

EFFECTS OF ETHANOL ON THE SHOALING BEHAVIOR OF ZEBRAFISH (*DANIO RERIO*)

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□ Ethanol (EtOH) often has stimulatory effects at low doses and inhibitory effects at high doses, affecting behavior and physiology of many organisms in a non-linear manner suggestive of hormesis. Zebrafish (*Danio rerio*) shoaling was studied in adult fish exposed to one of five different EtOH concentrations (v/v): 0.0% control, 0.125%, 0.25%, 0.5%, and 1.0%. Digital photographs of groups of four fish were taken every 2 min, with each trial lasting a total of 12 min. The median nearest neighbor distance and shoal area were calculated for each photograph. Exposure to 1.0% EtOH inhibited shoaling. In contrast, as predicted from hormesis, shoaling was significantly tighter (as measured by nearest neighbor distance) at low concentrations (0.125%, 0.25%) compared to the control, and a J-shaped dose-response curve was present. A similar pattern occurred for shoal area, but in this case the only statistically significant differences were between the high concentration and all others.

Keywords: alcohol, ethanol, hormesis, shoaling, zebrafish

INTRODUCTION

Results of previous experimental research indicate that many toxins have biphasic dose-response curves, characterized by low-dose stimulation and high-dose inhibition, a phenomenon known as hormesis (Calabrese 2002). Because of the implications for human health, a large body of literature exists on the effects of exposure to alcohol, specifically ethanol (EtOH), on humans and animal models. The dose-response curve for EtOH is typically biphasic rather than linear, showing the U- or J-shaped curve characteristic of hormesis (reviews in Pohorecky 1977; Phillips and Shen 1996; Calabrese and Baldwin 2003).

In zebrafish (*Danio rerio*), EtOH has been shown to have an inhibitory effect at high doses but a stimulatory effect at low doses on behaviors such as shoaling (grouping), startle reactions and locomotion (Gerlai *et al.* 2000; Dlugos and Rabin 2003). Gerlai and colleagues (2006) recently examined the effects of EtOH on behavioral responses of zebrafish toward a predator model. Higher acute doses of EtOH led to diminished predator avoidance, suggesting disrupted motor function, perception, or attention, and thus a reduced fear reaction (Gerlai *et al.* 2006). Zebrafish

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responses under chronic EtOH exposure were evocative of human drug use, in which prolonged exposure to a substance produces adaptation, where higher doses are required to achieve the same effects. Overall, these findings demonstrate that zebrafish can be an appropriate model to study the effects of chronic and acute exposure to EtOH, producing results correlative to other vertebrates.

This study was designed to determine how low-level doses of EtOH affect shoaling in zebrafish. Behavior was quantified by measuring the nearest neighbor distance and shoal area. Many species of fish keep a distance of approximately 0.5-1.0 body length from other shoal members, using vision and the lateral line system (Krause *et al.* 2000). Both sensory systems work together to allow for the high-speed transfer of information needed to stay in tight groups. Speed and direction of each fish are also influenced by the individual's nearest neighbors (Krause *et al.* 2000). It is thought that EtOH modifies shoaling behavior by altering levels of anxiety, perception, and/or motor mechanisms (Gerlai *et al.* 2000). This study differs from previous work on EtOH and shoaling in zebrafish by adding a low dose (0.125% EtOH) specifically to test for hormesis and to more fully characterize the dose-response curve.

MATERIALS AND METHODS

Animal model and treatment

Wild-type, adult zebrafish (*Danio rerio*) were purchased from a local pet store (Animal Pantry, Staten Island, NY, USA) and maintained in 10 and 20 gallon tanks containing gravel and artificial plants. The tanks were kept heated at approximately 24^o C and continuously aerated and filtered with standard aquarium pumps, air stones, and filters. The fish were fed tropical fish flakes daily, occasionally supplemented with brine shrimp (*Artemia* sp.). Food was purchased at local pet stores. Our facilities and procedures have been approved by the Institutional Animal Care and Use Committee of the New York State Institute for Basic Research in Developmental Disabilities.

The experiments, which took place in 2006 and 2007, required a total of 200 zebrafish: four fish per trial, ten trials per treatment, and five treatment groups. The five treatment groups correspond to solutions prepared with the following EtOH concentrations (v/v): 1.0%, 0.5%, 0.25%, 0.125%, and a 0.0% control. EtOH concentrations were chosen based on previously published data for zebrafish, as well as another cyprinid, the goldfish (*Carassius auratus*) (Ryback 1970; Gerlai *et al.* 2000; Dlugos and Rabin 2003). The 0.125% EtOH solution was added to this experiment to evaluate an additional low dose and thus allow a better test for possible hormetic effects.

No single fish was tested more than once. In all trials, the shoal consisted of two large fish and two small fish of the same sex, to ensure control for any sex-assortative shoaling (Etinger *et al.* in press). Preference for body size was not found in our previous studies (Kurta *et al.* 2007), thus segregation of fish by body length was not performed.

