al., 1999; Gerlai et al., 2000). In contrast to other studies using only one fish during experiments, placing three in the test tank allows the maintenance of their natural shoal behavior.

Shoal cohesion was measured individually by comparing to 'internal control' fish (i.e., a group of three untreated fish habituated in an independent tank) according to the following scores: 1—complete lack of group cohesion or fish interaction; 2—loose or partial shoaling behavior; 3—normal distance and shoaling behavior compared to 'internal control'; and 4—increased shoal cohesion. This score has a −0.81 correlation (Spearman test) with an objective measure of distance between the 3 fish (using Image J software) in pictures extracted from video recordings every 15 s for 5 min. This analysis was performed by separate and independent observers blinded to each others' results (n = 55).

### 2.6. GBT experiments

GBT experiments were repeated four times (for four groups of three fish), totaling 12 fish for each drug concentration.

#### 2.6.1. Experiment 1: effects of benzodiazepines, buspirone and ethanol in the GBT

Firstly, each drug was prepared directly in the test tank (2.7 L). Three fish were placed into a beaker filled with 300 mL of the water from the respective test tanks (with the corresponding drug) for a pretreatment of 10 min. After that fish were gently placed in the tank and observed for 10 min. A separate group underwent the same procedure in a test tank without drugs and was called 'water control'. Raters were blind to these treatment groups. Another group of three fish without drug treatment was used as a reference of normal behavior (e.g., locomotion and color) to help the rater and was called 'internal control'.

Behavioral characteristics of zebrafish were evaluated during exposure to the test tank after the pretreatment period. During the task, fish were observed by a rater blinded to treatments and 'water control' in minutes 1, 2, 3, 4, 5, and 10.

#### 2.6.2. Experiment 2: effect of exposure to drugs during the task in the GBT

The same apparatus of experiment 1 was used. After 10 min of drug pretreatment, three fish were transferred to the test tank and analyzed at minutes 1, 2, 3, 4, 5, and 10. The same procedure was also conducted in the presence (continuous treatment) or absence of drugs in the test tank during the experiment after 10 min pretreatments. This procedure was performed to examine if the presence of the drug during the task was required for the observed effect.

#### 2.6.3. Experiment 3: effects of drugs in the GBT during 15 min without pretreatment

Each drug was prepared directly in the test tank (2.7 L). Three fish were placed to the GBT and analyzed during 15 min with the anxiolytic drugs. This experiment was performed to evaluate the time required for drugs to induce their effects as an indirect measure of drug distribution.

#### 2.6.4. Experiment 4: effect of acute stress induced by a sinker on fish treated with buspirone in the GBT

Buspirone was prepared directly in the test tank (2.7 L). Three fish were placed to the GBT and analyzed during 10 min. After 5 min of observation a sinker (15 g) was dropped into the tank and behavior was analyzed for more 5 min. This procedure allowed assessing if the effect of buspirone on increasing exploration of the top of the tank was reversible and not due to an inability to swim at the bottom of the tank.

### 2.7. Light/dark task

Zebrafish show a marked preference for dark zones (Serra et al., 1999; Blank et al., 2009; Maximino et al., 2010). Based on a similar innate aversion of rodents to brightly illuminated areas (Bourin and Hascoët, 2003), the light/dark task is classically used to evaluate the effect of anxiolytics in rodents (Hascoët et al., 2001).

Fish were placed in the light zone of the apparatus with drug-free water and the following measures were recorded for 5 min: 1) latency to the first entry in the dark compartment; 2) time spent in the light compartment; and 3) number of crossings between compartments. The apparatus was filled with 3 cm of water. This shallow tank restricts bottom-dwelling, which is a well established anxiety behavior in a new environment. In this way, the main protective strategy is black preference, which is the measure used in this task.

#### 2.7.1. Experiment 5: effects of benzodiazepines, buspirone and ethanol in the light/dark task

After drug pretreatment of 10 min in a beaker, fish were individually placed at the white side of the tank and allowed to swim freely between compartments. The time spent on each compartment and the number of crossings were recorded during 5 min. The latency to first enter in the dark compartment was also measured.

### 2.8. Statistical analysis

All score data were expressed as median + interquartile range. Differences between control and treated groups were evaluated by a Kruskal–Wallis followed by a Mann–Whitney test. In the light/dark task, differences between control and treated groups were evaluated by one-way ANOVA followed by a Dunnett post hoc test. SPSS 16.0 for Windows was used, and a significance level of $p<0.05$ was adopted.

