Published in final edited form as:

Alcohol Clin Exp Res. 2009 April ; 33(4): 601–609. doi:10.1111/j.1530-0277.2008.00874.x.

Long-Term Behavioral Changes in Response to Early Developmental Exposure to Ethanol in Zebrafish

Yohaan Fernandes and Robert Gerlai

From the Department of Psychology, University of Toronto at Mississauga, Mississauga, ON, Canada.

Abstract

Background—Zebrafish is becoming an important research tool for the analysis of brain function and behavior. It has been proposed to model human alcoholism as well as fetal alcohol syndrome. Previous studies investigating the consequences of exposure to ethanol during early development of zebrafish employed robust dosing regimens (high ethanol concentration and long exposure) that may model a rare situation in the human clinic. These studies found major structural abnormalities developing in the exposed fish.

Methods—Here we hope to avoid such gross changes and administer only low doses of ethanol (0.00, 0.25, 0.50, 0.75, 1.00 vol / vol %) at 24-hour postfertilization and for only a short period of time (for 2 hours). We analyze the behavior of exposed fish at adult stage using computerized stimulus presentation and automated videotracking response quantification.

Results—Despite the short ethanol exposure period and the modest concentrations, significant behavioral alterations were found: fish exposed to higher doses of ethanol swam at an increased distance from a computer-animated zebrafish shoal while their activity levels did not change.

Conclusions—Although the interpretation of and the mechanisms underlying this finding will require further investigation, the results suggest that zebrafish will be an appropriate model organism for the analysis of the effects of moderate to mild prenatal ethanol exposure.

Keywords

Ethanol; Fetal Alcohol Syndrome; Prenatal Ethanol Exposure; Shoaling; Social Behavior; Zebrafish

Excessive alcohol (ETHYL alcohol, ethanol, EtOH) consumption can have deleterious effects on the human individual and society. Alcoholism and its related disorders are among the most devastating and costliest diseases (Harwood et al., 1998; Rice, 1999). Alcohol consumption by pregnant women can also significantly affect the developing fetus and can exert a lifelong effect on these children (Streissguth, 1997). Fetal alcohol syndrome is a severe form of this problem and is characterized by numerous robust physical malformations and functional abnormalities affecting approximately 1 in every 200 newborn (Stratton et al., 1996). Milder cases may be even more prevalent, and although they may present no obvious physical malformations, they may be associated with numerous significant behavioral impairments. Alcohol consumption during pregnancy can lead to hyperactivity, deficits in executive functioning, abnormal social skills, increased aggression, delinquency, self-injury, inappropriate sexual behavior, and enhanced fear responses or anxiety in the affected children

(for review and most recent examples see Arenzana et al., 2006; Green, 2007; also see Steinhausen, 1995). Concerted efforts have been made to discover the mechanisms of alcohol induced-changes and a large number of studies utilize animal models (e.g., Becker et al., 1996; Boehm et al., 1997; Guarnieri and Heberlein, 2003). Many of these studies employ genetic approaches (for review see Browman and Crabbe, 1999) first because a significant genetic component has been found influencing numerous aspects of alcohol-related disorders, including those of fetal alcohol syndrome (Streissguth and Dehaene, 1993) or alcohol abuse (Cloninger, 1987), and second because genetics offers a toolset that can open a window to the mechanisms of these diseases. Zebrafish is a relatively new and underutilized species in this research but appears to be a promising model organism.

Along with the fruit fly and the house mouse, zebrafish is the choice of species for geneticists, and a considerable amount of genetic information (e.g., genetic markers, sequencing of its genome, etc.) has been accumulated and numerous genetic tools have been developed for this species (Grunwald and Eisen, 2002). Given the power of genetics and the relevance of genetic manipulation in the discovery of the mechanisms and the modeling of human diseases and the fact that zebrafish is a vertebrate, this species has been proposed as a model organism important in translational research (Grunwald and Eisen, 2002; also see Sison et al., 2006). One of the diseases whose analysis may benefit from the use of zebrafish is alcohol-induced abnormalities (Gerlai et al., 2000). Indeed, zebrafish has been proposed as a model of fetal alcohol syndrome (Arenzana et al., 2006; Bilotta et al., 2004; Carvan et al., 2004; Matsui et al., 2006).

Bilotta and colleagues (2004) found that zebrafish embryos exposed to ethanol developed microphthalmia (decreased eye size and increased distance between the eyes), exhibited heart rate abnormalities, enlarged body cavities III and the exposed embryos also showed higher mortality rates. Carvan III and colleagues (2004) revealed that embryonic alcohol exposure led to increased cell death in the CNS, craniofacial and skeletal deformities, altered gene expression in the fore- and midbrain, as well as notochord and spinal cord structural abnormalities. Arenzana and colleagues (2006) found zebrafish embryos exposed to ethanol to develop numerous cytoarchitectural abnormalities along with cyclopia (fusion of 2 eyes). Finally, Matsui and colleagues (2006) found morphological as well as functional abnormalities of the visual system as a result of embryonic alcohol treatment.

A common thread in these zebrafish developmental alcohol exposure studies is the relatively high dose of alcohol and/ or the long duration of alcohol exposure employed. For example, Arenzana and colleagues (2006) treated their zebrafish embryos with 1.5 or 2.4% alcohol starting at 4.7 hour postfertilization and continued the alcohol treatment until 24 hour postfertilization. Bilotta and colleagues (2004) treated their fish with 1.5 or 2.9% alcohol for at least 8 hours varying the start of alcohol exposure from 0 hour postfertilization to 48 hour postfertilization. Carvan III and colleagues (2004) used some-what lower doses of alcohol (ranging between approximately 0.02% and 2.00%) but they exposed their fish for a prolonged period of time and within 4 hour postfertilization.

