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Stimuli affecting zebrafish (*Danio rerio*) behavior in the light/dark preference test

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ABSTRACT

Ethanol has been suggested to have an anxiolytic effect on zebrafish, primarily based on its disruption of the novel tank diving response and of some social behaviors. The light/dark preference test offers a complementary measure of anxiety-like behavior in fish, and the purpose of the current study was to determine the effects of acute ethanol exposure on behavior in the light/dark task. In Experiment 1, the stimuli used to induce light/dark preference in zebrafish were varied in order to determine how best to measure the behavior. Subjects exhibited phototaxis (preference for light) when illumination was manipulated, but scototaxis (preference for dark) when wall and substrate color were manipulated. There was a clear interaction between locomotor activity and color preference, with animals preferentially freezing in darker locations. Because of ambiguity in interpreting behavior in the open/covered version of the test, the black/white version was used in Experiment 2. In Experiment 2, zebrafish were exposed to ethanol (0.25%, 0.5%, or 1.0%) or water for 30 minutes, and then placed in a black/white preference tank containing either ethanol (same doses) or water for a 30-minute test. Ethanol exposure increased locomotor activity and reduced freezing. Additionally, there was a significant interaction between ethanol treatment and locomotor activity on side preference. Low doses of ethanol increased white avoidance in normally swimming fish, while high doses did not.

1. Introduction

As the zebrafish gains popularity in behavioral research, reliable and valid paradigms for measuring its behavior are increasingly essential [1–3]. Modeling fear and anxiety represents a major goal of behavioral research, and the purpose of the current study is to better understand behavior in one proposed test of anxiety in zebrafish, the light/dark preference test [4–8]. When given free choice between a black and a white chamber, zebrafish reliably demonstrate a preference for the black chamber, and in analogy to rodent models, it has been suggested that the degree of preference may be useful as a measure of anxiety [5–10]. This test has already been used to investigate the anxiolytic, or anxiogenic, properties of a variety of drugs [9]. Because ethanol has been suggested to have anxiolytic effects on zebrafish [9,11], the current study was designed to examine the effects of ethanol on behavior in the light/dark test.

The motivational effects of ethanol on zebrafish have been explored, but are not yet adequately characterized. Ethanol attenuates the diving response to a novel tank [11–15], which is generally taken to indicate an anxiolytic effect [15,16], although this effect appears sometimes to be quite robust (e.g. [11,13]), sometimes quite weak (e.g.[1]), is sometimes not observed (e.g. [9]), and appears to depend on strain (e.g. [14]). Ethanol also affects social behavior, including shoaling [1,17] and possibly aggression [12,18]. Studies of shoaling suggest that at low doses (e.g. 0.25%), ethanol either fails to affect shoaling or increases shoal cohesion, while at higher doses (e.g. 1.0%) it reduces shoal cohesion, although these effects also vary and appear to depend on strain [1,14,17,19,20]. Ethanol has also been observed to increase the time spent in the light compartment in a two-chambered light-dark apparatus [12,15,21] as well as a light/dark plus maze [9]. Taken together, the pattern of ethanol-induced behavioral change may be attributable to a general anxiolytic effect of ethanol, but interpretation is complicated by a robust preference for the light chamber by control fish in the Gerlai et al. study [12], by conflicting results in studies examining aggression [12,18], and by reported increases in shoal cohesion at low doses [20]. The purpose of the current study was to further examine the motivational effects of ethanol using the light/dark preference test.

The behavior of zebrafish in a light/dark preference test was first reported by Serra et al. [7]. Serra et al. found that in an aquarium with two chambers, one with black walls and one with white walls, zebrafish exhibited a robust preference for the black compartment. Shortly thereafter, Gerlai et al. published an apparently contradictory result, in which zebrafish significantly preferred a light compartment to a dark compartment in a similar preference task [4]. Unlike the black/white apparatus used by Serra et al., the one used by Gerlai et al. was transparent, with one chamber illuminated and the other covered with cardboard. Thus, the discrepancy may be attributable to differences in the apparatus used for preference testing. Since then, the results of Serra et al. have been replicated [5,6,22] using similar black/white tanks, and Sackerman et al. reported similar results in a modified plus maze, with

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transparent walls but black and white floor [9]. Conversely, in an apparatus with one transparent, illuminated chamber, and a second chamber with opaque black covering (walls, lid, floor), Champagne et al. found a preference for the light chamber similar to that reported by Gerlai et al. [8]. These differences are difficult to interpret, because multiple variables are confounded across laboratories; it is unclear whether floor (substrate) color, wall color, ambient luminosity, or some combination of stimuli is controlling behavior in the task. Additionally, since fish are commonly housed in transparent tanks, it may be that opaque or covered enclosures elicit a stronger neophobic response than transparent ones. Therefore, although scototaxis in zebrafish has been commonly reported, the stimuli controlling the behavior require closer analysis before the test can be used reliably.

Some attempts have already been undertaken to more fully characterize the behavior of zebrafish in the black/white test, and in doing so to ascertain its validity as a measure of anxiety [6,22]. Blaser et al. [22] demonstrated that fish showing high avoidance of white freeze more when confined to a white chamber than animals that show little avoidance. This was taken to indicate that at least in animals showing a strong preference, the avoidance may be driven by anxiety or aversion to the white chamber rather than approach to the black chamber. Maximino et al. [6] found that the avoidance of white does not significantly habituate with repeated testing, and both Maximino et al. and Blaser et al. found that forced exposure to the white chamber does not attenuate avoidance. Although these attempts at behavioral validation suggest that avoidance of the white chamber (rather than approach to the black chamber) may be the relevant drive, they are not by themselves conclusive.