## 3. Results

### 3.1. Experiment 1: effects of benzodiazepines, buspirone, and ethanol in the GBT

Buspirone significantly ($p<0.001$) increased height in the tank score across all minutes (Fig. 1A), i.e., fish spent more time exploring the upper part of the tank, without affecting shoal cohesion or locomotion. In contrast, all benzodiazepines (clonazepam, bromazepam and diazepam) and ethanol significantly ($p<0.001$) reduced

shoal cohesion (Fig. 1B) without affecting height in the tank and locomotion, except for a small effect of bromazepam reducing locomotion ($p<0.001$). These effects were more pronounced at 5 and 10 min of observation. Only ethanol increased color score (darker blue stripes, Fig. 1D). Propranolol had no effect on GBT endpoints (data not shown). These results suggest that height and shoal cohesion are anxiety parameters that are modulated by different neurotransmitter systems and drugs.

### 3.2. Experiment 2: effect of exposure to drugs during the task in the GBT

We also tested if drug pretreatment (10 min) without drug exposure during the task was sufficient to produce behavioral changes (Fig. 2). For simplicity, results are shown as median and interquartile range only at min 5. All benzodiazepines produced reduction of shoal cohesion with or without drug exposure during the task. The effect of buspirone on height and ethanol on shoal cohesion were more apparent with continuous treatment during the task ($p<0.001$ with X without treatment during the task), but were still significantly different from control ($p<0.001$) with their respective pretreatments without continuous exposure.

### 3.3. Experiment 3: effects of drugs in the GBT during 15 min without pretreatment

Buspirone significantly ($p<0.001$, Fig. 3A) increased height in the tank at all time points compared to control group. Diazepam significantly ($p<0.001$, Fig. 3A) reduced height in the tank after min 8, except at min 11. Shoal cohesion was reduced by all benzodiazepines after min 8 ($p<0.001$, Fig. 3B) and by ethanol at min 9, 10, 12, and 15. The insert (Fig. 3B) shows the medians of min 10–15 per

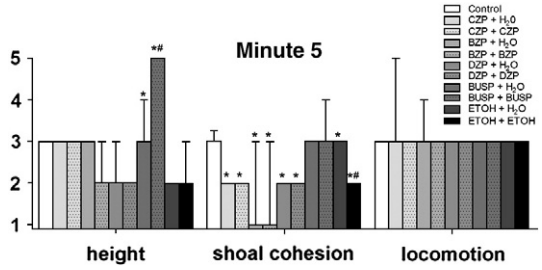


**Fig. 2.** Effects of clonazepam (CZP 0.3 mg/L), bromazepam (BZP 1.5 mg/L), diazepam (DZP 0.16 mg/L), buspirone (BUSP 3 mg/L), and ethanol (ETOH 0.5%) on height in the test tank, shoal cohesion, and locomotion in the GBT. The fish were pretreated during 10 min with the above drugs and after that observed during 10 min in the tank with or without drugs. Data of 5th min are expressed as median + interquartile range. *$p<0.001 \times$ control. Kruskal–Wallis followed by Mann–Whitney test. $n=12$.

treatment, including control group. No drug treatment significantly affected locomotion or color (data not shown, except for ethanol at all time points). These results suggest that the pretreatment of 10 min was appropriate.

### 3.4. Experiment 4: effect of acute stress induced by a sinker on fish treated with buspirone in the GBT

Since the robust and immediate effect of buspirone on height in the tank could be due to a non-specific effect on swimming behavior (e.g. direct effect on swimming bladder), we evaluated if this effect was reversible by acute stress (dropping a sinker in the tank at min 5), leading to a fear reaction that make fish go to the bottom of the tank (Egan et al., 2009). Buspirone treatment (only during the task)