Although the exact amount of alcohol reaching the zebrafish embryo has not been quantified in the above studies and the level of alcohol exposure in human fetal alcohol syndrome fetuses is also controversial, alcohol consumption by pregnant women may be rarely associated with such high levels of, or prolonged exposure to, alcohol in the fetus (e.g., see discussion of this topic in Matsui et al., 2006). Importantly, even short embryonic alcohol exposure has been shown to induce behavioral anomalies in rats (Vorhees and Fernandez, 1986) that parallel some aspects of the behavioral changes seen in fetal alcohol syndrome children. In our current paper, therefore, we decided to expose zebrafish to lower concentrations of alcohol and for a shorter period of time. This way we are hoping to mimic moderate drinking during pregnancy that is not expected to lead to major toxicity and structural deformities in the fetus but could still alter

brain development and cause a functional disruption resulting in behavioral problems one can detect at the adult stage. Briefly, we are hoping to induce changes in zebrafish that will parallel the finer but still problematic behavioral abnormalities seen in human children including hyperactivity, abnormal social behavior, and enhanced fear responses.

We chose these endpoints in our zebrafish behavioral analysis for practical reasons too. Hyperactivity has been described in zebrafish as a result of acute exposure to intermediate doses (0.25, 0.50 vol/ vol %) of alcohol and also as a result of acute withdrawal from alcohol. Hyperactivity has also been found induced by embryonic alcohol exposure in rats (Vorhees, 1988). Furthermore, activity levels of zebrafish can be quantified in a precise automated manner using videotracking (Blaser and Gerlai, 2006; Gerlai et al., 2000, 2006). Social behavior has also been shown to be affected (reduced) by acute alcohol treatment (Gerlai et al., 2000). Abnormal social behavior among peers (Lugo et al., 2003), altered olfactory (Vorhees and Fernandez, 1986) as well as mother offspring responses (Fernandez et al., 1983) all induced by embryonic alcohol exposure have been demonstrated in rats. In case of zebrafish, social behavior has been easily demonstrated and quantified with the use of computer animated (moving) images of zebrafish, an artificial shoal (Saverino and Gerlai, 2008). These images have been shown to elicit a robust preference (shoaling, a form of social behavior) from experimental zebrafish (Saverino and Gerlai, 2008) and this response can be precisely quantified by measuring the distance between the experimental subject and the computer image presentation screen using videotracking. Similarly, alcohol has been found to have both anxiolytic and anxiogenic properties depending on concentration and dosing regimen in mammals as well as in zebrafish (Gerlai et al., 2000 and references therein). Fetal alcohol exposure has been shown to alter anxiety-related behavioral and stress hormone (e.g., corticosterone) responses in rodents (Osborn et al., 1998). Last, fear responses could be elicited in zebrafish by the sight of a natural predator, the Indian leaf fish (Bass and Gerlai, 2008).

Thus, in our current study, we exposed zebrafish embryos to 5 concentrations of alcohol, ranging from 0 to 1.00 %, for only 2 hours at 24 hour postfertilization. We measured the potential effects of this manipulation in the adult zebrafish (6 to 7 month old) by quantifying certain swim characteristics of these fish, including their swimming activity and their distance from computer-animated stimulus fish images (conspecifics or a predator) using automated videotracking. We hope that by establishing a mild embryonic alcohol-exposure zebrafish model, we will facilitate the characterization of the molecular and neurobiological mechanisms underlying the behavioral changes seen in children whose mothers drank small to moderate amounts of alcohol during their pregnancy.

METHODS

Animals, Alcohol Treatment, and Housing

Adult zebrafish (*Danio rerio*) of the AB strain bred in our facility (University of Toronto Mississauga Vivarium, Mississauga, ON, Canada) were used for obtaining fertilized eggs. The progenitors of this population were obtained from the ZFIN Center (Eugene, Oregon). AB is one of the most frequently studied zebrafish strains which is often used in forward genetic (mutagenesis) studies (e.g., Lockwood et al., 2004). All parental fish were of the same age (7 months old sexually mature young adults), a consideration that has been shown to be important (maternal age influencing the effect of prenatal alcohol exposure) in mammals, e.g., rats (Vorhees, 1988). Eggs were collected 2.5 hour postfertilization. Approximately 300 fertilized eggs were randomly selected and were divided into 5 equal groups and placed in 100 ml Petri dishes containing system water [deionized and sterilized water supplemented with 60 mg/l Instant Ocean Sea Salt (Big Al's Pet Store, Mississauga, ON, Canada)].

A major advantage of zebrafish is that fertilization and development occur externally and thus the developing embryo can be directly exposed to drugs, alcohol in this case, without the complicating effects of the mother's physiology. At 24 hour postfertilization, each group of zebrafish embryos received one of the following concentrations of alcohol solution: 0.00%, 0.25%, 0.50%, 0.75%, or 1.00% (vol/ vol percentage). These concentrations were deliberately lower than those employed before by others. The timing of alcohol exposure at 24 hour postfertilization was chosen because by this stage of development most major organs have started to be formed but their development has not been finished. Last, the length of exposure to alcohol was chosen to be shorter than what was employed before: the treatment lasted for 2 hours after which the embryos were washed with system water. With the above alcohol treatment procedure, we were hoping to induce only mild developmental abnormalities resulting in lack of increased mortality or gross structural aberrations but leading to minor changes perhaps only detectable at the behavioral level. Our goal was to parallel the effects of mild to moderate alcohol consumption by pregnant women leading to abnormalities less severe than those seen in full fledged fetal alcohol syndrome.

