

## **A Y-maze Choice Task Fails to Detect Alcohol Avoidance or Alcohol Preference in Zebrafish**

**Stephanie L. Grella, Neeru Kapur, and Robert Gerlai**  
*University of Toronto, Canada*

The zebrafish has been proposed for the analysis of the neurobiological and behavioral effects of alcohol in vertebrates. Significant behavioral changes induced by acute alcohol treatment, adaptation to chronic alcohol exposure, and withdrawal induced behavioral responses have all been shown in zebrafish. Previously, a flow-through Y-maze paradigm was proposed to directly measure alcohol preference or avoidance in zebrafish without the need to train learning-based place preference. Here, we first demonstrate that this Y-maze paradigm is capable of quantifying preference for a positive stimulus (the sight of conspecifics) and also the avoidance of a negative stimulus, a noxious olfactory cue, denatonium benzoate. Second, we test whether naïve zebrafish avoid alcohol upon first encountering this substance, and whether fish chronically exposed to alcohol show preference, or acutely alcohol treated fish show signs of intoxication leading to random choice. Our results demonstrate that acute alcohol treated fish exhibit enhanced immobility and perform at chance but chronic alcohol treated fish are not intoxicated and swim as well as naïve fish, a finding compatible with the known intoxicating effect of acute alcohol and the adaptation expected after chronic alcohol exposure. However, despite the general feasibility of the task, neither alcohol preference, nor alcohol avoidance could be detected in any of our treatment groups. We discuss the possible reasons why differential alcohol vs. freshwater choice was not found in this task and propose follow up experiments.

Alcoholism, or alcohol dependence syndrome, is a complex behavioral disorder. Alcohol (ethanol, ethyl alcohol or EtOH) may be consumed excessively and a pattern of alcohol seeking associated with physical and psychological dependence may emerge. This major health problem has a substantial financial as well as overall negative impact on the human society (Harwood, Fountain, & Livermore, 1998). Due to high levels of relapse, the success of current treatment strategies is limited. Elucidation of the central neurobiological mechanisms underlying this disorder has been difficult due to the complexity of alcohol's actions in the brain but is believed to be necessary for the development of preventative strategies and effective treatment plans (Higuchi, Matsushita, & Kashima, 2006). The risk for developing alcoholism is influenced by genetic factors (Crabbe, 2002; Gianoulakis, 2001; Vanyukov & Tarter, 2000). For example, heritability estimates for this disorder range between 50-75% in traditional twin and adoption studies (McGue, 1999; Tyndale, 2004). Recently, increasing number of studies focus on the identification of candidate genes involved in alcohol related disorders. One approach that has proven beneficial in this research is the use of animal models. Mammalian model organisms including the rat and the mouse have been often employed (for review see Crabbe, Phillips, Buck, Cunningham, & Belknap, 1999) but even the fruit fly (*Drosophila melanogaster*) has been successfully utilized (e.g., Guarnieri & Heberlein, 2003).

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Recently, the zebrafish has also been proposed (Gerlai, Lahav, Guo, & Rosenthal, 2000) as an appropriate tool for the behavioral, biological and genetic analysis of the effects of alcohol.

There are numerous reasons why the zebrafish is believed to be a good tool for such purposes. This species may represent an optimal compromise between system complexity (it is a vertebrate with complex brain structure and function) and practical simplicity (it is small, easy to breed, and cheap to maintain in large numbers in the laboratory). The use of zebrafish has been particularly fruitful in forward genetic approaches where high throughput screening tools are employed to identify novel mutants and ultimately the genes behind the mutation (Ninkovic & Bally-Cuif, 2006). Such research is translationally relevant because zebrafish and human genes have high nucleotide sequence homology with each other (e.g., Lockwood, Bjerke, Kobayashi, & Guo, 2004). Furthermore, the major layout of the zebrafish brain is of a typical vertebrate (Tropepe & Sive, 2003). Last, alcohol delivery is simple and non-invasive: alcohol is water soluble and zebrafish take up the substance through the gills and body surface (Chatterjee & Gerlai, 2009; Gerlai et al., 2000).

Despite the cogent rationale for the use of the zebrafish in forward genetic analysis of behavior and brain function in general and alcohol research in particular, there is a serious bottleneck in these research areas: understanding or characterization of zebrafish behavior is in its infancy (Sison, Cawker, Buske, & Gerlai, 2006). Briefly, considerable need exists for the development of behavioral testing tools appropriate for high-throughput screening in zebrafish (Ninkovic & Bally-Cuif, 2006; Sison et al., 2006).

Many researchers investigating drug addiction have successfully used the conditioned place preference (CPP) paradigm to quantify the reinforcing properties of drugs in rodents. In this procedure the subject is expected to establish association between an environmental context or stimulus (the conditioned stimulus) and a drug (the unconditioned stimulus). Using this paradigm, alcohol has been shown to be rewarding in mice (e.g., Bechtholt & Cunningham, 2005; Hill, Alva, Blednov, & Cunningham, 2003) and rats (e.g., Green & Grahame, 2008). Ninkovic and Bally-Cuif (2006) have demonstrated a CPP effect for amphetamine in zebrafish, and Darland and Dowling (2001) have shown CPP for cocaine in mutagenized zebrafish with alterations in dopamine signaling, a neurotransmitter central to reward. Despite the frequent use of CPP tasks, however, these tests suffer from some drawbacks that may limit their utility for our purposes. First, CPP is a learning task that often requires multiple training trials, i.e., its throughput may not be appropriate for large scale mutagenesis screens. Second, learning, i.e., the acquisition, consolidation and recall of the association between the context and the drug, is a complex process. Drugs may alter mechanisms of learning and may have significant effects on CPP performance irrespective of their motivational properties. Alcohol, for example, is known to affect several neurotransmitter systems, and other molecular mechanisms (Hoek & Kholodenko, 1998), involved in learning, including, but not limited to, the glutamate receptors NMDA-R (Sircar & Sircar, 2006), AMPA-R (Vaglenova et al. 2008), and also the GABAergic system (Feller, Harris, & Crabbe, 1988; Kuziemka-Leska, Car, &

Wisniewski, 1999). In the current paper our main question is whether different prior experiences with alcohol could lead to alcohol preference vs. avoidance in zebrafish. That is, we are interested in the motivation altering properties of the substance. To answer this question we wish to test alcohol preference and avoidance directly, i.e. without the involvement of learning processes.