Procedure

Before each 12-min trial began, the test group of four was placed in a 1000 mL beaker for 60 min that contained EtOH diluted in distilled water to the concentration being tested. It was expected that during this period a stable brain alcohol level in the subjects could be attained and that the concentration of EtOH in the brain would be approximately 90% of the concentration in the water (Ryback 1970; Gerlai *et al.* 2000). Trials occurred between 1200 and 1600 h local time to avoid spawning behavior in the early morning (Spence *et al.* 2008), as well as any possible endogenous behavioral rhythm.

After 60 min of exposure to EtOH, the test group was transferred to the observation bowl, a round glass bowl with a diameter of approximately 19 cm. The bowl was characterized by smooth vertical sides and a flat bottom. No gravel was included, to ensure clarity of the photographs that were taken (see below). Water in the observation bowl had the same EtOH concentration as during exposure and was at a depth of 2 to 3 cm.

Using a Logitech ClickSmart 820 camera and Logitech QuickCapture software, digital pictures were taken every 2 min during each 12-min trial. A total of 10 trials were completed for each treatment; five trials included all females and the other five included all males. Image Tool software (<http://ddsdx.uthscsa.edu/dig/itdesc.html>) was used to analyze these recorded images by measuring the distance between each fish from head to head and by measuring the area of the shoal. The results were quantified in pixels, which later were converted to cm.

Statistical Analysis

The median nearest neighbor distance (NND) and the median shoal area were calculated for each trial. Medians were used to ensure that the presence of one fish distant from the shoal would not have a disproportionate effect on the results. The median values were then averaged together to obtain a single data point per trial. Because the frequency distribution of NND was skewed to the right, the square root transformation was used. Data were analyzed with a one-way ANOVA followed by Tukey's post hoc test (Zar 1999) using SPSS 13.0. An alpha level of 0.05 was used to determine statistical significance. A two-way ANOVA including sex as a variable was also performed, to test for possible sex differences.

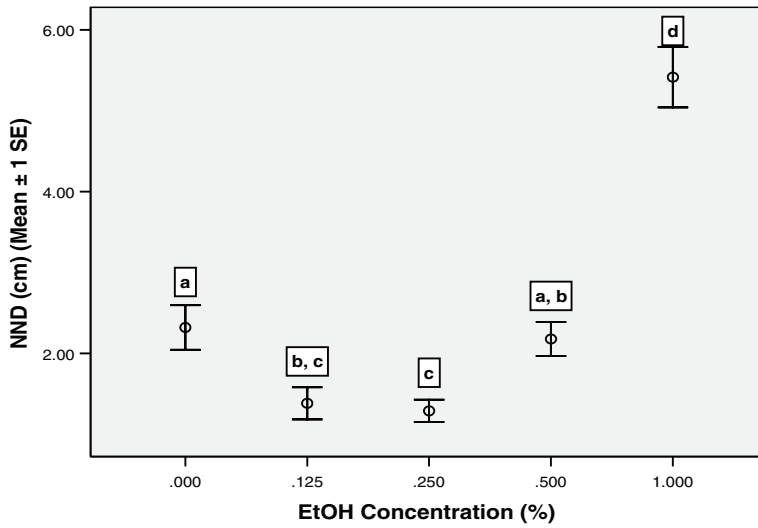


FIGURE 1. Nearest neighbor distance (NND) across treatments. Smaller numbers indicate tighter shoaling. Means that do not share the same letter are significantly different from each other (Tukey Test).

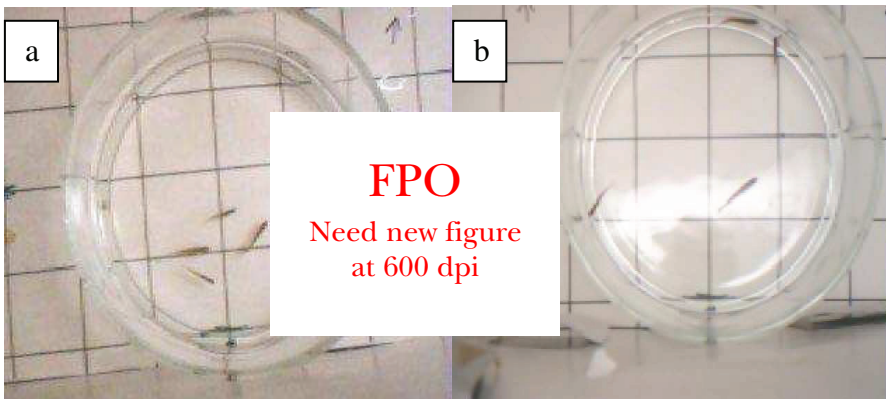


FIGURE 2. a) Treated with 0.125% EtOH. b) Treated with 1.00% EtOH.