After the alcohol treatment, one-third of the embryos were maintained in 100 ml Petri dishes for 5 days before being moved to 1.3 l nursery racks (Aquaneering Inc., San Diego, CA). Once in the nursery racks, the fish were fed Larval AP 100 (particle size below 100 μ m; ZeiglerBros. Inc., Gardners, PA). Three weeks later, the fish were moved into 2.8-l rearing tanks (20 embryo per tank) of a high density rack system (Aquaneering Inc.), which had a multistage filtration that contained a mechanical filter, a fluidized glass bed biological filter, an activated carbon filter, and a fluorescent UV light sterilizing unit. Zebrafish remained in these holding tanks until behavioral experimentation, i.e., were never isolated and were always exposed to approximately the same number of shoal members in their home environment. Every day 10% of the water was automatically replaced with fresh system water on the rack. The water temperature was maintained at 27°C. Illumination was provided by fluorescent light tubes from the ceiling with lights turned on at 08:00 hour and off at 19:00 hour. While the fish were in the high density racks, they received a mixture of dried fish food (4 parts of Nelson Silver Cup, Aquaneering Inc.) and powered spirulina (1 part, Jehmco Inc., Lambertville, New Jersey). Behavioral experiments were conducted after the fish reached 6 months of age (fully developed sexually mature young adults, 50 to 50% male–female). The sample sizes of treated fish were as follows: 0.00% EtOH control, $n = 22$; 0.25% EtOH $n = 20$; 0.50% EtOH $n = 21$; 0.75% EtOH $n = 21$; and 1.00% EtOH $n = 19$. The rest of the treated embryos (40 per EtOH treatment group) were not allowed to develop but were sacrificed to quantify the amount of EtOH inside the egg after the treatment.

Quantification of EtOH Content Inside the Egg

The external alcohol concentration may substantially differ from the alcohol concentration which reaches the developing embryo inside the egg. Quantification of the actual level of alcohol to which the zebrafish embryo is exposed has not been attempted in fetal alcohol syndrome studies. To measure the amount of alcohol inside the egg, we removed the eggs from the alcohol solution precisely after the 2-hour long exposure, washed the eggs in distilled water, and transferred them to micro centrifuge tubes (20 eggs per tube, i.e., 2 sets of 20 eggs per EtOH treatment concentration). The eggs were homogenized using sonication in 0.1 ml distilled water and the solution was centrifuged [1000 rpm (67 g)] for 10 minutes. Subsequently, the supernatant was transferred to another micro centrifuge tube and analyzed for alcohol concentration using the AM1 Alcohol Analyser (Analox Instruments, London, UK). The instrument works on the principle that alcohol–oxygen oxidoreductase catalyzes the enzymatic oxidation of ethyl alcohol to acetaldehyde, and the oxygen consumption by the enzyme reduces the oxygen content of the solution, which is quantified by a sensitive sensor. The instrument has been successfully utilized to measure small alcohol amounts from

mammalian tissue samples (e.g., Taylor et al., 2002). We analyzed the 2 separate samples (each based on the 20 embryos used) per EtOH treatment concentration.

Behavioral Apparatus

The experimental set up consisted of a 37-l tank (50 × 25 × 30 cm, length × width × height) with a flat LCD computer screen (17 inch Samsung SyncMaster 732N monitor) placed on the left and right side of the tank. Each monitor was connected to a Dell Vostro 1000 Laptop. Running a custom made software application (see Saverino and Gerlai, 2008) that allowed the presentation of animated fish images. The experimental tank was illuminated by a 15 W fluorescent light-tube placed directly above it and the back side and the bottom of the tank was coated with white corrugated plastic sheets to increase the contrast and to reduce glare and reflections for video-tracking analysis. Two identical experimental set ups were used in parallel. The behavior of experimental fish was recorded onto the hard drive of 2 videocameras (JVC GZ -MG37u and GZ-MG50) and the digital recordings were transferred to the hard drive of a desktop computer (Dell, Dimension 8400) and later replayed and analyzed using the Ethovision Color Pro Videotracking software (Version 3, Noldus Info Tech, Wageningen, the Netherlands).

Behavioral Test Procedure

Milder forms of abnormalities induced by prenatal alcohol exposure include numerous behavioral problems such as hyperactivity and abnormal social behavior. Because zebrafish has been found to alter its locomotory activity as well as its response to conspecifics as a result of alcohol treatment similarly to what has been seen in humans and other mammals (Gerlai et al., 2000), we have decided to measure these 2 endpoints. Previously we have developed a simple computerized stimulus presentation method and showed that zebrafish respond to an animated zebrafish group presented on a computer screen by swimming close to these images, a behavior termed group preference or shoaling (Saverino and Gerlai, 2008). Alcohol is also known to alter fear responses (the substance has anxiolytic and anxiogenic properties depending on dose) and such responses can be elicited and quantified using a predator stimulus paradigm in zebrafish (Gerlai et al., 2000). Furthermore, zebrafish has been shown to respond to its natural predator, the Indian leaf fish (*Nandus nandus*) (Bass and Gerlai, 2008). Thus, we decided to present images of a shoal (a group of zebrafish) or of the Indian leaf fish using computer animation. The advantage of using computer-animated images compared to presentation of live stimulus fish is that the former allows precise control and consistent stimulus delivery across multiple sessions and experimental subjects, whereas the latter is prone to vary in an uncontrollable manner.