To achieve this, we have developed a test paradigm, a flow-through Y-maze (Gerlai, 2001) that should allow us to test choice by zebrafish. The paradigm is, in principle, not different from the two bottle choice task employed in rodents (e.g., Taylor, Harris, & Vogel, 1990). Briefly, in this test zebrafish are expected to choose between two goal arms, say, a neutral and a stimulus arm of the Y-maze. The percent of time zebrafish spend in the stimulus arm quantifies whether they prefer or avoid the presented stimulus.

In this paper, we first utilize stimuli known to induce approach or avoidance and thus test the general concept, i.e. the ability of the Y-maze paradigm to detect preference or avoidance. Subsequently, we investigate the effect of different alcohol pre-exposure regimens on zebrafish. We hypothesize that, first, naïve zebrafish (previously not exposed to alcohol) will avoid the arm of the Y-maze from which alcohol solution flows (and will prefer the arm from which freshwater flows), second, zebrafish exposed to alcohol for prolonged period of time prior the Y-maze test (the chronic group) will show alcohol preference, and third, fish acutely exposed to alcohol immediately prior to the Y-maze test will show signs of intoxication (e.g. motor dysfunction and immobility) and may choose randomly.

## Method

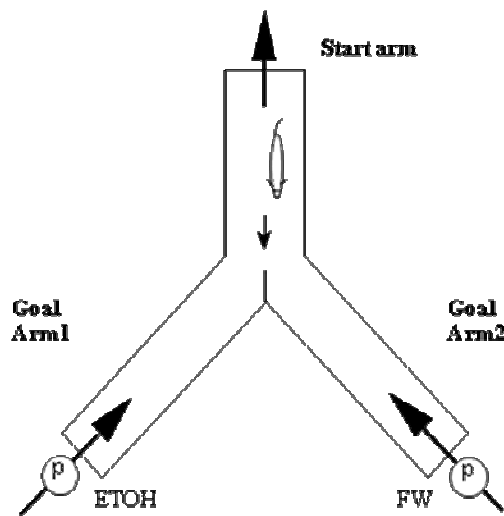
### *Animals and housing*

In the first set of experiments (testing the ability of the Y-maze paradigm to detect preference or avoidance) five month old (young adult) zebrafish of the AB strain were used (approximately 50-50% males and females). This strain is one of the most frequently utilized strains in forward genetic studies due to the fact that numerous genetic markers have been developed for it (e.g., Guo, 2004). All fish (sample sizes indicated in the figure legends) were housed in 1 L or 3 L transparent acrylic tanks at the same density (1 fish per liter). The difference in housing condition and its interaction with other factors were found non-significant and thus the data were pooled for housing condition. For the second set of the experiments, the short fin wild type (SF) population bred in our laboratory was used (5 month-old, 50-50% male-female). This population was hypothesized to be closer to the prototypical zebrafish (Bass & Gerlai, 2008), i.e. to resemble a natural genetically heterogeneous population found in nature. The appearance (size, color and pattern) of SF and AB fish is identical but AB fish, a standard strain with high homozygosity, originated from zebrafish purchased in pet stores 3 decades ago (e.g., Guryev et al., 2006) whereas the SF population, a currently genetically heterogeneous stock, is only being established in our laboratory. The SF fish used in the current study are the second filial generation bred in our vivarium originating from zebrafish purchased from Big Al's Aquarium Warehouse Inc (Mississauga, ON, Canada). Fish were kept in 3 L tanks (1 fish per liter density).

The holding tanks of the fish of both sets of experiments were filled with system water (deionized water supplemented with 60mg/L Instant Ocean Sea Salt [Big Al's Pet Store, Mississauga, ON, Canada]) kept at 27 C<sup>0</sup> and oxygenated via air stones connected to air pumps (Tetra USA, Blacksburg, VA). Ten percent of tank water was changed daily and fish were kept on a 12:12 Light/Dark cycle with lights turned on at 8:00 am. Fish were fed a mixture of ground freeze-dried krill and flake food (Tetramin Tropical Flakes, Tetra USA, Blacksburg, VA) once daily.

### *The Y-maze choice apparatus*

The Y-maze was a Y-shaped transparent acrylic tank with an 18 cm long and 10 cm wide start arm and two 30 cm long and 5 cm wide goal arms (Figure 1). The height of the tank was 20 cm but it was filled with water only to a depth of 7 cm. The water in the maze was identical to the system water, e.g., same temperature and salt content used for the home tanks of the fish. The maze was equipped with intake and outflow openings connected to polyethylene tubing (1 cm diameter) so that water could be made flowing from the beginning of the two goal arms and could exit at the end of the start arm (Figure 1). One flow meter at the intake to each goal arm allowed us to measure and control the rate of the fluid entry into the Y-maze (i.e. the flow rate). We have experimented with a range of flow rates and found that zebrafish can easily cope with flow speeds up to 12 cm/sec speed (note that the dimensions of the maze were established so that the flow rate was the same in the goal and the start arms). The pressure forcing the fluid into the maze was provided by elevated water cylinders (gravitation) connected to the tubing. The technical details and the concept of the maze have been described in more detail elsewhere (Gerlai, 2001). Briefly, shoaling fish living in freshwater streams and slowly moving waters are expected to orient towards and swim against water currents. Thus, the water flow in the maze is expected to orient the fish towards the choice point, the junction between the left and right goal arms. We expected zebrafish to show preference for, or avoidance of, one of the goal arms depending upon the differential stimuli provided in the goal arms.



**Figure 1.** The Y-maze. Pressure controlling flow meters indicated by the letter “p” allow the establishment of particular flow rates and the differential delivery of water soluble substances in the goal arms. In the current design, the flow rate was identical in the left and right goal arms and due to the dimensions of the maze (see Methods section) the flow rate was also the same in the start arm as in the goal arms. ETOH represents alcohol (ethyl alcohol or ethanol) delivered, and FW stands for freshwater. The large arrows show the direction of flow and the small arrow show the expected direction of zebrafish to swim. For additional details and behavioral procedures see Methods.

The Y-maze was placed on a styrofoam sheet to eliminate vibration and was positioned inside a 50 cm tall grey plastic box with open top so that zebrafish had no access to, and thus could not be disturbed by, external visual stimuli. The maze was illuminated from above by fluorescent light tubes on the ceiling. A video camera (Sony DCR-HC20; Sony Corporation, Japan) was positioned directly above the maze and recorded the behavior of the experimental zebrafish. Pilot

studies confirmed that even at such a low flow rate as 0.2 cm/sec the water marked by different food colors and flowing from the two goal arms did not mix between the left and right goal arms.