RESULTS

Analysis of nearest neighbor distance (NND) with ANOVA showed significant variation across EtOH concentrations ($F_{4, 45} = 44.655$, $p < 0.0001$). As shown in Figure 1, at 0.125% and 0.25% EtOH, NND decreased significantly relative to the control and thus shoaling was tighter. In the 0.5% concentration, results were similar to the control, and at the 1.0% concentration the NND was nearly three times greater than the control. Also note the difference between the photographs in Figures 2a and 2b, corresponding to 0.125% and 1.0% EtOH, respectively. Effects

TABLE 1. Two-way ANOVA with EtOH concentration and sex as the independent variables and nearest neighbor distance (square-root transformed) as the dependent variable.

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9.639	9	1.071	17.746	<.0001
Intercept	113.783	1	113.783	1885.294	<.0001
EtOH	9.318	4	2.329	38.597	<.0001
SEX	0.087	1	0.087	1.435	.238
EtOH * SEX	0.235	4	0.059	0.973	.433
Error	2.414	40	0.060		
Total	125.836	50			
Corrected Total	12.053	49			

R Squared = .800 (Adjusted R Squared = .755)

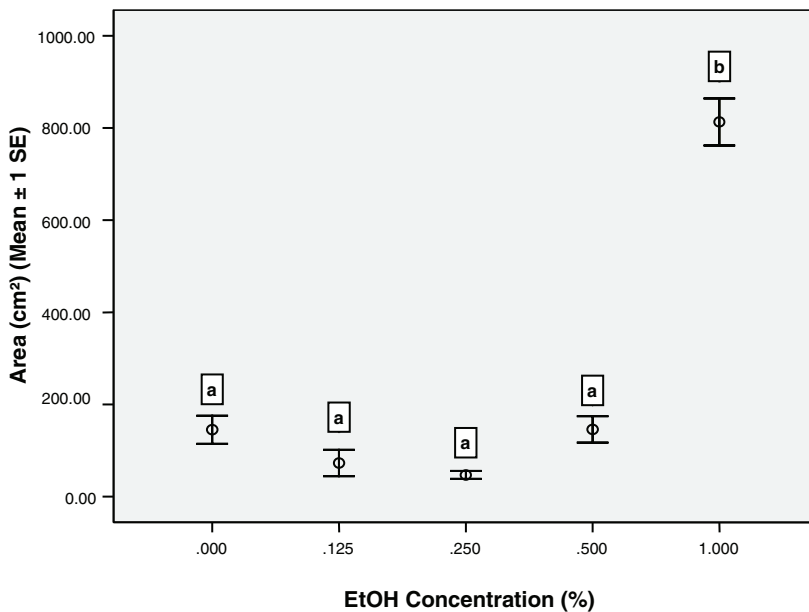


FIGURE 3. Shoal area across treatments. Smaller numbers indicate tighter shoaling. Means that do not share the same letter are significantly different from each other (Tukey Test).

of EtOH on NND were similar when controlled for sex in a 2-way ANOVA ($p < 0.0001$), and no significant sex difference ($p = 0.238$) or interaction ($p = 0.433$) was present (Table 1).

Results for shoal area were also found to have the same trend, indicating tight shoals at low doses and looser shoals at the highest dose ($F_{4,45} = 98.491$, $p < 0.0001$), but in this case the only statistically significant differences among treatments were between the high concentration and all others (Figure 3).

DISCUSSION

Zebrafish normally swim as a group and stay in close proximity to each other (Pritchard *et al.* 2001; Spence *et al.* 2008). In the present study, zebrafish shoaling behavior was used to examine the effects of EtOH on behavior. The results support the hormesis hypothesis, as a stimulatory effect of EtOH on shoaling was observed at low concentrations and an inhibitory effect was observed at the high concentration, resulting in a biphasic dose-response curve. It is possible that this tight shoaling at low doses results from the disinhibitory effects of EtOH, causing zebrafish to approach their shoal mates and allow their shoal mates to approach closer than they normally would. The increased NND and shoal area at the 1.0% EtOH concentration indicated that a general sedative response had occurred, limiting the fish's ability to shoal properly, as previously reported (Gerlai *et al.* 2000; Dlugos and Rabin 2003). Behavioral observations, although not quantified, also suggested that zebrafish were most active at low doses and were clearly inactive at the high dose.

This study demonstrates a hormetic effect of EtOH on behavior. Previous authors have also found nonlinear effects of EtOH on the behavior of zebrafish (Gerlai *et al.* 2000; Dlugos and Rabin 2003). Data gathered from these experiments can serve as valuable tools to use in future studies dealing with EtOH-induced changes at a behavioral and molecular level. Understanding how this compound affects CNS function and behavior in laboratory animals allows for comparison to humans, and fish (especially zebrafish) can be good models to study alcohol effects (Ryback 1970; Gerlai *et al.* 2000; Dlugos and Rabin 2003; Bilotta *et al.* 2004; Gerlai *et al.* 2006). More generally, this work contributes to the body of research on chemicals, including EtOH, that have opposing effects at low and high doses (Calabrese 2002; Calabrese and Baldwin 2003).

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