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Differences in Acute Alcohol-Induced Behavioral Responses Among Zebrafish Populations

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Abstract

Background—With the arsenal of genetic tools available for zebrafish, this species has been successfully used to investigate the genetic aspects of human diseases from developmental disorders to cancer. Interest in the behavior and brain function of zebrafish is also increasing as CNS disorders may be modeled and studied with this species. Alcoholism and alcohol abuse are among the most devastating and costliest diseases. However, the mechanisms of these diseases are not fully understood. Zebrafish has been proposed as a model organism to study such mechanisms. Characterization of alcohol's effects on zebrafish is a necessary step in this research.

Methods—Here, we compare the effects of acute alcohol (EtOH) administration on the behavior of zebrafish from 4 distinct laboratory-bred populations using automated as well as observation based behavioral quantification methods.

Results—Alcohol treatment resulted in significant dose-dependent behavioral changes but the dose–response trajectories differed among zebrafish populations.

Conclusions—The results demonstrate for the first time a genetic component in alcohol responses in adult zebrafish and also show the feasibility of high throughput behavioral screening. We discuss the exploration and exploitation of the genetic differences found.

Keywords

Alcoholism; Alcohol Abuse; Acute Alcohol Administration; Strain Comparison; Zebrafish; Zebra Danio

Alcoholism is an enormous problem, yet a clear picture for its mechanisms has not emerged. Alcohol (EtOH or ethyl alcohol) abuse cost more than \$150 billion yearly and resulted in 40,000 deaths in the United States of America, figures that are worsening (Harwood et al., 1998; Rice, 1999). The prevalence of alcohol abuse is staggering: 30 million people are afflicted with this disease only in the United States of America (Robbins et al., 1984; Sullivan and Handley, 1993). Detoxification or long-term rehabilitation programs have had limited success due to high relapse rate (Fuller and Hiller-Sturmhöfel, 1999; O'Brien et al., 1995). Pharmacotherapies have also been mainly unsuccessful (O'Brien et al., 1995) because alcohol acts through numerous molecular targets and in complex ways that remain to be elucidated (Crews et al., 1996; Deitrich et al., 1989; Hoek and Kholodenko, 1998; Koob, 1996; Lawrence, 2007; Lovinger et al., 1990; O'Brien et al., 1995).

To tackle this complexity, and because alcohol abuse has a significant genetic component (Cloninger, 1987), animal models have been employed using a range of quantitative genetic and molecular genetic approaches (Browman and Crabbe, 1999). A large number of these studies utilizes vertebrate model organisms such as the house mouse and the rat, but *Drosophila* has also been successfully employed (Guarnieri and Heberlein, 2003).

Zebrafish has also been proposed as a model organism for alcohol research (Gerlai et al., 2000). It is a small (4-cm long) and prolific vertebrate (400 eggs/female every other day) which, due to its social nature, can be housed in large numbers in a small room (1,500 zebrafish/m²), characteristics that make this species ideal for genetic screens. Zebrafish can be immersed into alcohol solutions and for prolonged periods of time if required (e.g., Gerlai et al., 2006) thus allowing precise and noninvasive drug delivery. Last, sophisticated genetic tools have been developed for zebrafish allowing both forward and reverse genetic approaches (Grunwald and Eisen, 2002).