***Procedure: can the Y-maze quantify preference or avoidance?***

Although the technical details (including the physical dimensions and shape of the maze, the optimal flow rate, etc.) have been worked out for the Y-maze before (Gerlai, 2001; Gerlai et al., preliminary unpublished studies), the question of whether the maze is actually capable of quantifying preference or avoidance has not been addressed. Thus before proceeding to the analysis of the effect of alcohol, we decided to test the Y-maze procedure with stimuli known to induce approach (preference) or avoidance. One stimulus that is well established to induce approach is the sight of conspecifics (Al-Imari & Gerlai, 2008; Fernandes & Gerlai, 2009; Saverino & Gerlai, 2008;). In fact this stimulus has been shown to have rewarding properties capable of supporting acquisition of association in learning paradigms (Al-Imari & Gerlai, 2008; Pather & Gerlai, 2009). Although this stimulus is different in modality from alcohol (visual vs. olfactory or taste), we decided to employ it for two reasons. First, it requires no prior deprivation as would be necessary if we had used, for example, food as a rewarding stimulus (for further discussion and rationale see Al-Imari & Gerlai, 2008). Second, our preliminary findings (Scerbina, Chatterjee, Yousuf, & Gerlai, unpublished results) showed that dopamine receptor antagonism disrupts responding both to alcohol and to the sight of conspecifics in a similar dose dependent manner, suggesting similar underlying reward mechanisms. The flow rate in the maze was set to 0 cm/sec. This was because the visual stimulus, unlike water dissolved olfactory cues, did not require a flow induced stimulus separation of stimuli in the water and we were interested in how zebrafish distributes themselves in the approximately three equal areas within the maze. A 3 l transparent acrylic tank was placed adjacent to the outside wall of one of the two goal arms. For experimental zebrafish in the control group, the stimulus tank remained empty. For experimental zebrafish in the stimulus choice group, the tank contained 10 stimulus fish (zebrafish of the same size as the experimental subject). The side of stimulus tank presentation (left vs. right goal arm) varied randomly across experimental subjects.

As a negative, aversive stimulus we decided to use a cue of olfactory modality. Denatonium benzoate (obtained from Sigma Aldrich, St. Louis, MO, USA) is a water soluble substance that may be detected by taste and olfaction in fish and has been shown to have aversive properties (Oike et al., 2007). This substance is one of the most bitter compounds known to date that is used for humans as an aversive agent to prevent accidental ingestion of poisonous substances and is also highly aversive for rodents (Glendinning, Yiin, Ackroff, & Sclafani, 2008). In our study, two groups of fish were tested: a control group, for which no denatonium benzoate was employed, and a stimulus choice group for which one of the goal arms of the maze dispensed denatonium benzoate at a concentration of 2.5 nM. For both groups, the flow rate was set to 0.4 cm/sec, a slow water speed that was found sufficient to separate the left and right goal arms for water dissolved substances. Again, the side of stimulus delivery varied randomly across subjects of the stimulus choice group. Between each subject the maze was emptied and thoroughly rinsed.

All experimental subjects were assigned to stimulus treatment groups randomly and the order of testing was also randomized across treatment groups and gender. Before experimental testing, all fish were habituated to the Y-maze apparatus by individually exposing them to the maze for 10 min for 3 consecutive days. For behavioral testing, which took place between 10:00 and 16:00 h, fish were placed individually behind a mesh sliding door at the beginning of the Y-maze and released within 30 sec, by which time the water flow was also started. The behavior of the fish was recorded by the overhead camera for 300 sec. Upon the conclusion of the test, the fish was returned to its home tank.

The recorded digital videofiles (AVI format) were transferred to external hard drives (Lacie 320 GB) and later replayed for behavioral quantification. Quantification was aided by the Observer event recording software application (Noldus, Wageningen, The Netherlands). The percent of time the fish spent in the stimulus arm was quantified and is used as a measure of preference for, or avoidance of, the stimulus employed. The stimulus arm is the arm where the stimulus tank or the denatonium benzoate was presented. For control fish in the denatonium benzoate experiment, the stimulus arm was the same arm where denatonium benzoate was presented for the fish immediately preceding the control fish, a yoked control. In addition to the location of the fish, the percent of time

the fish swim (defined as fast movement with the use of the caudal fin; Blaser & Gerlai, 2006) was also measured and analyzed.

***Procedure: do naïve, acutely, or chronically treated zebrafish prefer or avoid alcohol?***

The general holding and handling procedures were as described above. Experimental zebrafish were randomly assigned to three treatment groups. Fish in the naïve group received no alcohol before the test. Fish in the chronic group were exposed to alcohol for a prolonged period of time using an escalating dose increase procedure as described before (Gerlai, Chatterjee, Pereira, Sawashima, & Krishnannair, 2009). Briefly, these fish received an initial dose of 0.125% (v/v) alcohol in their holding tanks, a concentration that was increased by 0.125% once every four days until reaching the target dose of 0.500%. Subsequently, the fish were maintained in this final dose for two weeks before the Y-maze testing (note that the particular alcohol dose was achieved by replacing the water of the holding tank with the appropriate alcohol concentration once a day). Last, in the acute group, fish were exposed to 0.50% (v/v) alcohol for one hour immediately prior to the Y-maze testing, and are expected to enter the test ‘intoxicated.’ Fish in all three groups received the same handling procedure, which entailed changing the water (and in the case of the chronic group providing the appropriate alcohol concentration) once every day in the holding tank of the fish.

Fish in the three treatment groups were tested in the Y-maze in an identical manner and in a randomized order. The general procedure was similar to what is described above but here one of the goal arms expelled alcohol water (0.85%) while the other arm provided fresh system water (0.00% alcohol). The side of alcohol delivery was randomly changed across experimental fish. The rationale for using 0.85% in one of the goal arms was that SF fish has been found to tolerate alcohol well up to 1.00%, and the 0.85% test concentration represented a robust, and assumingly easily detectable, concentration difference between the goal arms for the experimental zebrafish. Furthermore, the delivery of 0.85% alcohol from one goal arm was expected to lead to absorption of sufficient amount of alcohol in the brain of the experimental zebrafish within the short test session (Chatterjee & Gerlai, 2009). Last, it also resulted in a 0.425% alcohol concentration in the start arm, a dose that was close to the concentration employed during both the acute and also the final stage of chronic alcohol dosing. The flow rate in the maze was set to 3 cm/sec, which was faster than what was employed in the first set of experiments. The rationale for this was that zebrafish were previously found to be able to cope with much faster speeds but if physically compromised, e.g., due to motor impairment resulting from alcohol induced intoxication, they were expected to show reduced ability to swim against this flow rate, an impairment that could stay hidden at lower flow rates.

