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Drinks Like a Fish: The Effects of Ethanol on Associative Learning in Zebrafish (*Danio rerio*)

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The present study sought to elucidate whether neural maturation has a mitigating effect on ethanol and its concomitant effects on memory. Three-month old zebrafish were acclimated to a plus maze using a habituation procedure. After acclimatization to the maze, associations between the red cue cards and reward were formed via a shaping procedure. Following the final shaping day, food was removed from the maze and red cues were only present in one arm. The time it took for the fish to go from the start box to the cued arm was then measured. Afterwards, fish were exposed to 0.00, 0.25, or 0.75% ethanol (v/v) for 72 hr. Post-exposure memory performance was tested at 0.5-day, 5-day, and 14-day endpoints. Three primary findings were noted. First, no significant differences in run time were found within the control group at any time point, suggesting an adept associative memory system in zebrafish. Second, no significant difference in run time was found when comparing 0.25 and 0.75% (v/v) ethanol groups. Therefore, these treatments were pooled for further analyses. Third, the most significant impairment was observed at the 0.5-day post exposure time point, indicating that ethanol has a significant impact on recently learned associations. Finally, no significant differences in run time were observed within the pooled treatment group on subsequent time points. This capacity for recovery was a key difference from what was observed in previous studies.

Keywords: zebrafish, associative learning, ethanol, lateral pallium

Alcohol is one of the most common drugs abused across the life span, usually initiated in adolescence, peaking through adulthood, and abated through later life (Zeigler et al., 2005). Between 1995 and 2000, the National Household Survey on Drug Abuse (NHSDA) found that the number of adolescents who began using alcohol, ages 12-17, increased from 2.2 to 3.1 million (Office of Applied Studies; Substance Abuse and Mental Health Services Administration, 1997, 2001). Premature use of alcohol in adolescence can result in alcohol dependency and neurological damage due to alcohol consumption. During adolescence, increased myelination occurs. This increase in myelination is also associated with concomitant increases in synaptic pruning, whereby synapses are selectively removed (Brown, Tapert, Granholm, & Delis, 2000). Particularly, the hippocampus and prefrontal cortex develop significantly during adolescence. The prefrontal cortex becomes increasingly efficient with developmental maturity, which enables enhanced performance on complex tasks, such as planning, integrating information, abstract thinking, problem solving, judgement, and reasoning. This pattern of neurodevelopment requires a substantial amount of time and energy expenditure, and thus does not taper off until about 20 years of age. These dynamic

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brain changes make the adolescent brain more susceptible to permanent damage than the more stable and hardwired adult brain (Zeigler et al., 2005).

The teratogenic effects of ethanol and the diagnosis of fetal alcohol syndrome are relatively well-understood, although the mechanisms by which ethanol or metabolites induce developmental toxicity are not completely clear (Chernoff, 1977; Clarren & Smith, 1978; Fernandes, Tran, Abraham, & Gerlai, 2014; Jones & Smith, 1973). Less understood than the teratological effects of early developmental exposure are the cognitively deleterious effects that ethanol may have on the developing brain. Rats, mice, and zebrafish have been utilized in research for understanding the effects of ethanol on the mechanisms of development and subsequent cognitive capacity. The prominence of lab rats in pharmaceutical and other scientific research is well established; however, the zebrafish has become an extremely popular model to use in high-throughput behavioral assays and comparative cognition (Kalueff & Stewart, 2012). The emergence of zebrafish models can be attributed to the convenience and simplicity of their care and culturing as well as their mechanistic simplicity (Grunwald & Eisen, 2002). Zebrafish and humans share relatively similar physiology, brain anatomy, and neurochemistry. More importantly, research has shown profound nucleotide sequence homology (70-80%) between zebrafish (*Danio rerio*) and *Homo sapiens*, and the homology of certain proteins has shown to be even more conserved (Gerlai, 2010). Identification of the mechanism of action or gene responsible for a given malformation or aberrant behavior in the zebrafish likely can be used to identify human homologs. Thus, zebrafish represent a reasonable balance between mechanistic complexity and practical simplicity that is capable of modeling mammalian abnormalities efficaciously (Sison & Gerlai, 2010).