To explore the potential effect of early developmental exposure to alcohol, we placed our adult experimental zebrafish to the test tank singly, and 1 minute later we started a 30-minute long recording session. During particular intervals of the recording session, the subject was presented with animated images of either a group of zebrafish or a single Indian leaf fish. The custom software application that allowed us to present the images was first used and described in Saverino and Gerlai (2008). The sequence of stimulus presentation was as follows: 10-minute no stimulus (acclimation period), 10-minute zebrafish group [images of 5 zebrafish moving in realistic manner with a speed similar to that of live zebrafish (ranging between 1.5 and 4 cm/s)], 1-minute no stimulus interval, 1-minute predator (animated Indian leaf fish image moving with a speed of 0.3 cm/s), 3-minute no stimulus interval, 1-minute predator, 3-minute no stimulus interval, 1-minute predator. The stimuli were presented only on one side for each experimental fish but the side of presentation alternated randomly across experimental subjects.

Quantification of Behavior and Statistical Analysis

The digital video files (AVI format) were later replayed and analyzed by Ethovision as described in detail by Gerlai and colleagues (2008). This tracking system allowed us to precisely quantify the swimming activity of the experimental fish (cumulative distance swam measured in cm) as well as the distance the fish maintained from the stimulus presentation computer screen adjacent to their experimental tank (average distance from stimulus in cm). The former measure may reflect alcohol-induced activity changes, while the latter may quantify the level of preference to conspecifics (group preference) or avoidance of the predator (the operational definition of fear in this case). In addition, we also quantified the cumulative turn angle (i.e., the absolute degree of turn without reference to the direction of turn) for the last 10 minutes of the recording session (during which the predator image was shown) because our preliminary results (unpublished data) suggested that this measure was particularly sensitive to the appearance of the predator. Occasionally (less than 3 % of the tracks) small errors were detected (erroneous tracks due to floating debris, reflections, etc.). These tracks were omitted from analysis.

Data were analyzed using SPSS (version 14.1) for the PC. Repeated measure variance analysis (ANOVA) was used to investigate the effect of interval (30 1-minute intervals, the repeated measure factor), the effect of alcohol treatment (5 doses), and the interaction between these factors. Nonrepeated measure ANOVA was also used for measures derived from the interval data. In case of significant effects, post hoc Tukey Honestly Significant Difference (HSD) multiple comparison test as well as paired *t*-tests were conducted as appropriate.

RESULTS

Results of the analysis of the alcohol content inside the eggs are shown (Fig. 1) as a bar graph with each bar representing a particular alcohol concentration. The results demonstrate a significant [ANOVA $F(4, 5) = 326.575$, $p < 0.0001$] dose-dependent increase in alcohol concentration inside the egg (with each dose group differing from the other, Tukey HSD test, $p < 0.05$) but also show that only about 1/ 25 to 1/ 30 of the external alcohol concentration employed reached the embryo inside the egg. Notably, no physical abnormalities were observed in the developing or adult fish exposed to this level of alcohol. The mortality rate of the exposed fish was also not increased.

Statistical analysis of the behavior of the fish demonstrated no significant gender differences [$F(1, 96) = 0.188$, $p > 0.65$] and the interaction between gender and interval [$F(29, 2784) = 0.871$, $p > 0.65$] and among gender, interval, and alcohol treatment [$F(116, 2784) = 0.751$, $p > 0.95$] was also found nonsignificant for any behavioral measure quantified. Thus the data were pooled for genders.

The effect of the employed early developmental alcohol exposure on the distance the fish stayed from the stimulus presentation computer screen was significant and is shown in Fig. 2. Zebrafish normally swim close to their conspecifics. Upon exposure of a single subject to the sight of conspecifics, the subject usually attempts to join the group, i.e., will approach the group and will stay within close proximity of the group (Saverino and Gerlai, 2008). Conversely, zebrafish are expected to avoid their natural predator (Bass and Gerlai, 2008) and thus should move away from the stimulus screen when this image is shown.

The conspecifics-induced approach (shoaling) response is evident on Fig. 2. Particularly robust is the response seen in fish exposed to 0.00% alcohol (control) during development. However, this response appears to diminish in a dose-dependent manner in fish exposed to alcohol. The results of statistical analysis are in accordance with this observation. ANOVA revealed a significant Interval effect [$F(29, 2929) = 18.699$, $p < 0.0001$] suggesting that the distance of

fish from the screen varies across different intervals. ANOVA found a nonsignificant Alcohol treatment effect [$F(4, 101) = 0.411, p > 0.80$]. But the significant Interval $\times$ Alcohol treatment interaction [$F(116, 2929) = 1.392, p < 0.01$] confirmed that the alcohol treatment affected the distance of fish from the screen in an interval dependent manner. Post hoc multiple comparison tests such as Tukey HSD is inappropriate for repeated measure designs but pair-wise comparison of all possible interval pairs would increase type-I error. Therefore, to explore the significant Interval $\times$ Alcohol interaction and because the reduction of distance from the no-stimulus acclimation period (first 10 minute of the session) to the conspecific presentation period (subsequent 10 minutes of the session) is evident in Fig. 2, we calculated the difference between the distances during these periods. First, we calculated the average distance from the stimulus screen for the entire 10 minute no-stimulus period and the entire 10-minute conspecific presentation period, and then we subtracted the value of the no-stimulus period from the value of the conspecific presentation period (Fig. 3) and analyzed the results using univariate ANOVA.