***Statistical analysis***

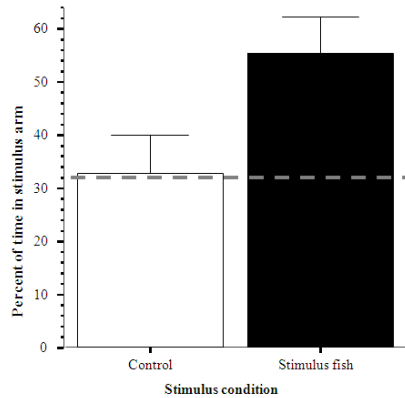
For all analyses, the SPSS statistical package (version 14) was used. Independent samples *t*-tests were performed to investigate the difference between control and stimulus presented groups. Univariate ANOVA was conducted to analyze the effect of alcohol treatment condition. In case of significant effect, the Tukey Honestly Significant Difference multiple comparison *post hoc* test was employed. Where appropriate, comparison to random chance level performance was made using the one sample *t*-test.

## **Results**

***The sight of conspecifics is preferred and denatonium benzoate is avoided***

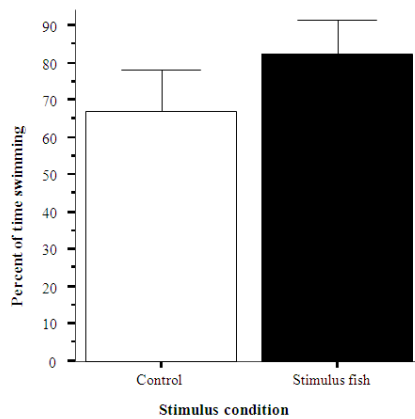
Experimental zebrafish spent significantly more time in the stimulus arm when the stimulus tank contained conspecifics (Figure 2; effect of stimulus condition,  $t = 2.93$ ,  $df = 26$ ,  $p < 0.01$ ). The increased time in the stimulus arm is also significantly above random chance (chance = 31.5%, based upon the area of the different arms of the maze) when conspecifics were in the stimulus tank ( $t = 3.44$ ,  $df = 13$ ,  $p < 0.01$ ) but not when the stimulus tank was empty ( $t = 0.14$ ,  $df =$

13,  $p > 0.05$ ). These findings confirm that zebrafish prefer staying close to their conspecifics (shoaling; Miller & Gerlai, 2007; Saverino & Gerlai, 2008) and that in the Y-maze this preference is clearly quantifiable.



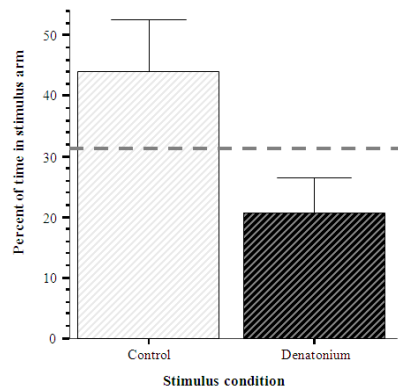
**Figure 2.** The percent of time zebrafish spend in the stimulus arm significantly increases when the stimulus is a tank with conspecifics. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{control}} = 14$ ,  $n_{\text{stimulus\_fish}} = 14$ . Random chance (31.5%, based upon the area of the stimulus arm vs. the area of the maze) is shown by the grey broken line.

Analysis of the swimming activity of experimental zebrafish revealed no significant differences between control fish and the fish that were presented with the stimulus fish (Figure 3;  $t = 1.38$ ,  $df = 26$ ,  $p > 0.05$ ). This finding implies that the increased time spent in the stimulus arm with stimulus fish adjacent to it was not an artifact, e.g., was not due to enhanced immobility or fear induced by the stimulus, but rather was indeed the result of preference exhibited by actively moving experimental zebrafish).

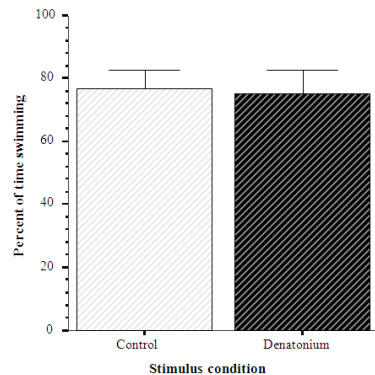


**Figure 3.** The percent of time zebrafish actively swam does not depend upon stimulus condition. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{control}} = 14$ ,  $n_{\text{stimulus\_fish}} = 14$ .

Denatonium benzoate, a noxious substance, induced significant avoidance (Figure 4). Fish that had a choice between the denatonium benzoate injected stimulus arm and the freshwater arm spent significantly less time in the denatonium stimulus arm compared to those fish whose choice was between the yoked control side and the other arm (both of which contained freshwater) ( $t = 2.31$ ,  $df = 33$ ,  $p < 0.05$ ). Also importantly, the value obtained for the denatonium benzoate group was significantly below ( $t = -3.67$ ,  $df = 18$ ,  $p < 0.01$ ) random chance (31.5%) whereas the value obtained for the control fish was not significantly different from random chance ( $t = 0.87$ ,  $df = 15$ ,  $p > 0.05$ ). These findings demonstrate that zebrafish, when given a choice between denatonium benzoate and freshwater, will choose the latter and that this choice is quantifiable in the current Y-maze paradigm. It is also unlikely the demonstrated avoidance reaction is due to abnormal motor function, increased fear or immobility, as the analysis of the percent of time the fish were swimming (Figure 5) showed no differences between the groups ( $t = 0.18$ ,  $df = 33$ ,  $p > 0.05$ ).



