

Ethanol's influence on Habituation and Stress Response in *Danio rerio*

Daniel Barba

ABSTRACT

Danio rerio are fast becoming a premiere model organism for neurobehavioral research. Ethanol has been shown to have an anxiolytic effect when administered to *Danio rerio*, elucidating behaviors like reduced stress and increased habituation. The dive test works as a measure of stress for *Danio rerio*. In the dive test, the fish showed a noticeable decrease in all areas that would point to stress when going from the test without ethanol to the test with ethanol. The 3-Chamber Maze is a test to measure the *Danio rerio*'s ability to avoid a negative stimulus. The fish were able to make the correct decision after only two encounters with the negative stimulus when not under the influence of ethanol. While under the influence of ethanol, the fish took more encounters with the negative stimulus and did not learn to make the correct decisions. The results of both tests lead to the conclusion that while under treatment of ethanol at a concentration of 0.05% locomotor activity increased and their decision making process was inhibited.

Keywords: *Ethanol, Habituation, Danio rerio*

INTRODUCTION

Habituation is the increasing loss of response based on the amount of exposure to a stimulus. The more frequently the stimulus is presented, without warranting any necessary threat, the less the brain will provide a reaction to it. Response to stress and habituation are directly related. (Thompson, 1966) Zebrafish, *Danio rerio* are relatively new subject in the field of neurobehavioral research, which has been dominated by mice and rats for much of the last 50 years. (Gerlai, 2006) Testing the anxiological and habituation behaviors of *Danio rerio* is starting to become increasingly popular, especially when *Danio rerio* are under the influence of ethanol. (Mathur, 2010) Although there is still debate and confusion on what behaviors are normal, reports of *Danio rerio* behavior under the influence of ethanol have produced conflicting results. (Blaser, 2011)

Danio rerio are a model vertebrate organism whose behaviors can be monitored on a scale more easily than many species of fish or animals. Studying the effects of ethanol on the habituation behaviors and stress responses of *Danio rerio* can provide a basis for future neurobehavioral research. Ethanol's supposed anxiolytic effects on the behavior and motivation of *Danio rerio* is worth examining because of ethanol's ability to change an animal's normal cognitive processes. (Blaser, 2011) Through testing on habituation responses and stress stimuli, with and without ethanol, I will be able to contribute to the growing database of behavioral neuroscience experiments involving *Danio rerio*.

To establish the *Danio rerio*'s habituation and response to stress stimuli I will employ a number of tests that have proven successful in other experiments. I will employ tests such as a dive test to evaluate stress, and a three-chamber maze to estimate learning and memory. All tests will involve

two runs, one with exposure to ethanol and one without exposure. I expect results to show that ethanol will lower stress response, dull habituation ability, and impair ability to learn and memorize patterns. My research will set out to evaluate the influence of ethanol on the habituation and stress of zebrafish through tests designed to evaluate such, with the end result being a contribution to the growing field of neurobehavioral research of *Danio rerio*.

MATERIALS AND METHODS

Fifteen (15) male and female wild-type adult *Danio rerio*, approximately 4-5 months old were used in this study. They were obtained from an online source (aquariumfish.net). The subjects were housed in two different aquaria (each 6 gallons in size, dimensions of Tank 1 were 34.2 cm length, 24.7 cm width, 26.8 cm height, dimensions of Tank 2 were 38.3 cm length, 18.8 cm width, 28.5 cm height), with 10 fish in one aquarium and 5 in the other. Conditions were the same for each tank; they were maintained at a temperature of 28 + or - 0.05 degrees Celsius and received a 14 L:10 D light cycle. Feeding took place 1-2 times daily with a diet of Tetra tropical flake food and supplemented at least twice a week with brine shrimp.

The subjects treated with ethanol before testing were moved to a 2L tank filled with an ethanol concentration of 0.05% (vol/vol) for a 30 minute period before testing. This amount of time was selected based on earlier behavioral studies involving *Danio rerio*. (Blaser, 2011) All 15 *Danio rerio* were individually identified as to see the progress from the test without ethanol to the test with ethanol. Through hours of observation and the creation of a flowchart I was able to separately identify every *Danio rerio*

based on characteristics such as number of bands, gender, and distinguishing blemishes.