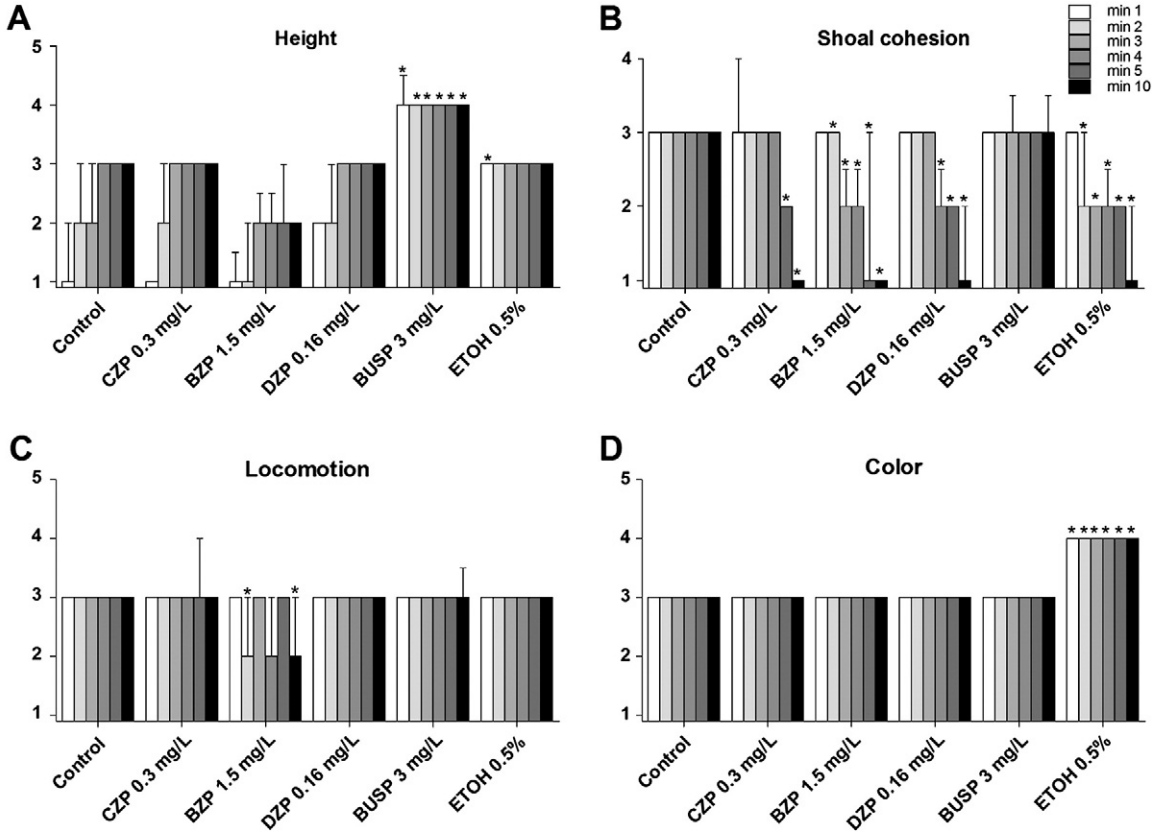


**Fig. 1.** Effects of clonazepam (CZP 0.3 mg/L), bromazepam (BZP 1.5 mg/L), diazepam (DZP 0.16 mg/L), buspirone (BUSP 3 mg/L), and ethanol (ETOH 0.5%) on height in the test tank (Fig. 1A), shoal cohesion (Fig. 1B), locomotion (Fig. 1C) and color (Fig. 1D) in the GBT. The fish were pretreated during 10 min with the above drugs and after that observed during 10 min in the tank with the same drug. Data are expressed as median + interquartile range. *$p<0.001 \times$ control. Kruskal–Wallis followed by Mann–Whitney test. $n=12$.