The current study evaluated the effects of ethanol on associative learning in zebrafish and whether these effects can be attenuated by enhanced neural maturation. Cognitive research on humans has shown that certain memories and different types of learning can be mapped and attributed to different parts of the brain (Squire, 2003). Several studies were able to conclude that different types of memory and learning occur in different neural areas (Gaffan, 1974; Milner, Corkin, & Teuber, 1968; O'Keefe & Nadel, 1978). This is important to the present study for two different but related reasons. First, there are several analogous brain areas in the zebrafish brain. For instance, the lateral pallium is considered the teleost analog of the mammalian hippocampus (Portavella, Vargas, Torres, & Salas, 2002). Second, the metabolic degradation of ethanol is very similar in zebrafish and humans (Reimers, Hahn, & Tanguay, 2004). Ethanol is metabolized by alcohol dehydrogenase (ADH) by converting ethanol into acetylaldehyde. The alcohol dehydrogenase present in zebrafish, ADH5A, and the alcohol dehydrogenase present in humans, ADH5, share a homology rate of 91% (Reimers et al., 2004).

Sison and Gerlai (2010) attempted to determine the learning capacities of zebrafish. To do this they established two learning tasks, the simple associative task and the spatial learning task. Using a similar model as Sison and Gerlai (2010), Fernandes et al. (2014) evaluated the effects of low doses of ethanol on zebrafish embryos for 2 hr to mimic fetal alcohol syndrome. Results from these studies suggest that short-duration, low doses cause permanent associative learning deficits in

zebrafish. Delay conditioning in teleost fish, wherein the onset of the conditioned stimulus precedes the presentation of the unconditioned stimulus and continues to overlap the unconditioned stimulus in time, is heavily reliant on the cerebellum and does not necessarily require telencephalic structures like the hippocampus or amygdala. However, trace conditioning in teleost fish, wherein the end of the conditioned stimulus and the onset of the unconditioned stimulus are separated by a time gap, requires recruitment of telencephalic structures, such as the hippocampus (Salas et al., 2006). Taken together, associative learning in zebrafish may be a more complex behavior than was previously thought. Ultimately, preventing aberrations in the development of these brain areas during adolescence and into adulthood may be pivotal for preventing the deleterious effects that ethanol has on memory.

In the present study, the effects of acute ethanol exposure on mature zebrafish were investigated in order to explore the potential impact that ethanol has on the learning ability of developmentally mature zebrafish. Since the fish in the present study were developmentally mature, they had already undergone significant changes in neural maturation, synaptic pruning, and white matter structure. Thus, we hypothesized that these dynamic changes in neural microstructure would attenuate ethanol-induced learning impairments after the cessation of ethanol.

Method

Test Organisms and Materials

Research was conducted in compliance with Section 8.09 (Humane Care and Use of Animals in Research) of the APA Ethical Principles of Psychologists and Code of Conduct and other federal statutes and regulations regarding the ethical treatment of animals. This project was approved by the Southwestern College Human and Animal Subjects Research Committee.

Thirty juvenile male AB-strain zebrafish (3-mo post-fertilization) were obtained from an established colony (Fort Environmental Laboratories, Inc., Stillwater, OK). Zebrafish were maintained in 10-L flow-through tanks containing dechlorinated tap water. To control photoperiod, a 16hr-8hr light/dark cycle was maintained throughout the study. All fish were communally housed in tanks prior to associative learning measurements. Prior to habituation to the plus maze, fish were assigned a specimen number. Fish were then individually placed into partitioned tanks. Each partition housed one fish. Fish were fed freeze-dried brine shrimp flakes (Aquatic Ecosystem, Apoka, FL) ad libitum twice daily. Habituation to the food took approximately 2 weeks after introduction to the aquarium. Ethanol (CAS no. 64-17-5, Pharmaco, Brookville, CT, 99.9% pure) was stored at room temperature. The test learning apparatus was a plus-shaped, 4-armed Plexiglas maze designed by MazeEngineers, Inc (Boston, MA). The dimensions of the arms were 70 cm in length by 50 cm in width with a height of 10 cm. At the center of the maze, a start box was positioned such that the fish could be placed in the maze to initiate the trial (Figure 1).