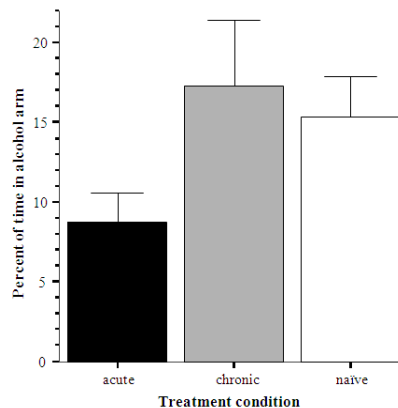
**Figure 4.** The percent of time zebrafish spent in the stimulus arm is significantly reduced by denatonium benzoate. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{control}} = 16$ ,  $n_{\text{denatonium}} = 19$ . Random chance (31.5%, based upon the area of the stimulus arm vs. the area of the maze) is shown by the grey broken line.



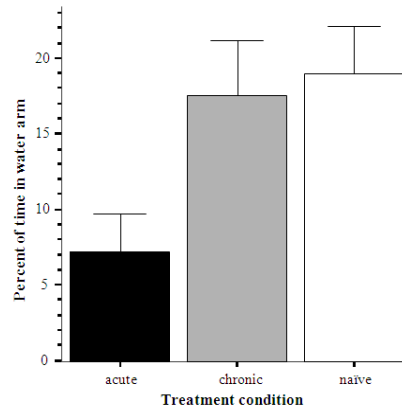
**Figure 5.** The percent of time zebrafish actively swam does not depend upon stimulus condition. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{control}} = 16$ ,  $n_{\text{denatonium}} = 19$ .

### ***Alcohol preference or avoidance could not be detected***

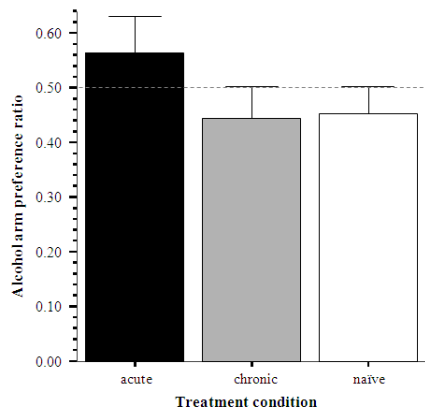
The treatment condition did not have a significant effect on the percent of time zebrafish spent in the alcohol arm but the  $p$  value was bordering significance [ $F(2, 92) = 2.66, p = 0.08$ ]. Figure 6 also shows an apparent difference between the fish of the acute group, spending an apparently smaller amount of time in the alcohol goal arm, as compared to fish in the other two groups. Analysis of the percent of time in the water goal arm confirmed this observation (also see Figure 7) and found a significant treatment effect [ $F(2, 92) = 4.110, p < 0.05$ ]. Tukey HSD demonstrated a significant ( $p < 0.05$ ) difference between the acute alcohol group and the other two groups. It is notable, however, that although the fish in the acute group did differ from fish in the other groups, this difference does not appear to be related to choosing the alcohol vs. the water arm. Figure 8 shows the alcohol goal arm preference ratio calculated as follows:  $A_R = T_A / (T_A + T_W)$ , where  $T_A$  is the time spent in the alcohol goal arm and  $T_W$  is the time spent in the water goal arm. Analysis of this ratio, which reflects the differential preference for alcohol vs. freshwater, showed no significant treatment effect [ $F(2, 92) = 1.30, p > 0.05$ ]. Also importantly, none of the groups differed from 0.50 random chance (acute  $t = 0.95, df = 29, p > 0.05$ ; chronic  $t = -0.96, df = 28, p > 0.05$ ; naïve  $t = -0.99, df = 33, p > 0.05$ ) suggesting that the location of fish in all treatment groups was random as far as the alcohol vs. water goal arm was concerned.



**Figure 6.** The effect of alcohol treatment condition on the percent of time zebrafish spent in the alcohol goal arm. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{acute}} = 30$ ,  $n_{\text{chronic}} = 29$ ,  $n_{\text{naïve}} = 34$ . Note that the apparent decrease seen in the acute group does not reach significance. For methodological details and statistical analyses, see Methods and Results.



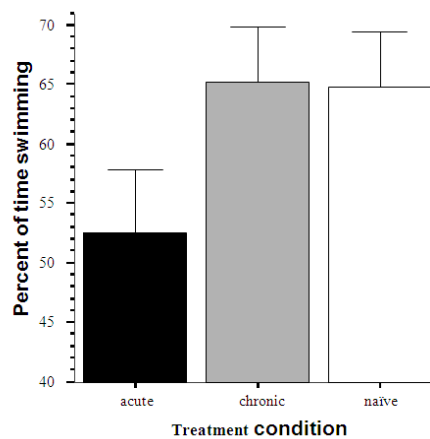
**Figure 7.** The effect of alcohol treatment condition on the percent of time zebrafish spent in the freshwater goal arm. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{acute}} = 30$ ,  $n_{\text{chronic}} = 29$ ,  $n_{\text{naive}} = 34$ . Note that the decrease seen in the acute group is significant. For methodological details and statistical analyses, see Methods and Results.



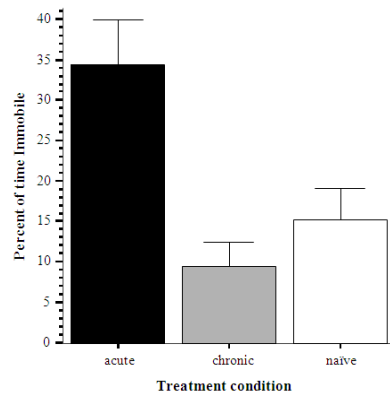
**Figure 8.** Alcohol treatment had no significant effect on the Alcohol arm preference ratio. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{acute}} = 30$ ,  $n_{\text{chronic}} = 29$ ,  $n_{\text{naive}} = 34$ . The preference ratio is calculated as follows:  $A_R = T_A / (T_A + T_W)$ , where  $T_A$  is the time spent in the alcohol goal arm and  $T_W$  is the time spent in the water goal arm. Random chance (0.50) is indicated by the grey broken line.