A significant bottleneck in neurobehavioral genetics with zebrafish is the lack of understanding of its behavior in general (Sison et al., 2006) and the paucity of information on the effects of alcohol in particular (Gerlai et al., 2000, 2006). Nevertheless, interest in this question has been increasing. For example, behavioral effects of alcohol exposure during early development of zebrafish have been shown and in fact zebra-fish has been proposed as a potential model for fetal alcohol syndrome (Carvan et al., 2004). Strain differences in the alteration of development of zebrafish induced by early ethanol exposure have been demonstrated (Loucks and Carvan, 2004). Although most studies focused on developmental aspects of ethanol exposure, strain differences in alcohol responses have also been investigated in the adult zebrafish. Dlugos and Rabin (2003) have revealed differences between 2 distinct zebrafish populations in shoaling behavior in response to acute and chronic alcohol exposure, and concluded that the results demonstrated genetic influence. While this latter study was suggestive, the 2 zebrafish populations were obtained from pet stores and thus differential environmental effects could not be ruled out. Furthermore, the fish were obtained from multiple stores and the origin (genetic make-up) of fish deemed to belong to a particular population could be speculated on only on the basis of superficial visible markers (fin length or body coloration). Briefly, the contribution of environmental and genetic factors to the observed population differences could not be dissociated.

In this study, we investigate the effects of acute alcohol administered to adult zebrafish that were bred, raised, and tested under controlled laboratory conditions and at the same time. We chose the analysis of adults because alcoholism and alcohol abuse starts during late teenage years or in adulthood in humans. We compare the alcohol responses of 4 populations of zebrafish and investigate whether there are alcohol dose-dependent or -independent differences among these populations for 2 main reasons. First, one of the populations we test in this study is the AB strain which is often used in forward genetic (mutagenesis) studies but has not been characterized for its alcohol responses at its adult stage before. We plan to use this strain for our forward genetic studies thus its analysis is important for our future work. Second, discovery of population differences in alcohol responses would imply genetic differences and may open the avenue toward the identification of genetically variable loci in zebrafish.

In addition, another goal of this study is to investigate some procedural and technical aspects of behavioral testing. Behavioral testing of zebrafish is much less frequently employed (Sison et al., 2006) than that of other laboratory organisms including the house mouse (Gerlai, 2002), and thus exploring different methods is of importance. Although here we study the behavioral effects of alcohol using previously developed behavioral paradigms (Gerlai et al., 2000), we employ some modifications in an attempt to refine the tests. Furthermore, and more importantly, we now utilize automated quantification techniques and investigate whether such

methods are sensitive enough to detect acute alcohol effects and genetic differences in these effects in zebrafish.

METHODS

Animals and Housing

A total of 202 zebrafish were tested in this study. Four distinct populations of fish were used. One of these populations was the AB, a genetically well-defined strain maintained in the Zebrafish Center ZFIN (Eugene, OR; <http://zfin.org/>). This strain is bred using a “Round Robin” mating system designed to preserve genetic variability. Nevertheless, due to decades of closed breeding, only less than 25% of the loci of the genome of AB are heterozygous (Guryev et al., 2006). An advantage of this strain is that a large number of genetic markers have been developed for it and thus AB has become the strain of choice in forward genetic studies in which linkage analysis-based mapping is employed (e.g., Guo, 2004). Behavioral responses of this strain to alcohol treatment in the adult have not been tested. We plan to use this strain for future mutagenesis studies. In addition to AB, we chose 3 populations of zebrafish whose progenitors we obtained from a local pet store (Big Al’s Aquarium Warehouse Services Inc., Mississauga, ON, Canada): Short fin wild type (SFWT), Long fin wild type (LFWT) and Leopard danio (LD). Two of these populations (SFWT and LFWT) correspond to those tested for their shoaling behavior in response to acute and chronic alcohol treatment by Dlugos and Rabin (2003) and have also been used in a number of other behavioral studies (Al-Imari and Gerlai, 2008; Speedie and Gerlai, 2008; Bass and Gerlai, 2008; Miller and Gerlai, 2007). The third population, the LD, was chosen because of its broad availability from pet stores and because of its long breeding history as a color variant of zebrafish (Hamilton, 1822). Based on the above, one may expect genetic differences among these populations.