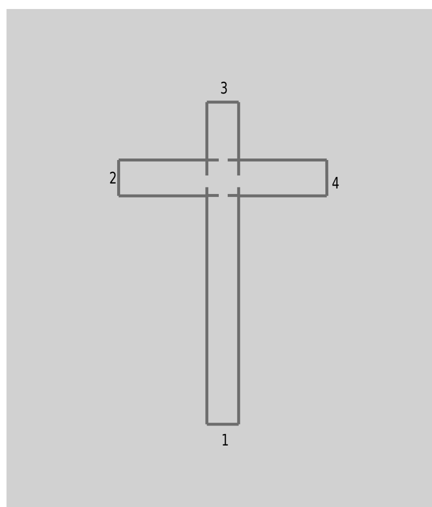

Figure 1. **Diagram of four-armed plus maze.** The dimensions of the arms were 70 cm in length by 50 cm in width with a height of 10 cm. At the center of the maze, a start box with sliding plexiglass doors was positioned such that the fish could be placed in the maze to initiate the trial.

Water-quality parameters. All water-quality characteristics fell within the predetermined ranges for zebrafish culture. All water temperatures fell within the range of 25°-28°C, pH was maintained between the range of 6.5-8.5, dissolved oxygen (DO) was > 3.5 mg/L, and light intensity ranged from 600-1000 lux.

Survival and clinical signs. No zebrafish died prior to ethanol exposure, during ethanol exposure, or during the post-exposure ethanol examination phase. No clinical signs of toxicity or abnormal behavior were noted.

Experimental Design

Three-month-old zebrafish were introduced to the laboratory culture tanks and allowed to acclimate to the conditions. Following acclimation, the fish were habituated to the plus maze. After habituation, associations between the red cue cards and reward were formed by the shaping procedure. Shaping was used to facilitate acquisition of the association of the cue, a red cue card, and reward. After the final shaping day, the food was removed from the maze and red cue cards were only present in one arm. The fish were then timed from the start box to the cued arm. Immediately following this, they were placed into a previously assigned ethanol concentration for 3 days. Upon the completion of exposure, fish were reintroduced to the maze at 0.5-days post-exposure, 5-days post-exposure, and 14-days post-exposure endpoints using the same associative learning procedure to determine if ethanol exposure altered learning in the juvenile fish and if effects are reversible at some point after the cessation of ethanol exposure.

Procedure

Habituation. To further acclimatize the fish to the maze, a habituation procedure was utilized. It consisted of two hour-long habituation trials, one trial per day for four consecutive days. On Day 1, all 30 experimental fish were placed in the maze for 2 hr. On Day 2, two groups of 15 fish were placed in the maze for habituation. On Day 3, six groups of 5 fish placed in the maze for habituation. On the final habituation day, all 30 fish were placed individually in the maze. Fish were randomly assigned to groups

during the habituation procedure. During habituation trials, food reward was accessible in all arms of the maze, and no red cues were present.

Shaping. To facilitate acquisition of the association between red cues and food reward, a shaping procedure was implemented following maze habituation. This consisted of 5-min trials, in which each fish explored the maze individually. On Day 1, all arms of the maze were marked with red cues and food reward. This color cue was chosen due to its distinguishability by the tetrachromatic vision of zebrafish, which has been utilized previously by other investigators (Sison & Gerlai, 2010 Williams, White, & Messer, 2002). On Day 2, three arms were marked with red cues and food. On Day 3, two arms were marked with red cues and food, and, on Day 4, only one arm was marked with red cues and food reward. Each subject was given four 5-min trials on each shaping day for a total of 20 min of exposure at each shaping day. The purpose of the habituation procedure was to gradually decrease the natural shoaling behavior of the zebrafish, thereby reducing the stress associated with placement in a novel testing environment. Shaping gradually aided in the association between food and red cue. The arms that were marked on each shaping day were based on the numerical outputs of a random number generator in Microsoft Excel to reduce preferential exploration of the maze. The maze was cleaned between shaping days but not between habituation and shaping. Since food was present in all arms during habituation and also present in all arms during the first shaping day, it was unlikely this cleaning process affected the results of the study. The order in which fish were placed into the maze for shaping was randomized prior to each shaping day.

Associative learning trials (ALTs). To determine whether the fish learned the association between the red cue card and food reward, probe trials were conducted in which a single fish was placed in the plus maze with the red cue card present, but no food reward was given. The location of the cue card in the maze was randomized between trials for each fish. The amount of time required for the fish to go from the start box to the box at the end of the cued arm was recorded. Timing was initiated once the doors to the start box were lifted and stopped when the fish reached the box at the terminal end of the cued arm. As a measure of quality control, a digital video (Sony HD Handcam HDR-CX210) of each ALT was recorded to verify ALT measurements. In each case, digital video clips were consistent with initial ALT measurements. Therefore, results presented in this paper are based off of the initial ALT measurement. The maze was drained and cleaned following each shaping day and ALT procedure to remove olfactory cues. Additionally, the order in which fish were placed into the maze during ALT was randomized prior to ALT measurement.