In addition to the location of the fish we also measured the percent of time the fish swam. Although analysis of this measure did not reveal a significant treatment effect [ $F(2, 92) = 2.18$ ,  $p > 0.05$ ], Figure 9 shows an apparent reduction of activity in the acute group as compared to the other two groups. To further explore the potential effects of alcohol treatment on motor function, we also quantified the percent of time our fish remained completely immobile. Immobility can arise as a result of increased fear but can also appear due to the sedative effects of high doses of acute alcohol (e.g., Gerlai et al., 2000). Figure 10 demonstrates that immobility indeed was increased in the fish of the acute alcohol group, an

effect that was found significant [ANOVA  $F(2, 92) = 8.76, p < 0.001$ ]; Tukey HSD, acute group differs from the other two ( $p < 0.05$ ), but the other two treatment groups do not differ significantly ( $p > 0.05$ ) from each other). In summary, it appears that acute alcohol exposure just prior to the Y-maze task led to significant increase of immobility and interfered with the ability of the fish belonging to this treatment group to swim into and stay in the goal arms. It is also important to note that fish in the chronic group also received a strong concentration (0.50%) of alcohol just prior to the Y-maze (the chronic dose), but their performance was indistinguishable from that of the fish in the naïve group, a result that is in line with the known adaptation that develops in zebrafish after chronic exposure to alcohol (Gerlai et al., 2009; Gerlai, Lee, & Blaser, 2006). Last, the results demonstrate no alcohol preference or avoidance in any of our treatment groups.



**Figure 9.** Percent of time zebrafish swam is not significantly affected by alcohol treatment. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{acute}} = 30, n_{\text{chronic}} = 29, n_{\text{naive}} = 34$ .



**Figure 10.** Percent of time zebrafish stayed completely immobile is significantly affected by alcohol treatment. Mean  $\pm$  SEM are shown. Sample sizes are as follows:  $n_{\text{acute}} = 30, n_{\text{chronic}} = 29, n_{\text{naive}} = 34$ . Note that immobility is significantly ( $p < 0.01$ ) increased in fish exposed to alcohol acutely as compared to fish in the other two groups, which do not significantly differ ( $p > 0.05$ ) from each other.

## Discussion

Zebrafish have been successfully utilized in developmental biology and genetics for the past three decades (Grunwald & Eisen, 2002) but unlike the rat or the mouse, there is only a small number of zebrafish behavioral tests available (Sison et al., 2006), and even those that have been published on are often not well characterized and used highly infrequently. For example, the key words “rat” and “conditioned place preference” returns 1261 papers using PubMed., whereas a similar search but with the keywords “zebrafish” and “conditioned place preference” gives only 6 publications. The key words “rat” and “alcohol preference” provides 1341 publications, whereas the keywords “zebrafish” and “alcohol preference” returns only 2 papers, one of which is a recent gene expression analysis (Kily et al., 2008) of the effect of alcohol and nicotine using a conditioned place preference task, and the other (Lockwood et al., 2004) is a study of the effect of acute alcohol on the zebrafish larva. Briefly, unlike for rodents, an alcohol choice task capable of quantifying alcohol preference or avoidance does not exist for adult zebrafish.

In the current paper we were hoping to fill this void and establish that the Y-maze paradigm proposed before (Gerlai, 2001) is capable of quantifying preference as well as avoidance in zebrafish. Briefly, the preference for the sight of conspecifics was clearly reflected by how much time experimental zebrafish spent in particular arms of the Y-maze: experimental zebrafish exposed to this stimulus chose to stay in the stimulus arm longer. Similarly, a negative stimulus, denatonium benzoate, induced a quantifiable avoidance reaction as the exposed experimental zebrafish stayed away from the denatonium benzoate arm. These results are noteworthy because quantification of choice responses in zebrafish may not be trivial. Zebrafish is a highly social species: individuals prefer staying in close proximity to each other and form shoals, aggregates of multiple fish (Miller & Gerlai, 2007; Saverino & Gerlai, 2008). However, most behavioral tasks require the testing of single subjects. Under this artificial condition, one may expect zebrafish behavior to be significantly driven by fear. Nevertheless, the current Y-maze paradigm demonstrates that testing individual zebrafish in an active choice task is possible. Elimination of potentially disturbing external stimuli (e.g., noise, vibration and visual stimuli) and appropriate habituation to the apparatus and proper handling of the experimental zebrafish resulted in lack of fear responses, such as erratic movement and jumping, and allowed our subjects to swim actively and make choices based upon the stimuli provided to them.

Despite this demonstrated general feasibility of the Y-maze paradigm, however, our current findings could not reveal alcohol avoidance or preference. None of the treatment groups exhibited a differential choice for alcohol vs. freshwater. Perhaps the most robust finding with regard to alcohol was associated with the behavior of fish in the acute alcohol group. These fish spent significantly less time in both goal arms compared to the naïve and the chronic alcohol treated fish and also stayed immobile for longer periods of time. These findings were expected as alcohol is known to have sedative, e.g. motor impairing, effects at higher doses (Phillips & Dudek, 1991). For example at 1% (v/v) concentration

alcohol has been shown to increase immobility in zebrafish (Gerlai et al., 2000). Intoxication induced by the high dose of acute alcohol administered was expected to lead to random choice between the freshwater and alcohol goal arms of the maze and indeed this is what we found in the fish of the acute group. However, naïve fish were expected to avoid and chronic fish were expected to prefer alcohol. Naïve wild type rats or mice, when given an unforced choice between an alcohol solution and freshwater, show robust avoidance of the substance (for recent review see Green & Grahame, 2008). After chronic exposure to alcohol rodents have been shown to develop not only tolerance but also preference for the substance (for examples see Green & Grahame, 2008). Why could not we see such avoidance or preference responses in zebrafish using the current Y-maze paradigm?

The pioneering aspect of this work makes it difficult to answer this question with certainty but there may be several possibilities. Previously, we found a robust acute alcohol effect both in AB and in SF zebrafish (Gerlai et al., 2009). We also discovered that neurochemical changes induced in the brain of zebrafish by acute alcohol treatment require at least 20-40 min immersion (depending on the neurochemical tested) in the alcohol solution (Chatterjee & Gerlai, 2009). These neurochemical changes included increased level of dopamine, a neurotransmitter involved in reward (e.g., Wise & Bozarth, 1985). The surge of dopamine in response to acute alcohol treatment is one of the mechanisms believed to underlie the rewarding aspects of alcohol consumption. However, exposing the naïve fish of the current study to alcohol for 5 min in the Y-maze may not have been long enough for alcohol to enter the brain of these fish and induce this potential rewarding effect. Thus, given that alcohol is known to have an aversive taste, at least in rodents (Green & Grahame, 2008; Liu, Showalter, & Grigson, 2009), we expected the naïve fish to show alcohol avoidance. We have no evidence, however, that zebrafish taste receptors can detect alcohol, and if they do, we do not know whether alcohol is indeed perceived as aversive by zebrafish. It is also interesting to note that being immersed in alcohol may have some negative effects the fish experience as irritation to their skin and gills. Our current results, however, do not support this as no irritation related behavioral responses (rubbing against solid objects in the tank or shaking the body or fins; see Parra, Adrian, & Gerlai, 2009) were observed and even the naïve fish did not avoid the alcohol arm.