Five breeding pairs from each of the 4 populations were mated in our facility (UTM Vivarium) and their offspring were raised under identical conditions and at the same time. Equal number of offspring from each mating was selected for behavioral experimentation. The experimental fish were raised and housed in 3 l transparent acrylic tanks (15 fish per tank) that were part of a zebrafish rack system (Aquaneering Inc., San Diego, CA) with multistage filtration that contained a mechanical filter, a fluidized glass bed biological filter, an activated carbon filter, and a fluorescent UV light sterilizing unit. Every day 10% of the water was replaced with fresh system water [deionized water supplemented with 60 mg/l Instant Ocean Sea Salt (Big Al’s Pet Store)]. The water temperature was maintained at 27°C. Illumination was provided by fluorescent light tubes from the ceiling with lights turned on at 8 AM and off at 7 PM. Fish were fed a mixture of ground freeze-dried krill and flake food (Tetramin Tropical Flakes; Tetra USA, Blacksburg, VA). Behavioral experiments were conducted when the fish reached 4 months of age (fully developed sexually mature young adults).

Experimental Design and Procedure

Approximately 50 randomly chosen subjects from each of the 4 zebrafish populations were divided and randomly assigned to one of the following 4 alcohol dose treatment groups: EtOH 0.00% (control), EtOH 0.25%, EtOH 0.50%, and EtOH 1.00% (vol/vol %). The exact sample sizes used in the behavioral tests were as follows: AB 0.00% $n = 10$, AB 0.25% $n = 15$, AB 0.50% $n = 11$, AB 1.00% $n = 15$, LD 0.00% $n = 9$, LD 0.25% $n = 12$, LD 0.50% $n = 14$, LD 1.00% $n = 15$, LFWT 0.00% $n = 11$, LFWT 0.25% $n = 15$, LFWT 0.50% $n = 14$, LFWT 1.00% $n = 13$, SFWT 0.00% $n = 12$, SFWT 0.25% $n = 15$, SFWT 0.50% $n = 10$, SFWT 1.00% $n = 11$. Experimental zebrafish were placed in the corresponding alcohol dose for 60 minutes prior to behavioral testing, an immersion period known to be sufficient to achieve maximal blood and brain alcohol levels. The fish continued to be exposed to the corresponding alcohol dose during the behavioral tests. Notably, these acutely administered alcohol concentrations have

been found not to lead to mortality or to lasting physiological changes (for review see Gerlai et al., 2000). Indeed, this dosing regimen has been successfully utilized in adult zebrafish (Gerlai et al., 2000; Dlugos and Rabin, 2003; Blaser and Gerlai, 2006) and the blood alcohol levels achieved with it are expected to be in the range seen in the human clinic after mild to moderate acute alcohol consumption (for references see Le and Mayer (1996). Experiments were conducted between 10^{AM} and 5^{PM} in 4 test paradigms as described previously (Gerlai et al., 2000): first fish were tested in the novel open tank, then in the group preference paradigm, followed by an aggression (mirror) test, and finally in a predator exposure task. The 4 behavioral tests were originally designed to tap into a range of behavioral responses that one may expect to be affected by alcohol (Gerlai et al., 2000). The use of multiple tests and multiple behavioral measures is essentially the same approach that is employed in phenotyping of drug or mutation effects. In these studies test batteries are utilized to cover the range of potential functional alterations (for references and comparison of different test strategies in phenotyping see Gerlai, 2002).

The order of fish tested in the 4 test paradigms was random in terms of their population origin and alcohol dose. In each test, fish were placed individually into the experimental tank (50 × 25 × 30 cm, length × width × height) and were monitored for 10 minutes. Inter-test interval was 2 minutes. Upon conclusion of all 4 tests, the fish were returned to their home tank and were kept there for future experimentation.

Behavioral Tests

Novel Open Tank—Exposure to a novel test chamber, as well as handling by the experimenter, is an inherent part of most laboratory animal behavioral tests. The novel open tank task is intended to analyze behavior in response to these factors. In this task, zebrafish may exhibit elevated activity that habituates with time (Gerlai, 2003) and they may also show fear related behavioral responses (Gerlai et al., 2000). These behavioral responses were previously shown to be altered by alcohol in a dose-dependent manner in an outbred population of zebrafish (Gerlai et al., 2000).