Ethanol exposure. Immediately following training, groups of 10 fish were exposed to dechlorinated tap water (control), 0.25% ethanol, or 0.75% (v/v) ethanol for 3 days. A 24-hr static renewal procedure was utilized. This procedure renewed the ethanol solution in each treatment group daily to account for the potential degradation of ethanol over time. The ethanol concentrations utilized were based on an initial range-finding test which established the maximum tolerable concentration (Fort & Fort et al., unpublished data). During and immediately following exposure, the fish were examined for signs of overt toxicity. Fish displaying signs of overt toxicity including aberrant or peculiar activity, impaired swimming behavior, or external abnormality were removed from the study and humanely euthanized in 200 mg MS-222 (pH 7).

Post-exposure ALT assessment. Following 3 days of exposure in the ethanol solutions, all fish were subjected to the same associative learning procedure (ALT) used prior. The fish were timed at three post-exposure endpoints: 0.5 days, 5 days, and 14 days post exposure. During the periods between the trials, the fish were maintained in separate 10-L aquaria containing dechlorinated tap water and fed brine shrimp flakes twice daily. The maze was drained and cleaned following each post-exposure ALT procedure to remove olfactory cues. Additionally, the order in which fish were placed into the maze during post-exposure ALT was randomized prior to ALT measurement.

Data analysis. All data were compared within given treatment groups. Each treatment group and the controls were assessed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's

test). Because the data sets failed tests for normality, data sets were evaluated using a Kruskal-Wallis ANOVA (KW-ANOVA) with Tukey's HSD post-hoc comparisons. In addition to Tukey-HSD post-hoc comparisons, estimates of effect size were also generated by adapting Cohen's d to evaluate the differences between cell medians (Grissom & Kim, 2011; Wuensch, 2012). Alpha was 0.05 for all analyses performed.

Results

Results for ALT performance of the control and treatment groups are presented in Figure 2. Zebrafish run times in the control group (*n* = 10) showed no significant differences at any timing point (KW-ANOVA, *p* = 0.48, *d* = .37).

Initial statistical testing revealed that there was no significant difference between the 0.25% (*n* = 10) or 0.75% (*n* = 10) treatment groups at any time point. This lack of significance was used as a reason to pool the treatment groups to increase statistical power (*n* = 20). Results of the KW-ANOVA indicated that there was a significant difference in the pooled treatment median run time across the days that ALT was measured (*p* = 0.02). To probe these differences, a Tukey HSD multiple comparison procedure was conducted. The results of the Tukey test indicated that pooled treatment run time at the 0.5-hr (*Mdn* = 300.00, *SD* = 110.71) time point was significantly higher than the initial pooled treatment (*Mdn* = 99.41, *SD* = 101.83) run time (*p* = 0.01, *d* = .83). However, run times at the 5-day time point (*Mdn* = 222.96, *SD* = 101.81) were not significantly different from the initial pooled treatment (*Mdn* = 99.41, *SD* = 101.83) run time (*p* = 0.06, *d* = .64). Finally, run times at the 14-day time point (*Mdn* = 147.99, *SD* = 91.13) were not significantly higher when compared to the initial pooled treatment (*Mdn* = 99.41, *SD* = 101.83) run time (*p* = 0.07, *d* = .51). Additionally, after the initial to 12-hour time point, no subsequent time point was significantly different from the preceding time point ($p < 0.05$). These results are presented in Figures 2 and 3.