The change of distance between the no-stimulus period and the conspecific stimulus period showed a quasi-linear dose–response trajectory with the biggest apparent reduction of distance in the control and the smallest apparent reduction of distance in the highest dose group. Supporting this observation, the alcohol treatment effect was found to be significant [ANOVA $F(4, 101) = 2.457, p < 0.05$]. Post hoc Tukey HSD test confirmed this result and showed that the highest dose group (1.00% alcohol) and the control differed significantly ($p < 0.05$). In addition, we also analyzed whether the apparent reduction of distance in response to the conspecific stimulus was significant, i.e., compared the value of each treatment group to the value “0” (no reduction). One-sample t -tests confirmed that fish from all but the 1.00% alcohol group significantly reduced their distance to the stimulus screen upon conspecific stimulus presentation (control $t = -5.886, df = 21, p < 0.0001$; 0.25% EtOH $t = -3.620, df = 20, p < 0.01$; 0.50% EtOH $t = -2.555, df = 20, p < 0.05$; 0.75% EtOH $t = -2.747, df = 21, p < 0.05$; 1.00% EtOH $t = -0.879, df = 19, p > 0.35$).

The effect of alcohol treatment during early development of zebrafish on the locomotory activity of zebrafish is shown in Fig. 4. Although some variation is evident, the alcohol treatment groups (control group included) do not appear to be different from each other. This is confirmed by ANOVA. It showed a significant Interval effect [$F(29, 2842) = 35.870, p < 0.0001$], but no significant Interval $\times$ Alcohol treatment interaction [$F(116, 2842) = 0.889, p > 0.75$] or Alcohol main effect [$F(4, 98) = 2.153, p > 0.05$] was found. These results are notable because they suggest that zebrafish from all groups changed (reduced) their locomotory activity (length of distance swam) in response to the sight of conspecifics but the alcohol treatment had no effect on this response. Because of the absence of Interval $\times$ Alcohol treatment interaction, we calculated the total distance traveled during the entire session and plotted (Fig. 5) and analyzed these data. Although the group treated with 0.25% alcohol appears to be slightly more active than the fish in the other groups, the difference is not significant [$F(4, 98) = 2.153, p > 0.05$] and the other groups show very similar activity levels to each other.

The last point we want to focus on is the effect of the predator stimulus. Perusal of Fig. 2 suggests that the presentation of an image of the predator increased the distance the experimental fish swam from the presentation screen compared to the distance the fish were from the screen during the 1 minute period immediately preceding the predator stimulus presentation, but embryonic exposure to alcohol did not modify this response. To confirm this and to explore whether embryonic alcohol exposure had any differential effects on the predator image-induced responses, we calculated the ratio between the distance measured during the 1-minute predator presentation period and the 1-minute no-stimulus period immediately preceding it (distance value obtained for the 1-minute predator period divided by the distance value obtained for the 1-minute no-stimulus period prior to the predator image presentation).

We computed this ratio for each of the 3 predator presentations. We expected an increase of distance from the stimulus screen upon presentation of the predator, i.e., a ratio value larger than 1. As there were 3 predator presentation periods, we obtained 3 values, which are shown in Fig. 6. The results confirm that the predator image presentation led to an increase of distance from the presentation screen, i.e., values are significantly above 1 for each of the 3 predator presentation periods (0.00% control $t_1 = 3.542$, $t_2 = 4.983$, $t_3 = 8.269$, $df = 21$, $p < 0.01$ for each t ; 0.25% EtOH $t_1 = 2.780$, $t_2 = 6.861$, $t_3 = 3.273$, $df = 20$, $p < 0.05$ for each t ; 0.50% EtOH $t_1 = 3.470$, $t_2 = 4.069$, $t_3 = 3.615$, $df = 20$, $p < 0.01$ for each t ; 0.75% EtOH $t_1 = 1.945$, $t_2 = 4.694$, $t_3 = 4.743$, $df = 20$, $p < 0.01$ for t_2 and t_3 and $p = 0.065$ for t_1 ; EtOH 1.00% $t_1 = 3.349$, $t_2 = 2.563$, $t_3 = 2.910$, $df = 19$, $p < 0.05$ for each t). However, the increase was consistent across intervals and treatment groups: ANOVA found no significant effect of Interval [$F(2, 200) = 2.761$, $p > 0.05$], Alcohol treatment [$F(1, 100) = 0.499$, $p > 0.70$], and the Interval $\times$ Alcohol treatment interaction was also nonsignificant [$F(8, 200) = 0.643$, $p > 0.70$]. In addition, we also investigated the effect of predator image on a number of other behavioral measures that we previously found sensitive to quantify antipredatory responses (Terence Perreira, Yohaana Fernandes, and Robert Gerlai, unpublished data), including the turn angle of swim, swim speed, distance from bottom, and the temporal variability of these responses within each fish. Essentially we found the same results as with distance from stimulus: the predator image was effective and elicited a significant change compared to the prior no-stimulus interval, but embryonic alcohol treatment had no effect on this response (data not shown).

DISCUSSION

The issue of whether any alcohol consumption during pregnancy is safe or how much alcohol is deleterious for the fetus is controversial (see e.g., Eustace et al., 2003; Henderson et al., 2007a,b). Nevertheless, it is clear that full-fledged fetal alcohol syndrome is on one extreme of the continuum and is due to unusually heavy drinking, i.e., regular consumption of more standard drinks daily (at least 9 g alcohol per day). More moderate drinking is also believed to have significant negative effects on the fetus that, although may not manifest as gross anatomical abnormalities, may lead to significant aberrations detectable at the behavioral level when the exposed children grow up (e.g., Redgrave et al., 2003). The goal of the current study was to examine whether smaller concentrations of alcohol administered for shorter periods of time than before could lead to significant alterations detectable in the behavior of zebrafish. Briefly, our question was whether zebrafish could be utilized as a tool for the analysis of the effects of moderate to mild prenatal alcohol consumption. The answer appears to be yes. Zebrafish not exposed to alcohol showed a robust decrease of distance from the stimulus screen upon presentation of the conspecific group, an animated image of a shoal of zebrafish but this decrease was blunted by early alcohol exposure.