To evaluate stress, a dive test was performed. When a perceived threat presents itself, in this case introduction into a new aquarium, fish will dive to the bottom of the tank. As they become more comfortable and stress starts to subside they will gradually rise in the tank. (Levin & Cerutti, 2009) I performed this test in six minute intervals on one fish at a time. The same test was performed with fish under the 30 minute pre-exposure to ethanol. The same 15 fish were tested without ethanol exposure and then with ethanol exposure. The testing aquarium was split into three sections (Dimensions of the tank were 11.1 cm length, 11.1 cm width, and 20.5 cm height). Variables tested were freezing (lack of movement for more than 2 seconds), time spent in the bottom third of tank, time taken to leave bottom third of tank, time spent in the middle third of tank, time taken to enter top third of tank, and time spent in the top third of tank. This allows us to understand the individual effects ethanol had on each fish.

A three chamber maze was made to estimate to the learning and memory ability of the *Danio rerio*. Again, I followed the techniques laid out by Levin and Cerutti (2009) and the original test by Levin (2001). Using a 5.5 gallon tank I constructed (Dimensions were 40.0 cm length, 20.8 cm width, 22.6 cm height), which was split into three chambers (two large on the outside and a smaller one in the middle), I introduced the fish into the middle chamber. The fish was allowed to move about for one minute and then I slid open the partitions of the two outside chambers, allowing the fish the choice of either. When the fish has swam to one side I closed the chambers and showed whether or not the right decision was made. If the fish went to the side I had determined to be correct (the correct side was chosen prior to the test) it was allowed to swim freely for 2 minutes, if the incorrect decision was made I made the chamber it chose smaller as to restrict its swimming space (where it was left for one minute). This test was repeated for six trials with each fish and was performed by fish with the 30 minute pre-ethanol exposure and those without it. As with the previous test, all 15 fish were tested without ethanol exposure and then with ethanol exposure.

RESULTS

The overview of the data suggests that that the *Danio rerio* showed varied behavior in response to the ethanol in the dive test. The most common responses to ethanol exposure were decreased freezing (no swimming, nearly completely still), increased movement, and more time spent in the upper third of the tank. The data points represented read from left to right, freezing, left bottom third, time in bottom third, entered top third, time in middle third,

and time in top third.

An average of all the data is presented in this graph.

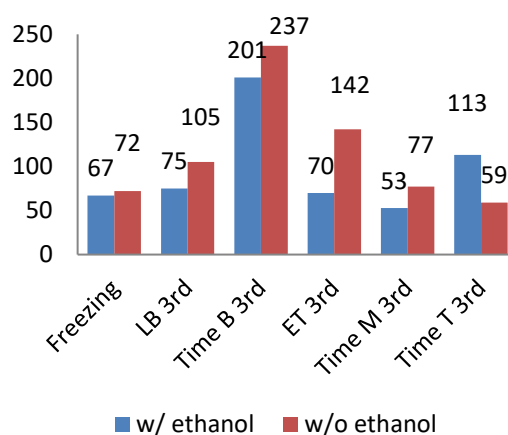


Figure 1: Showing average variation in freezing time, time it took to leave bottom 3rd of tank (LB 3rd), time spent in bottom 3rd (Time B 3rd), time it took to enter top 3rd of tank (ET 3rd), Time spent in Middle 3rd (Time M 3rd), and lastly time it spent in top 3rd (Time T 3rd).

The graph with the averages of the test presents a few conclusions. Ethanol reduced freezing behavior of *Danio rerio* by 5 seconds. *Danio rerio* under the influence of ethanol left the bottom third of the tank earlier, by 30 seconds. Time spent in the bottom third of the tank was reduced by 26 seconds after the ethanol treatment. *Danio rerio* entered the top third of the tank much sooner when treated with ethanol, by 72 seconds. Time spent in the middle third of the tank was also reduced by 24 seconds. Time in the top third of the tank was greatly increased when under the influence of ethanol, a difference of 54 seconds. I performed a paired t-test for every data point tested. Using the paired t-test allowed me to compare whether or not there was a significant relationship between the time values of without ethanol to with ethanol. The p-value for freezing came out to 0.898, which suggests that there is no significant difference between the two. The p-value for left bottom third came to 0.406, which suggests no significant difference. The p-value for time spent in bottom third came out to 0.396, which suggests no significant difference. The p-value for entered top third came to 0.068, which suggests no significant difference. The p-value for time spent in middle third came out to 0.117, which show no significant difference. The p-value for time spent in top third came out to 0.185, which suggests no significant difference. From the resulting data of the paired t-tests I am forced to conclude that the ethanol had no significant difference on any of the variables that I

was testing for of.