We also assumed that fish treated with alcohol chronically should develop preference for alcohol. This assumption was based upon the fact that after prolonged exposure to alcohol, alcohol tolerance and dependence develops. Such adaptation to alcohol has been demonstrated in numerous species including the zebrafish (e.g., Gerlai et al., 2009; Gerlai et al., 2006). Furthermore, withdrawal from alcohol has also been shown to have a significant effect leading to the development of hyperactivity and impaired social behavior in zebrafish (Gerlai et al., 2009; Gerlai et al., 2006). Therefore it seemed reasonable to assume that after chronic alcohol treatment, zebrafish should continue to seek alcohol and avoid the freshwater arm. Although this assumption may be correct, the current experimental design may have had a temporal confound. We have recently demonstrated that the effect of a 60 min alcohol withdrawal induces significant changes (Gerlai et al., 2009) but we do not know how soon withdrawal from alcohol may have this effect.

It is likely that the short excursions into the freshwater arm of the Y-maze during the 5 min Y-maze test were not sufficient for the chronic alcohol treated fish to experience the effects of withdrawal and thus to start alcohol seeking. Perhaps a freshwater pre-exposure before the Y-maze paradigm could enhance the withdrawal effect and strengthen alcohol seeking and thus alcohol preference.

It is also notable that although the origin of AB and SF fish is similar (both are from pet store kept populations), AB fish are highly inbred as a result of three decades of breeding in the laboratory. Due to random fixation of alleles and accumulation of mutations during this inbreeding process the genotype of AB may have changed. Notably, while the current study was being conducted, we completed a strain comparison analysis between AB and SF zebrafish (Gerlai et al., 2009) and discovered that SF fish are less sensitive to withdrawal from alcohol as compared to AB. Briefly, it is possible that SF was not the optimal strain to choose for our alcohol experiments and AB would have been more responsive to alcohol withdrawal and thus might have shown a measurable alcohol preference in the Y-maze after chronic alcohol treatment.

Last, it is also possible that certain parameters of the current Y-maze design made the task insensitive to detecting alcohol choice. Although we have found zebrafish to be able to cope with a broad range of flow speeds and move around the maze even when the flow rate was as high as 12 cm/sec, we have never systematically tested this parameter in the context of alcohol. It is possible that the small Y-maze in which the water flow was relatively slow (3 cm/sec in our alcohol experiment) did not represent a strong enough demand and the experimental zebrafish could easily move around and enter all parts of the maze quickly without having to actually make a decisive choice. Increasing the stringency of the test, i.e. the consequences of making a choice, may make the task more sensitive. This may be achieved by increasing the flow rate, or by increasing the length of the arms, or by establishing a physical barrier at the choice point. We are also developing an entirely new maze design, a simple tube divided longitudinally by a perforated center piece. This design is based upon the preliminary data (not shown) we obtained from the Y-maze that suggested that alcohol concentration in the goal arm remains different on the right and left side of the arm and zebrafish may differentiate the sides.

In summary, establishment of an alcohol choice task that is capable of measuring alcohol preference or avoidance directly, i.e., without the need of multiple learning trials, is of importance as it may represent a good high throughput screening tool employable in mutagenesis or drug screens for zebrafish. Although the current paper provides evidence that, in principle, the Y-maze paradigm may be such a task, our results with alcohol show that it is premature to employ this paradigm for alcohol research. Additional experiments must follow the work presented above before an alcohol choice test can be conducted for zebrafish with confidence.

## References

- Al-Imari, L., & Gerlai, R. (2008). Conspecifics as reward in associative learning tasks for zebrafish (*Danio rerio*). *Behavioural Brain Research*, *189*, 216-219.
- Bass, S. L. S., & Gerlai, R. (2008). Zebrafish (*Danio rerio*) responds differentially to stimulus fish: The effects of sympatric and allopatric predators and harmless fish. *Behavioural Brain Research*, *186*, 107-117.
- Bechtholt, A. J., & Cunningham, C. L. (2005). Ethanol-induced conditioned place preference is expressed through a ventral tegmental area dependent mechanism. *Behavioral Neuroscience*, *119*, 213-223.
- Blaser, R., & Gerlai, R. (2006). Behavioral phenotyping in zebrafish: Comparison of three behavioral quantification methods. *Behavior Research Methods*, *38*, 456-469.
- Chatterjee, D., & Gerlai, R. (2009). High precision liquid chromatography analysis of dopaminergic and serotonergic responses to acute alcohol exposure in zebrafish. *Behavioural Brain Research*, *200*, 208-213.
- Crabbe, J. C. (2002). Alcohol and genetics: New models. *American Journal of Medical Genetics*, *114*, 969-974.
- Crabbe, J. C., Phillips, T. J., Buck, K. J., Cunningham, C. L., & Belknap, J. K. (1999). Identifying genes for alcohol and drug sensitivity: Recent progress and future directions. *Trends in Neuroscience*, *22*, 173-179.
- Darland, T., & Dowling, J. E. (2001). Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 11691-11696.
- Feller, D. J., Harris, R. A., & Crabbe, J. C. (1988). Differences in GABA activity between ethanol withdrawal seizure prone and resistant mice. *European Journal of Pharmacology*, *157*, 147-154.
- Fernandes, Y., & Gerlai, R. (2009). Long-term behavioral changes in response to early developmental exposure to ethanol in zebrafish. *Alcoholism: Clinical and Experimental Research*, *33*, 601-609.
- Gerlai, R. (2001). Alcohol rewarded place preference (ARPP) in zebra fish (*Danio rerio*): A novel paradigm in development. *Cognitive Processing*, *2*, 215-220.
- Gerlai, R., Chatterjee, D., Pereira, T., Sawashima, T., & Krishnannair, R. (2009). Acute and chronic alcohol dose: Population differences in behavior and neurochemistry of zebrafish. *Genes, Brain, and Behavior*, *8*, 586-599.
- Gerlai, R., Lahav, M., Guo, S., & Rosenthal, A. (2000). Drinks like a fish: Zebrafish (*Danio rerio*) as a behavior genetic model to study alcohol effects. *Pharmacology, Biochemistry, and Behavior*, *67*, 773-782.
- Gerlai, R., Lee, V., & Blaser, R. (2006). Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). *Pharmacology, Biochemistry, and Behavior*, *85*, 752-761.
- Gianoulakis, C. (2001). Influence of the endogenous opioid system on high alcohol consumption and genetic predisposition to alcoholism. *Journal of Psychiatry & Neuroscience*, *26*, 304-318.
- Glendinning, J. I., Yiin, Y. M., Ackroff, K., & Sclafani, A. (2008). Intra-gastric infusion of denatonium conditions flavor aversions and delays gastric emptying in rodents. *Physiology & Behavior*, *93*, 757-765.
- Green, A. S., & Grahame, N. J. (2008). Ethanol drinking in rodents: Is free-choice drinking related to the reinforcing effects of ethanol? *Alcohol*, *42*, 1-11.
- Grunwald, D. J., & Eisen, J. S. (2002). Timeline: Headwaters of the zebrafish – emergence of a new model vertebrate. *Nature Reviews Genetics*, *3*, 717-724.