Zebrafish swim in shoals in nature (Engeszer et al., 2007) and have been also shown to exhibit strong preference for their conspecifics in the laboratory (e.g., Gerlai et al., 2000; Saverino and Gerlai, 2008). The image of a group of conspecifics has been demonstrated to elicit the species-typical shoaling behavior: the experimental subject approaches the group of zebrafish images and subsequently swims parallel in close proximity to the moving images (Saverino and Gerlai, 2008; R. Gerlai, personal observation). The dramatic reduction of distance between the experimental subject and the image presentation screen during conspecific presentation is likely to reflect this shoaling tendency: the onset of the response precisely coincides with the appearance of the conspecific images and its offset with the disappearance of the images (Fig. 2). More importantly, this response to the conspecifics is diminished in fish that received exposure to alcohol 24-hour post-fertilization. Also notable is that the severity of the effect appears linearly correlated with the concentration of alcohol employed.

The interpretation of this finding is speculative at this point. Given that alcohol is known to affect locomotory function in zebrafish and may alter numerous molecular mechanisms associated with motor function (for review and examples see Gerlai et al., 2000), one may suggest that the above behavioral effect is due to disrupted motor function and hypothesize that the affected fish could not approach the images fast enough or could not swim parallel to them well enough. Similarly, exposure to alcohol during the development of zebrafish is known to impair vision (see e.g., Arenzana et al., 2006; Matsui et al., 2006) and one may argue that perhaps the behavioral effects were due to abnormal vision. While these hypotheses will have to be systematically analyzed in the future, our current results suggest that they are not likely to be correct.

Analysis of the effect of alcohol exposure on the level of activity (distance swam, Fig. 4 and Fig. 5) demonstrated that motor function was unaffected by the treatment. Figure 4 also reveals that fish from all groups responded with decreased activity to the presentation of conspecifics, and the nonsignificant Interval $\times$ Alcohol interaction shown by ANOVA confirmed that this reduction in activity was not affected by alcohol treatment. Briefly, it appears that all fish perceived the stimulus and exhibited a consistently reduced activity in response to it, irrespective of prior alcohol treatment. Given that the only perceptual modality the experimental fish could use to detect the stimulus was vision, we conclude that the subjects could see the stimulus and the early alcohol treatment did not disrupt their vision. Because the very same stimulus (sight of conspecifics) induced the alcohol exposure-dependent decrease of responses in the distance from the stimulus screen, the most likely hypothesis is that social behavior, and not vision, was disrupted by early alcohol treatment.

Importantly, we could not demonstrate a similar alcohol effect in the distance from stimulus screen when the stimulus shown was the image of the predator. Fish from all groups significantly increased their distance from the computer screen upon the presentation of the predator image irrespective of whether or how much alcohol they received during embryonic development. This result again confirms that all fish could see but it also demonstrates that the avoidance reaction of our experimental fish remained unaffected by the alcohol treatment they received. Given that the general method of stimulus presentation for the shoal and the predator images was the same (computer animation shown on the same screen), the test environment was also identical and the same fish were tested in the same manner using these stimuli, one may argue that embryonic alcohol treatment selectively affected functions associated with responding to the former (shoal) but not the latter (predator) stimulus.

However, the lack of effects of embryonic alcohol treatment on fear and anxiety may need to be carefully evaluated and the above conclusion may be premature. Anxiety and fear responses have been thoroughly characterized for rodents and numerous well-established paradigms have been developed over the past 5 decades (for examples see Blanchard et al., 2003; Gerlai, 1998; and references therein). Zebrafish is a novel study species in this regard and although some fear inducing stimuli [e.g., alarm substance (Hall and Suboski, 1995; Speedie and Gerlai, 2008) or predatory fish (Bass and Gerlai, 2008)] as well as the responses of zebrafish to these stimuli, have been described, it is too early to tell what an optimal fear paradigm sensitive to modifications of brain function by embryonic alcohol treatment may be.

The last point we would like to briefly discuss are the methodological aspects of this study and their relevance to high throughput screening. It is notable that both the stimulus presentation and the quantification of the responses of the experimental zebrafish to the stimuli were computerized and required minimal experimenter interference. The advantage of this is 2 fold. First, the precision of the experiment is increased. Second, the paradigm becomes scalable, i.e., can be run in a massively parallel manner. These 2 aspects are crucial criteria for high throughput screening. Given that zebrafish is particularly amenable to genetic screens (forward

genetics) as well as drug screening due to its small size, our current work suggests that it will be a promising study species from this perspective.

In summary, although activity levels and fear responses were not found to be altered by early alcohol exposure, response to the sight of conspecifics was. The employed low level of alcohol treatment during early development of zebrafish induced no physical abnormalities and the mortality rate of the exposed fish was also not increased. Thus the long-lasting effect of early alcohol exposure on the social behavior of zebrafish is notable. It suggests that this lower order vertebrate may be a promising model with which mechanisms of the behavioral changes seen in the more frequent mild to moderate prenatal alcohol exposure cases may be investigated.

ACKNOWLEDGMENTS

We would like to thank Rajesh Krishnannair and Terence Perreira for their technical help. This work was supported by an NIH/NIAAA grant (#1R01AA015325-01A2) to RG.