When testing for the three-chamber maze (without ethanol) 11/15 *Danio rerio* were able to remember the negative stimulus after only two trials and consequently avoided that chamber when given the choice. When faced with round two of testing (with ethanol) only 3/15 *Danio rerio* remembered the negative stimulus after 2 encounters with it. Most of the *Danio rerio* would actually go back to the chamber with the negative stimulus after experiencing the positive side, which is something that was not common in the first round of testing.

Table 1: P-Value Results of the 3-Chamber Maze without ethanol

	Trial 1	Trail 2	Trail 3	Trail 4	Trail 5	Trail 6
w/o eth	33%	53%	86%	73%	86%	93%
P-value	.5	.25	.125	.063	.031	.016

The results show that as the test went on the majority of the fish chose the correct side, with 93% by Trail 6. This leads us to conclude that the fish were avoiding the negative stimulus.

Table 2: P-Value Results of the 3-Chamber Maze with ethanol

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
w/ eth	46%	46%	60%	53%	60%	46%
P-value	.5	.25	.125	.063	.031	.016

The results of the test show that while under the influence of ethanol, *Danio rerio* were not able to understand the negative stimulus throughout all six trials.

The data from the dive test without ethanol shows a significant difference in the number of fish that chose the correct side from Trial 1 to Trial 6. During Trial 1 only 5/15 fish chose the correct side (although at this point there is still a 50:50 chance that either side will be chosen). By Trial 6, 14/15 fish had chosen the correct side. By using the binomial probability formula I determined that there is only a 34% chance that the fish will choose the correct side in 4/6 trials if the $P=0.5$. There is only an 11% chance that the fish will choose the correct side in 5/6 trials if $P=0.5$. The data and results lead to the assumption that the *Danio rerio* began to avoid the negative stimulus as the trials progressed. The data from the dive test with ethanol did not show a significant difference between the first and last trials. In Trial 1 7/15 fish chose the correct side and by Trail 6 still only 7/15 chose the correct side. In all of the other

trials there was not a significant difference, with the most being 9/15 choosing correct. These results suggest that the ethanol had an effect on the fish's ability to learn from the negative stimulus, which led to the largely even results throughout the trials.

DISCUSSION

The idea of what is normal *Danio rerio* behavior is still up for debate. While the 3-chamber maze test showed that *Danio rerio* can be made to make the right choice when faced with negative stimulus, the results of the dive test raised questions. Expected behavior was reduced freezing, increased movement, and more time spent in the upper third of the tank, but the results showed differently. The paired t-test showed that the ethanol had no effect on freezing in *Danio rerio*, but did have an effect on all other variables. Some *Danio rerio* will react differently than others to ethanol. This calls for future studies to perform more replicates, with more fish and different concentrations of ethanol. An ethanol concentration of 0.05% perhaps isn't enough to elucidate a response in the fish that didn't have the expected results. I believe the true merit of this project is tracking of individual *Danio rerio* progress from a test without ethanol to one with ethanol. Individual studies are the norm in just about every other animal study, but *Danio rerio* are difficult to distinguish from one another and sample sizes are usually very large so it is not often used.

LITERATURE CITED

- Blaser, R., and YM Penalosa. 2011. Stimuli affecting zebrafish (*Danio rerio*) behavior in the light/dark preference test. *Physiology and Behavior* 104:831-837.
- Gerlai, R., and V Lee, R Blaser. 2006. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). *Biochemistry and Behavior* 85: 752-761.
- Levin, ED., and DT Cerutti. 2009. Behavioral Neuroscience of Zebrafish. *Methods of Behavioral Analysis in Neuroscience* 2: Ch 15.
- Levin, AD. 2001. Spatial and non-spatial discrimination learning in zebrafish. *Animal Cognition* 4:125-131.
- Mathur, P., MA Berberoglu, and S Guo. 2010. Preference for ethanol in zebrafish following a single exposure. *Behavioral Brain Research* 217:128-133.
- Thompson, RF, WA Spencer. 1966. Habituation: a model phenomenon for the study of neuronal substrates of behavior. *Psychological Review* 73: 16-43.