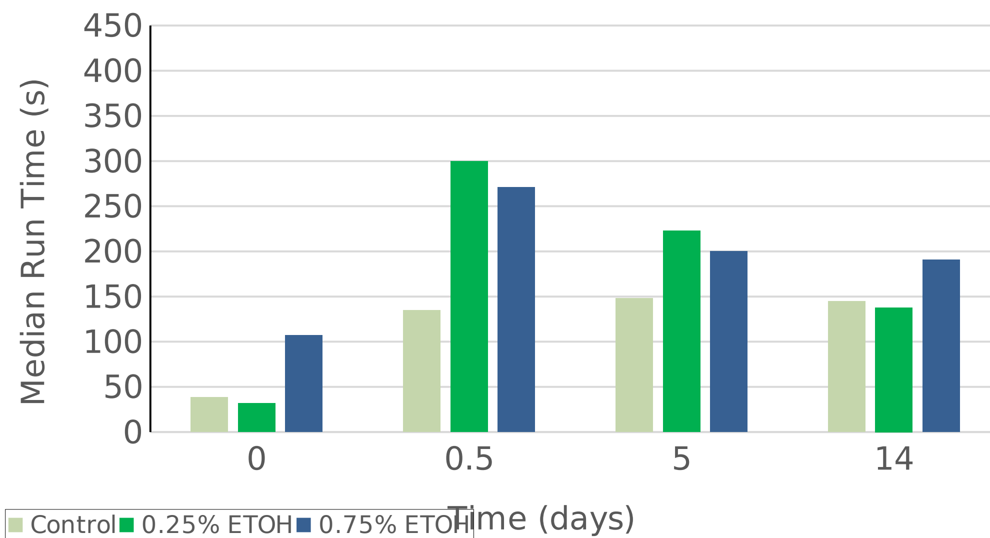

Figure 2. **Median run time (s) of the control group and each treatment group at all four time points.** No significant differences between the control or treatment groups at any time point were observed. There were no significant differences between the 0.25% or 0.75% ETOH treatments at any time point. Thus, these treatments were pooled for later analyses. The error bars denote symmetrical 95% confidence intervals. Alpha was set to 0.05 for all analyses

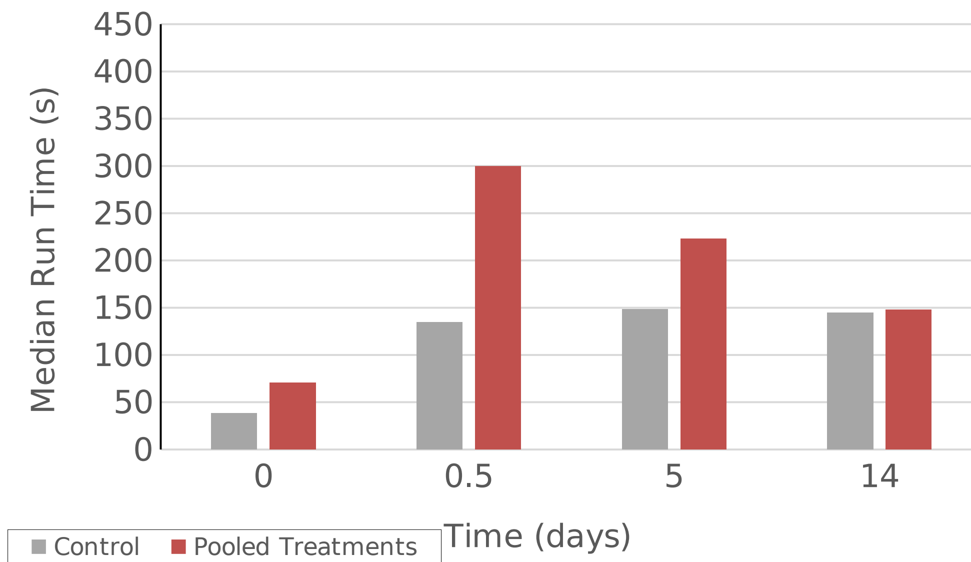

Figure 3. **Run time from control and pooled treatments over the time points used in this study.** Pooled treatment groups at each time point were significantly different when compared to the initial time point. After the 0.5-day time point, no significant difference was noticed when comparing subsequent days. The general trend of the pooled treatments demonstrated that some recovery is being made over time. * indicates a significant difference in pooled treatment run time at the 0.5-day time point when compared to the initial pooled treatment run time (p = .011 , d = .83). Error bars denote symmetical 95% confidence intervals. Alpha was set to .05 for all analyses.

Spatial memory. In addition to the investigation of associative learning, the spatial learning capacity of zebrafish was also qualitatively observed in this study. In the 0.5-day timing group, fish spent a substantial amount of time in the arm that was cued most recently. For instance, on the first time trial day, a substantial number of fish left the start box and immediately swam to the arm cued the previous day. This pattern was observed at the pre-exposure, 0.5-day, and 5-day time points but not at the 14-day time point.