- Guarnieri, D. J., & Heberlein, U. (2003). *Drosophila melanogaster*, a genetic model system for alcohol research. *International Review of Neurobiology*, *54*, 199-228.
- Guo, S. (2004). Linking genes to brain, behavior and neurological diseases: What can we learn from zebrafish? *Genes, Brain, and Behavior*, *3*, 63-74.
- Guryev, V., Koudijs, M. J., Berezikov, E., Johnson, S. L., Plasterk, R.H., van Eeden, F. J., & Cuppen, E. (2006). Genetic variation in the zebrafish. *Genome Research*, *16*, 491-497.
- Hoek, J. B., & Kholodenko, B. N. (1998). The intracellular signaling network as a target for ethanol. *Alcoholism: Clinical and Experimental Research*, *22*, 224S-230S.
- Harwood, H. J., Fountain, D., & Livermore, G. (1998). Economic costs of alcohol abuse and alcoholism. *Recent Developments in Alcoholism*, *14*, 307-330.
- Higuchi, S., Matsushita, S., & Kashima, H. (2006). New findings on the genetic influences on alcohol use and dependence. *Current Opinion in Psychiatry*, *19*, 253-265.
- Hill, K. G., Alva, H., Blednov, Y. A., & Cunningham, C. L. (2003). Reduced ethanol-induced conditioned taste aversion and conditioned place preference in GIRK2 null mutant mice. *Psychopharmacology*, *169*, 108-114.
- Kily, L. J., Cowe, Y. C., Hussain, O., Patel, S., McElwaine, S., Cotter, F. E., & Brennan, C. H. (2008). Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. *Journal of Experimental Biology*, *211*, 1623-1634.
- Kuziemka-Leska, M., Car, H., & Wisniewski, K. (1999). Baclofen and AII 3-7 on learning and memory processes in rats chronically treated with ethanol. *Pharmacology, Biochemistry, & Behavior*, *62*, 39-43.
- Liu, C., Showalter, J., & Grigson, P. S. (2009). Ethanol-induced conditioned taste avoidance: Reward or aversion? *Alcoholism: Clinical and Experimental Research*, *33*, 522-530.
- Lockwood, B., Bjerke, S., Kobayashi, K., & Guo, S. (2004). Acute effects of alcohol on larval zebrafish: A genetic system for large-scale screening. *Pharmacology, Biochemistry, & Behavior*, *77*, 647-654.
- McGue, M. (1999). Phenotyping alcoholism. *Alcoholism: Clinical and Experimental Research*, *23*, 757-758.
- Miller, N., & Gerlai, R. (2007). Quantification of shoaling behaviour in zebrafish (*Danio rerio*). *Behavioral Brain Research*, *184*, 157-166.
- Ninkovic, J., & Bally-Cuif, L. (2006). The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. *Methods*, *39*, 262-274.
- Oike, H., Nagai, T., Furuyama, A., Okada, S., Aihara, Y., Ishimaru, Y., et al. (2007). Characterization of ligands for fish taste receptors. *Journal of Neuroscience*, *27*, 5584-5592.
- Parra, K. V., Adrian Jr., J. C., & Gerlai, R. (2009). The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (*Danio rerio*). *Behavioral Brain Research*, *205*, 336-341.
- Pather, S., & Gerlai, R. (2009). Shuttle box learning in zebrafish. *Behavioral Brain Research*, *196*, 323-327.
- Phillips, T. J., & Dudek, B. C. (1991). Locomotor activity responses to ethanol in selectively bred long- and short-sleep mice, two inbred mouse strains, and their F1 hybrids. *Alcoholism: Clinical and Experimental Research*, *15*, 255-261.
- Saverino, C., & Gerlai, R. (2008). The social zebrafish: Behavioral responses to conspecific, heterospecific, and computer animated fish. *Behavioral Brain Research*, *191*, 77-87.
- Sircar, R., & Sircar, D. (2006). Repeated ethanol treatment in adolescent rats alters cortical

- NMDA receptor. *Alcohol*, 39, 51-58.
- Sison, M., Cawker, J., Buske, C., & Gerlai, R. (2006). Fishing for genes of vertebrate behavior: Zebrafish as an upcoming model system. *Lab Animal*, 35, 33-39.
- Taylor, J., Harris, N., & Vogel, W. H. (1990). Voluntary alcohol and cocaine consumption in "low" and "high" stress plasma catecholamine responding rats. *Pharmacology Biochemistry and Behavior*, 37, 359-363.
- Tropepe, V., & Sive, H. L. (2003). Can zebrafish be used as a model to study the neurodevelopmental causes of autism? *Genes, Brain, and Behavior*, 2, 268-281.
- Tyndale, R. F. (2004). Genetics of alcohol and tobacco use in humans. *Annals of Medicine*, 35, 94-121.
- Vaglenova, J., Pandiella, N., Wijayawardhane, N., Vaithianathan, T., Birru, S., & Breese, C. (2008). Aniracetam reversed learning and memory deficits following prenatal ethanol exposure by modulating functions of synaptic AMPA receptors. *Neuropsychopharmacology*, 33, 1071-1083.
- Vanyukov, M. M., & Tarter, R. E. (2000). Genetic studies of substance abuse. *Drug and Alcohol Dependence*, 59, 101-123.
- Wise, R. A., & Bozarth, M. A. (1985). Brain mechanisms of drug reward and euphoria. *Psychiatric Medicine*, 3, 445-460.