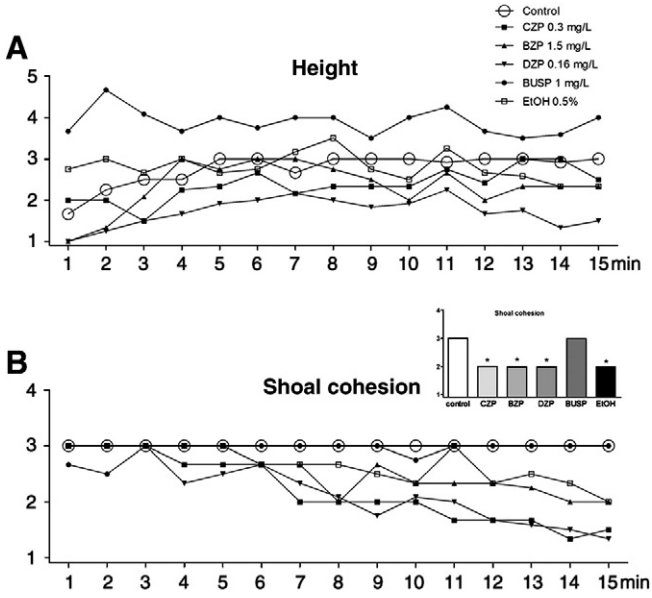


**Fig. 3.** Effects of clonazepam (CZP 0.3 mg/L), bromazepam (BZP 1.5 mg/L), diazepam (DZP 0.16 mg/L), buspirone (BUSP 3 mg/L), and ethanol (ETOH 0.5%) on height in the test tank (Fig. 3A) and shoal cohesion (Fig. 3B) in the GBT during 15 min with the above drugs. Data are expressed as median + interquartile range. *$p<0.001 \times$ control. Kruskal–Wallis followed by Mann–Whitney test. n = 12. The insert (Fig. 3B) shows median of min 10–15 per treatment, including control group.

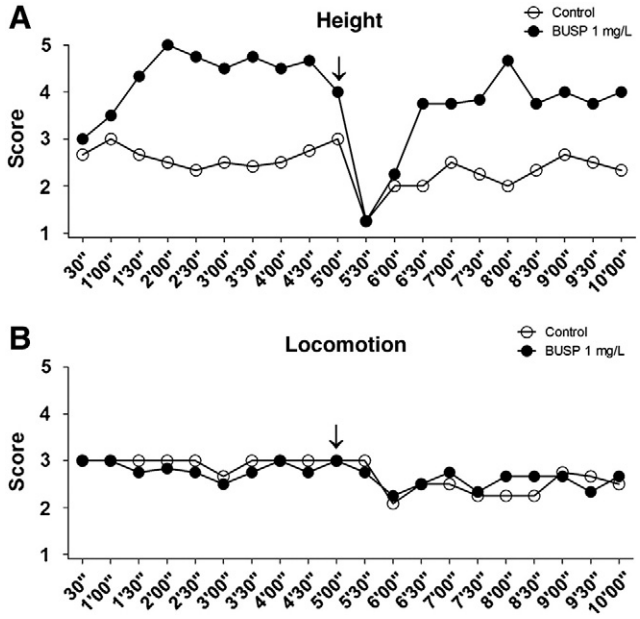


**Fig. 4.** Effects of buspirone (1 mg/L) on height in the test tank (Fig. 4A) and locomotion (Fig. 4B) and in the GBT during 10 min. After 5 min of observation a sinker was dropped into the tank and behavior was analyzed for a further 5 min. Data are expressed as median. Kruskal–Wallis followed by Mann–Whitney test. n = 12.

significantly increased height in the tank after min 1 without pretreatment ($p<0.001$, Fig. 4A). Immediately after stress induced by a sinker (5'30" and 6'), fish moved towards the bottom of the tank both in control and buspirone groups (Fig. 4A), returning to their baseline levels afterwards. Compared to min 5, locomotion was slightly, but significantly reduced after dropping the sinker (Fig. 4B) in both groups compared to their respective controls at min 6 only. Other parameters were not affected (data not shown). This result shows that the effects of buspirone were reversible by an anxiogenic behavioral intervention, suggesting that the increased exploration of the upper part of the tank is not due to non-specific effects of buspirone on swimming behavior.

### *3.5. Experiment 5: light/dark task*

In the light/dark task, the control group spent $33.3 \pm 14.4$ s of 300 s in the light compartment. All control fish spent less than 60 s in the light compartment (Fig. 5A).

After 10 min of drug pretreatment, all benzodiazepines, buspirone, the combination of diazepam and buspirone, and ethanol 0.5%, but not 0.25%, increased time spent in the light compartment ($F_{(8,150)} = 13.9$, $p<0.001$, Fig. 5A). After treatment with anxiolytics or 0.5% ethanol, fish typically spent more than 60 s and very rarely less than 30 s in the light compartment. Propranolol failed to affect behavior in this task.

The latency to the first entry in the dark compartment and the number of crossings was not altered by treatments (Fig. 5B and C, respectively).

## 4. Discussion

The current study demonstrated that benzodiazepines and ethanol specifically decreased shoal cohesion, whereas buspirone specifically increased height in the tank in the GBT. These effects were present at doses that did not significantly affect other behavioral parameters, such as locomotion and color (except for darker color with ethanol). In contrast, the light/dark task was sensitive for all anxiolytics and ethanol. Propranolol failed to affect anxiety-related behaviors in both tasks. Thus, the light/dark task may be an interesting screening task for anxiolytics, whereas shoal cohesion and height in the tank could be useful endpoints to differentiate the type of anxiolytic response.