REFERENCES

- Arenzana FJ, Carvan MJ III, Aijon J, Sanchez-Gonzalez R, Arevalo R, Porteros A. Teratogenic effects of ethanol exposure on zebrafish visual system development. *Terat Neurotox* 2006;28:342–348.
- Bass SLS, Gerlai R. Zebrafish (*Danio rerio*) responds differentially to stimulus fish: The effects of sympatric and allopatric predators and harmless fish. *Behav Brain Res* 2008;186:107–117. [PubMed: 17854920]
- Becker HC, Díaz-Granados JL, Randall CL. Teratogenic actions of ethanol in the mouse: a minireview. *Pharmacol Biochem Behav* 1996;55:501–513. [PubMed: 8981580]
- Bilotta J, Barnett JA, Hancock L, Saszik S. Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome. *Neurotoxicol Teratol* 2004;26:737–743. [PubMed: 15451038]
- Blanchard DC, Griebel G, Blanchard RJ. Conditioning and residual emotionality effects of predator stimuli: some reflections on stress and emotion. *Prog Neuropsychopharmacol Biol Psychiatry* 2003;27:1177–1185. [PubMed: 14659473]
- Blaser R, Gerlai R. Behavioral phenotyping in Zebrafish: Comparison of three behavioral quantification methods. *Behav Res Meth* 2006;38:456–469.
- Boehm SL, Lundahl KR, Caldwell J, Gilliam DM. Ethanol teratogenesis in the C57BL/6J, DBA/2J, and A/J inbred mouse strains. *Alcohol* 1997;14:389–395. [PubMed: 9209555]
- Browman KE, Crabbe JC. Alcohol and genetics: new animal models. *Mol Med Today* 1999;5:310–318. [PubMed: 10377523]
- Carvan MJ III, Loucks E, Weber DN, Williams FE. Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis. *Neurotoxicol Teratol* 2004;26:757–768. [PubMed: 15451040]
- Cloninger C. Neurogenetic adaptive mechanisms in alcoholism. *Science* 1987;236:410–416. [PubMed: 2882604]
- Engeszer RE, Patterson LB, Rao AA, Parichy DM. Zebrafish in the wild: A review of natural history and new notes from the field. *Zebrafish* 2007;4:21–40. [PubMed: 18041940]
- Eustace LW, Kang DH, Coombs D. Fetal alcohol syndrome: a growing concern for health care professionals. *J Obstet Gynecol Neonatal Nurs* 2003;32:215–221.
- Fernandez K, Caul WF, Haenlein M, Vorhees CV. Effects of prenatal alcohol on homing behavior, maternal responding and open-field activity in rats. *Neurobehav Toxicol Teratol* 1983;5:351–356. [PubMed: 6877476]
- Gerlai R. Contextual learning and cue association in fear conditioning in mice: A strain comparison and a lesion study. *Behav Brain Res* 1998;95:191–203. [PubMed: 9806439]
- Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: Zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. *Pharmacol Biochem Behav* 2000;67:773–782. [PubMed: 11166068]
- Gerlai R, Lee V, Blaser R. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). *Pharmacol Biochem Behav* 2006;85:752–761. [PubMed: 17196640]

- Gerlai R, Prajapati S, Ahmad F. Differences in acute alcohol induced behavioral responses among zebrafish populations. *Alcohol Clin Exp Res* 2008;32:1763–1773. [PubMed: 18652595]
- Green J. Fetal alcohol spectrum disorder: Understanding the effects of prenatal alcohol exposure and supporting students. *J Sch Health* 2007;77:103–108. [PubMed: 17302851]
- Grunwald DJ, Eisen JS. Timeline: headwaters of the zebrafish—emergence of a new model vertebrate. *Nat Rev Genet* 2002;3:717–724. [PubMed: 12209146]
- Guarnieri DJ, Heberlein U. *Drosophila melanogaster*, a genetic model system for alcohol research. *Int Rev Neurobiol* 2003;54:199–228. [PubMed: 12785288]
- Hall D, Suboski MD. Visual and olfactory stimuli in learned release of alarm reactions by zebra danio fish (*Brachydanio rerio*). *Neurobiol Learn Mem* 1995;63:229–240. [PubMed: 7670836]
- Harwood HJ, Fountain D, Livermore G. Economic costs of alcohol abuse and alcoholism. *Recent Dev Alcohol* 1998;14:307–330. [PubMed: 9751951]
- Henderson J, Gray R, Brocklehurst P. Systematic review of effects of low-moderate prenatal alcohol exposure on pregnancy outcome. *BJOG* 2007b;114:243–252. [PubMed: 17233797]
- Henderson J, Kesmodel U, Gray R. Systematic review of the fetal effects of prenatal binge-drinking. *J Epidemiol Community Health* 2007a;61:1069–1073. [PubMed: 18000129]
- Lockwood B, Bjerke S, Kobayashi K, Guo S. Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. *Pharmacol Biochem Behav* 2004;77:647–654. [PubMed: 15006478]
- Lugo JN Jr, Marino MD, Cronise K, Kelly SJ. Effects of alcohol exposure during development on social behavior in rats. *Physiol Behav* 2003;78:185–194. [PubMed: 12576115]
- Matsui JI, Egana AL, Sponholtz TR, Adolph AR, Dowling JE. Effects of ethanol on photoreceptors and visual function in developing zebrafish. *Inv Ophthalm Visual Sci* 2006;46:4589–4597.
- Osborn JA, Yu C, Gabriel K, Weinberg J. Fetal ethanol effects on benzodiazepine sensitivity measured by behavior on the elevated plus-maze. *Pharmacol Biochem Behav* 1998;60:625–633. [PubMed: 9678645]
- Redgrave GW, Swartz KL, Romanoski AJ. Alcohol misuse by women. *Int Rev Psychiatry* 2003;15:256–268. [PubMed: 15276964]
- Rice DP. Economic costs of substance abuse, 1995. *Proc Assoc Am Physicians* 1999;111:119–125. [PubMed: 10220806]
- Saverino C, Gerlai R. The social zebrafish: Behavioral responses to con-specific, heterospecific, and computer animated fish. *Behav Brain Res* 2008;191:77–87. [PubMed: 18423643]
- Sison M, Cawker J, Buske C, Gerlai R. Fishing for genes of vertebrate behavior: Zebra fish as an upcoming model system. *Lab Anim* 2006;35:33–39.
- Speedie N, Gerlai R. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). *Behav Brain Res* 2008;188:168–177. [PubMed: 18054804]
- Steinhausen HC. Children of alcoholic parents. A review. *Eur Child Adolesc Psychiatry* 1995;4:143–152. [PubMed: 8846203]
- Stratton, K.; Howe, C.; Battaglia, F. Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention and Treatment. Washington, DC: National Academy Press; 1996. p. 213
- Streissguth, AP. Fetal Alcohol Syndrome: A Guide for Families and Communities. Baltimore, MD: Paul H. Brookes Publishing Co; 1997. p. 306
- Streissguth AP, Dehaene P. Fetal alcohol syndrome in twins of alcoholic mothers: concordance of diagnosis and IQ. *Am J Med Genet* 1993;47:857–861. [PubMed: 8279483]
- Taylor AN, Romeo HE, Beylin AV, Tio DL, Rahman SU, Hovda DA. Alcohol consumption in traumatic brain injury: Attenuation of TBI-induced hyperthermia and neurocognitive deficits. *J Neurotrauma* 2002;19:1597–1608. [PubMed: 12542860]
- Vorhees CV. Maternal age as a factor in determining the reproductive and behavioral outcome of rats prenatally exposed to ethanol. *Neurotoxicol Teratol* 1988;10:23–34. [PubMed: 3352565]
- Vorhees CV, Fernandez K. Effects of short-term prenatal alcohol exposure on maze, activity, and olfactory orientation performance in rats. *Neurobehav Toxicol Teratol* 1986;8:23–28. [PubMed: 3703092]