Discussion

The approach used in the present study sought to elucidate whether the changes neural microstructure present in post-adolescence would have a neuroprotective role on previously learned associative memories. This method differed from Fernandes et al. (2014) by exposing fish with advanced neural maturity to ethanol, rather than during embryo-larval development, with the objective of

elucidating whether a more advanced developmental stage allows the mitigation of memory impairments after the cessation of ethanol. In the present study, no significant differences within the control group over the time points utilized were found. The relatively stable run times of the control group was suggestive of an adept associative memory system in zebrafish, particularly considering that the cue-reward association was never re-paired after the end of the shaping phase. The current study suggests that zebrafish are able to recall appetitive associations at a relatively stable rate over time. The associative learning performance was similar to that found by Sison and Gerlai (2010). The literature on zebrafish associative memory systems has primarily focused on vision. However, previous associative learning literature has examined the role of olfactory cues in associative learning in molluscs (Sahley, Gelperin, & Rudy, 1981). In mammals, fMRI studies have shown that the olfactory system projects to other brain regions, such as the amygdala and hippocampus (Herz, Eliassen, Beland, & Souza, 2004; Poellinger et al., 2001). Thus, examining the role of olfaction in zebrafish associative memories could further explicate zebrafish brain regions analogous to the amygdala and hippocampus, as well as discerning whether the same systems are implicated in zebrafish associative memories pertaining to olfaction.

The second key finding of the present study was that ethanol treatment had a significant effect on the run time of zebrafish at the 0.5-day time point; however, this effect dissipated over the 5-day and 14-day time points. This effect was most pronounced when comparing the initial and 0.5-day post-exposure run times. These results suggested that acute ethanol exposure has significant effects on the ability of zebrafish to recall previously learned associations. However, the results suggested that the deleterious effects of ethanol might be hindering the retrieval of the memory trace and not the storage or consolidation of the associative memory. Taken together, these results may help ameliorate the hypothesis that the maturations in neural microstructure present in adult fish help protect previously learned associative memories. These results were likely due to the deleterious effect of ethanol on memory and not due to the lack of repeated pairing of food and reward because there were no significant differences in the run times of the control group across all time points.

The third key finding of this study was that there were no significant differences within the pooled treatment group on subsequent time points. Although the ethanol-treated fish did not return to a state comparable with their baseline, a trend towards some level of recovery was observed by 14-day post-exposure (Figure 3). To evaluate this trend more definitively, a longer post-exposure monitoring period would be required. However, it is plausible that there is a point at which ethanol alters the memory trace irreparably, precluding a full recovery of the associative memory.

The observations of possible place preference could serve as evidence that zebrafish are able to recall the environment and surroundings in which they had previously received reward. However, this was observational and was not quantified for statistical analysis. Spatial memory has long been attributed to the function of the mammalian hippocampus (Dusek & Eichenbaum, 1997). The hippocampus encodes spatial information through specific trisynaptic circuitry (dentate gyrus, pyramidal

neurons CA3, and pyramidal neurons CA1; Salas et al., 2006). Although this trisynaptic circuitry is absent in the teleost lateral pallium, its absence does not preclude teleost fishes from encoding spatial memories. Cyprinid species, such as the carp and goldfish, have shown a robust capacity for spatial memory (Salas, Rodriguez, Vargas, Duran, & Torres, 1996). Despite the differences in neural physiology between teleost fish and mammals, the two organisms are both capable of encoding spatial information. Perhaps spatial learning is at least partly reliant on lower levels of the brain. Perhaps there are certain homologous molecular pathways or synaptic processes that are implicated in both species' ability to form spatial memories. Regarding the identification of this lower level mechanism of action in teleost spatial memory, the same mechanism of action could likely be identified in the mammalian neural circuitry due to the relatively high homology rate of 70-80% (Gerlai, 2010). Such a finding would further ameliorate the hypothesis that the lateral pallium present in teleost fish was the evolutionary precursor to the mammalian hippocampus. Further, the mechanisms implicated in teleost spatial memory are still involved in the formation of spatial memories in the hippocampus. The elucidation of the specific mechanisms of spatial learning in zebrafish should be a target of future research (see Sison & Gerlai, 2010, for a spatial memory task method).

Overall, this study found that ethanol had a deleterious effect on associative memory capacity shortly after exposure. However, the present study also demonstrated that despite the inexorable effects of ethanol in memory, neural maturity associated with late-stage development alleviates the effects over time. More broadly, this study served to further validate established methodologies for the assessment of associative learning in zebrafish.

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