Zebrafish have a natural tendency to initially remain at the bottom of a novel environment (e.g., a test tank) and then gradually, over a few minutes, explore the higher portions of the test tank (Levin et al., 2007; Egan et al., 2009). The fear response of zebrafish also includes forming stronger shoal cohesion, freezing, and becoming pale (Rehnberg and Smith, 1988; Gerlai et al., 2000). The exposure to the new environment of a tank is not particularly alarming to produce such behaviors, but the effects of benzodiazepines and ethanol on shoal cohesion became apparent only after 3–4 min in the tank, whereas the effect of buspirone is readily observed in the first minute. These distinct time courses and sensitivities to different drugs suggest that the neurobiological systems underlying height in the tank and shoal cohesion are quite independent, but differential kinetics of the drugs tested may play a role in their observed behavioral profile.

Bencan et al. (2009) first showed that buspirone significantly increased zebrafish exploration of the higher portions of the tank in a 5 min task at doses that did not have sedative effects. Moreover, in this study chlordiazepoxide failed to affect this parameter and diazepam slightly reduced bottom-dwelling, but not in a dose–response fashion. Levin et al. (2007) also showed that nicotine induced zebrafish to stay in the upper part of the tank in a 5 min task, and Egan et al. (2009) found that exposure to an alarm pheromone led fish towards the bottom part. Our study indicates that height and shoal cohesion represent distinct and independent anxiety/ defensive behaviors.

The results of Fig. 3 showed that a 10 min exposure to drugs was sufficient to exert behavioral effects for all drugs. Also, our preliminary results comparing 10 and 30 min of pretreatment did not show significant differences (data not shown). Of note, buspirone had a surprisingly rapid behavioral effect on height in the tank (since the first minute of exposure). However, buspirone produced significant effects even when absent in the task apparatus both in the GBT and in the light/dark task. Moreover, the effect of buspirone on height in the tank was transiently reversed by an acute stress induced by dropping a sinker in the tank, suggesting that it was not a non-specific