Fig. 1. Concentration of alcohol (EtOH) inside the zebrafish egg after 2 hours of alcohol exposure at 24-hour postfertilization. The results show the average of 20 eggs per sample per alcohol treatment and suggest an alcohol treatment dose-dependent increase in concentration inside the egg reaching about 1 / 25 to 1 / 30 of the external alcohol dose employed.

Fig. 2.

Average distance between the adult experimental zebrafish and the stimulus presentation computer screen plotted for 1-minute intervals of the 30-minute behavioral recording session. Mean $\pm$ SEM are shown. Sample sizes are given in the Methods section. The timeline of stimulus presentation is shown by the horizontal bar above the x axis (white, no stimulus; black, image of a group of 5 conspecifics; gray, image of the predator). The concentration of alcohol treatment (started at 24th hour postfertilization and lasting for 2 hours) is shown above the graphs. Note the dramatic reduction in distance from the stimulus screen upon presentation of the conspecifics and the increase in distance upon removal of conspecifics. Also note that this response diminishes with increasing concentrations of alcohol treatment.

Fig. 3.

Reduction of distance between the adult experimental zebrafish and the stimulus screen in response to conspecific stimulus presentation. Mean $\pm$ SEM are shown. Sample sizes are given in the Methods section. Values represent the difference between the distances fish were from the stimulus presentation screen before and during conspecific stimulus presentation. Larger negative values mean larger reduction of distance, i.e., stronger response to the conspecifics. The concentration of alcohol (EtOH %) represents the alcohol treatment administered at 24th hour postfertilization and lasting for 2 hours. Note the dose-response relationship: increasing concentrations of alcohol led to diminished responses (smaller reduction of distance in response to the conspecifics).

Fig. 4.

Total distance swam by the adult experimental zebrafish plotted for 1-minute intervals of the 30-minute recording session. Mean $\pm$ SEM are shown. Sample sizes are given in the Methods section. The timeline of stimulus presentation is shown by the horizontal bar above the x axis (white, no stimulus; black, image of a group of 5 conspecifics; gray, image of the predator). The concentration of alcohol treatment (started at 24th hour postfertilization and lasting for 2 hours) is shown above the graphs. Note the dramatic reduction of distance swam (reduced activity) upon presentation of the conspecifics. Note that this response is independent of the concentration of alcohol.

Fig. 5. Total distance swam by the adult experimental zebrafish during the entire 30-minute recording session. Mean $\pm$ SEM are shown. Sample sizes are given in the Methods section. The concentration of alcohol treatment (started at 24th hour postfertilization and lasting for 2 hour) is shown under the x axis. Note that early developmental exposure to alcohol had no significant effect on general activity of the adult fish.

Fig. 6.

Increase of distance from stimulus screen in response to predator image presentation relative to distance from stimulus screen during the 1-minute interval immediately preceding the stimulus presentation. The ratio of distance values (distance from stimulus screen during the 1-minute predator stimulus presentation divided by the distance from stimulus screen during the 1-minute period preceding the predator presentation) is calculated and plotted. Mean $\pm$ SEM are shown. Note that there were 3 predator stimulus presentations and the values are shown separately for each of these. Sample sizes are given in the Methods section. The concentration of alcohol treatment (started at 24th hour postfertilization and lasting for 2 hours) is shown above each corresponding graph. A lack of change from the no-stimulus interval to the predator presentation interval would give a ratio value of 1, and this is represented by the dashed line on each graph. Also note that while the predator had a significant effect and increased the distance from stimulus screen (ratio above 1) early developmental exposure to alcohol did not modify this effect.