Our goal with the current studies was to better characterize normal behavior of wild-type zebrafish in the light/dark preference test, before testing the effects of several doses of ethanol on this behavior. In Experiment 1a, fish were given standard preference tests for black and white, but also with combinations including transparent and grey chambers. In Experiment 1b, fish were tested for light/dark preference, while illumination, floor, and wall color were varied. In Experiment 2, fish were pre-exposed to 0.0%, 0.25%, 0.5%, and 1.0% ethanol for 30 minutes, and then tested with either 0.0%, or the pre-exposure dose of ethanol, for 30 minutes in a black/white preference tank.

2. Experiment 1

2.1. Methods

2.1.1. Subjects

Subjects were 28 adult wild-type (AB) zebrafish (16 in Experiment 1a, 12 in Experiment 1b), raised in the laboratory from a line originally obtained from the University of Oregon breeding facility. Subjects were housed in an Aquaneering table-top housing rack, with a recirculating filtration system using mechanical, biological, and chemical filtration. The subjects were housed in groups of 20–25, in 3 L system tanks. For Experiment 1a, each subject was run in a single session; they were removed from the group of 20–25 naïve fish, and then returned to a separate, identical tank containing experienced fish. For Experiment 1b, subjects were separated into 1 L tanks in groups of four one week prior to the experiment, for identification purposes. The temperature of the tanks was held at 25° C, and the room was maintained on a 14/10 light/dark cycle. Subjects were fed 1–2 times daily on a mixed diet of live brine shrimp, freeze-dried brine shrimp, and Tetra-Min flake food. The housing conditions and protocols for Experiments 1 and 2 were approved by the University of San Diego IACUC.

2.1.2. Apparatus

The preference tanks were 2 L rectangular tanks (20×12×10 cm; length x width x depth to water surface) divided into two chambers with a clear divider (for details, see [22]). A 5×5 cm opening in the clear acrylic divider allowed the fish to swim freely between both chambers of the tank. In the center of the tank, near the water's surface, was a start-box that opened into both chambers: both doors were opened to allow the fish an initial side choice. Light levels were measured using an Extech Instruments 403125 Light ProbeMeter.

Experiment 1a: The bottom of both chambers was filled with identical grey gravel (to ensure consistent video-tracking), while the walls of each chamber were either transparent, or painted matte black, matte white, or matte middle grey (6 preference tanks instantiated all possible color pairs: Black/White, Black/Grey, Black/Transparent, White/Grey, White/Transparent, Grey/Transparent). The experimental setup was lit by ambient room lights plus additional diffuse lighting approximately 1 meter above the tanks; light levels inside the tanks ranged from 300 lux (in black chambers) to 450 lux (in white chambers). A video-camera suspended approximately one meter above the testing tanks was used to monitor the location and activity of the fish. The video-camera fed into a desktop computer using Noldus Ethovision® to track the swim-patterns of the fish at a rate of 10 samples/sec.

Experiment 1b: For Tank 1, a transparent tank was used, but half of the tank was surrounded (three outer walls and lid) by brown cardboard, as described by Gerlai et al. [1]. Testing was in room lit normally by overhead lighting (250 lux), but no additional lighting was added to the transparent side. For Tank 2, a transparent tank was used in a completely dark room, while a 10 Watt light bulb was placed over one side of the tank to create a luminosity gradient. Light levels in the apparatus ranged from 28 lux on the side near the light to 9 lux on the far side. For Tank 3, both the walls and floor of one chamber were painted black, and the walls and floor of the other chamber were painted white. For Tank 4, the walls of one chamber were painted black, and the walls of the other chamber were painted white, and grey gravel covered the floor of both chambers (as in Experiment 1a, and [22]). Tanks 3 and 4 were also placed in a diffusely lit room at about 250 lux. For Tanks 3 and 4, which had opaque walls, the video-camera was suspended above the tanks as described in Experiment 1. For Tanks 1 and 2, which had transparent walls, the video-camera recorded from the side of the tank. Because of differences in background lighting and contrast across tanks and chambers, video-tracking was not used, and all videos were scored by hand. A stopwatch was used to time the duration in the brighter half of each tank, in one minute intervals, which were then recorded in an Excel worksheet.

2.1.3. Procedure

Experiment 1a: Each subject was observed individually in a single session lasting approximately 1.5 hours. Within the session, each animal was given six 15-minute tests, one in each of the six preference tanks. The order of testing was counterbalanced across animals, such that each tank appeared equally often in each serial position. Subjects were initially placed in the start-box of the preference tank. All doors between the chambers were opened to allow the fish to make an initial choice of side, and then to shuttle freely between the two chambers. The subjects' behavior was recorded by video-tracking for the entire 15 minutes of each test. Behavioral measures included: Proportion in Color – the absolute duration in each colored side, divided by the total recording duration in seconds; Proportion Freezing – the absolute duration spent immobile (mean velocity<1.0 cm/sec) divided by the total recording duration in seconds; Thigmotaxis – the mean distance from the nearest wall, averaged across all samples (with lower values indicating a higher degree of thigmotaxis); Locomotor Activity – the total path length in cm divided by recording duration in seconds; Turn Angle – the mean angle created by 3 adjacent samples, averaged across all samples. Proportion in Color was of primary interest, used to measure preference. Freezing, Thigmotaxis, Locomotor Activity, and Turn Angle were used to further characterize behavior in this task. Freezing may indicate stress/anxiety, and Locomotor Activity is useful for interpreting preference behavior. Thigmotaxis and Turn Angle are difficult to interpret, but were included in order to more fully characterize behavior in this task.