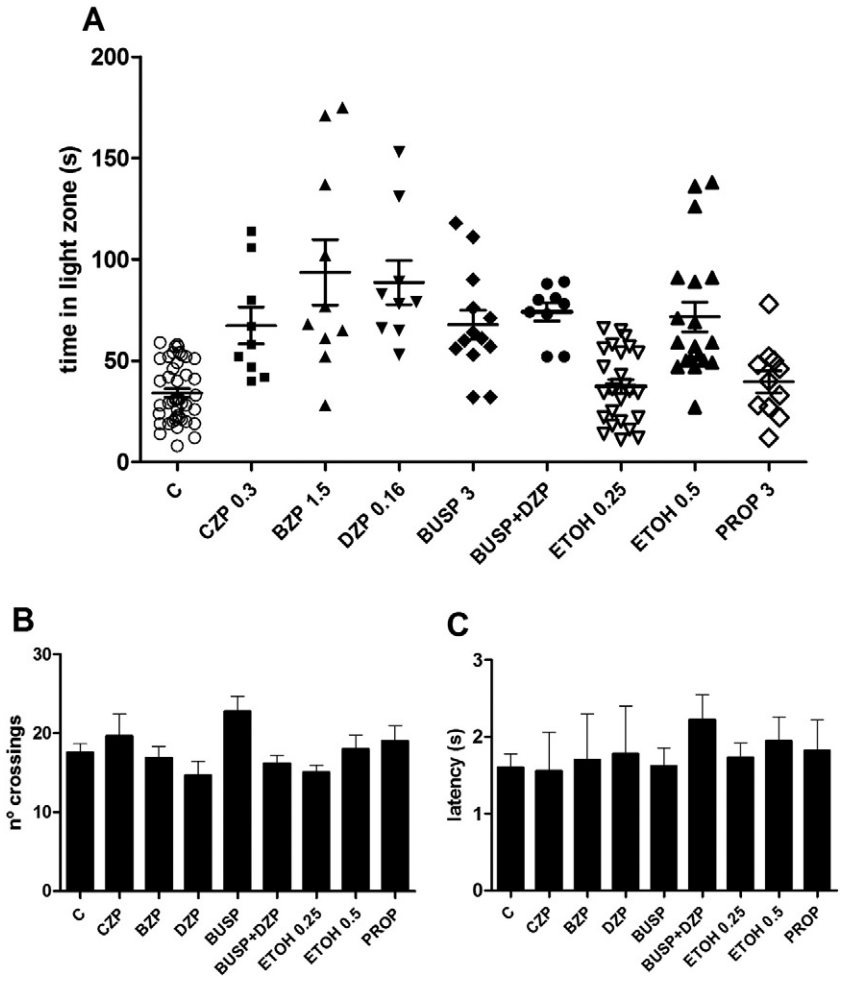


**Fig. 5.** Effect of clonazepam (CZP 0.3 mg/L), bromazepam (BZP 1.5 mg/L), diazepam (DZP 0.16 mg/L), buspirone (BUSP 3 mg/L), buspirone plus diazepam (BUSP + DZP, 3 mg/L and 0.16 mg/L, respectively), ethanol (ETOH 0.25% and 0.5%), and propranolol (PROP 3 mg/L) on time spent in the light compartment (Fig. 5A), number of crossings (Fig. 5B), and latency for first crossing (Fig. 5 C) in the light/dark task. Fig. 5A shows dot plot and means (—), where each dot represents one fish. Groups with filled dots were significantly different from the control group. Data are expressed as mean ± S.E.M. One-way ANOVA followed by Dunnett post hoc test. n = 9–19 for all groups, except for n = 26 in 0.25% ethanol group and n = 45 in the control group.

stereotypic swimming behavior. Further experiments are necessary to understand such a rapid onset of behavioral effects of buspirone in zebrafish.

Color was not a useful parameter for anxiety. Gerlai et al. (2000) reported that increased color intensity was associated with aggressiveness in zebrafish, resembling the alcohol-induced increase in aggression reported in rodents and primates (Miczek et al., 1993). In the present study, alcohol induced an increase in the intensity of color in zebrafish, but aggression parameters were not evaluated.

The light/dark task has been classically used as an anxiety test in rodents. Anxiolytics have been found to increase time spent in the light zone whereas anxiogenic drugs decrease it (Imaizumi et al., 1994). Zebrafish also prefer dark environments (Serra et al., 1999; Egan et al., 2009; Blank et al., 2009), which make this parameter potentially useful to assess the effects of anxiolytics. Our results confirmed this dark preference of zebrafish and showed that benzodiazepines, buspirone and ethanol were effective in increasing time in the light zone. In contrast, propranolol, which is not clinically effective as an anxiolytic, produced no effect in this task, suggesting some specificity of the light/dark task for anxiolytics. The combination of diazepam and buspirone failed to produce an additive effect, suggesting that in the light/dark task these drugs may have a similar neural substrate. Another possible explanation for this result is a ceiling effect on the range of this behavior. It should be noted that in the light/dark task the apparatus was quite different from the GBT and that fish were tested alone. These results suggest that the light/dark task may be useful for behavioral high-throughput screening of new anxiolytic compounds, since it is quick, easily performed and allows automated detection methods (e.g., videotracking). Besides predictive validity, Maximino et al. (2010) also point out the face and construct validity of this task.

For all drugs and in both tasks, pretreatment for 10 min in a beaker and behavioral evaluation for 5–10 min without drug in the test tank were adequate. However, ethanol and buspirone showed more pronounced effects in the GBT with continuous treatment. These results suggest that the protocol with pretreatment only can be used for anxiolytic screenings (as for the light/dark task), but there may be some effect reduction for some drugs in the GBT for pharmacokinetic reasons.

## 5. Conclusion

Rodents have been preferred for anxiety models due to its genetic and physiological similarities to the human system (West et al., 2000). On the other hand, cheaper and more easily handled organism models, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, are invertebrates. The ease of observation of various parameters, genetic manipulation, and low cost may be advantages of the

zebrafish to cover this gap between model animals for neuroscience research (Guo, 2009). Moreover, the behavioral methods employed here (the GBT and the light/dark task) generate different types of data, in a practical, simple, inexpensive, safe, and reproducible manner. The simplicity of these behavioral parameters and their capacity to detect distinct and common behavioral changes with different anxiolytic drugs should make them useful for anxiety assessment in zebrafish. Further studies are necessary to evaluate the effect of other drug classes in these behavioral parameters in zebrafish.

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