The experimental tank was filled with system water mixed with alcohol to match the pretest alcohol concentration. The system water was previously exposed to zebrafish, thus all experimental fish experienced the same olfactory milieu. The tank was illuminated from above by two 13W fluorescent light bulbs. Three sides of the tank were covered by a gray cardboard paper (partitions). The experimental fish was placed singly in the experimental tank and after a 20-second period its behavior was recorded for 10 minutes. Following the recording, the test fish was left in the tank undisturbed for a 2 minutes long inter-test interval.

Group Preference—Zebrafish is a highly social species. In nature and in the aquarium it forms schools, a group of individuals that swim close to one another (Engeszer et al., 2007; Miller and Gerlai, 2007). Individual zebrafish are expected to be motivated to join a school. This preference for the group, also termed as group cohesion, formed the basis of a behavioral test in which the effect of alcohol was previously investigated (Gerlai et al., 2000). In the latter study, multiple subjects were used at a time. In the current study, we used a single fish per observation to aid automated behavior quantification with the use of videotracking. The partitions on the right and left sides of the experimental tank were removed to allow unobstructed view of 2 adjacent stimulus tanks. One of these tanks contained 10 stimulus fish, conspecific zebrafish of the same population origin as the given experimental fish, and the other just fresh water. The positioning of the stimulus fish, i.e., whether they were presented on the left or the right side of the experimental tank varied randomly among test fish but was balanced across experimental groups. Behavior was recorded for 10 minutes. Following the

recording, the grey partitions were lowered on the side of the experimental tank, the test fish was left in the tank undisturbed for a 2 minutes inter-test interval.

Aggression Test—Solitary zebrafish encountering another individual often exhibit agonistic behavior, a response different in form and alcohol dose–response characteristics from social behavior (Gerlai et al., 2000). Agonistic behaviors were tested following the group preference task. A mirror was placed on one side of the test tank behind the partition. The side of mirror presentation varied randomly among test fish but was balanced according to experimental groups. Subsequently, the partitions were removed and the mirror was made visible to the test fish. Recording started 20 seconds later. As solitary fish of the same gender encountering each other often exhibit agonistic behaviors rather than group cohesion, the “approaching” mirror image is expected to elicit aggression. Behavior of zebrafish was monitored in this test again for 10 minutes. Following the recording, the partitions were lowered on the side of the experimental tank, the test fish was left in the tank undisturbed for a 2 minutes inter-test interval.

Predator Test—Antipredatory behavior of zebrafish is believed to be adaptive, thus likely to be under the influence of genetic factors (Bass and Gerlai, 2008; Speedie and Gerlai, 2008). Furthermore, predator model elicited behavioral responses were shown to be dependent upon acute alcohol dose (Gerlai et al., 2000). Thus, predator elicited responses may allow the detection of mutation effects on alcohol-dependent functional alterations in the brain.

The features that characterize a dangerous predator for zebrafish have not been identified yet. In this study, live stimulus fish were used instead of a predator model employed before (Gerlai et al., 2000). The “predator” stimulus was 2 goldfish (*Carassius auratus*) of the “black moor” strain (each 15-cm long). This stimulus was chosen for the following reasons. This variety of goldfish is black (the color of the predator model successfully employed before by Gerlai et al., 2000). It has large “telescope” eyes. Eyes and eye-like spots were previously shown to serve as key stimuli in antipredatory behavior in numerous species ranging from fish to mammals (for examples, see Gerlai et al., 2000). Finally, the stimulus fish was used in pairs because in isolation these fish were found to freeze and provide insufficient stimulation for the experimental zebrafish.

Upon completion of the aggression test, the stimulus tank containing the “predatory” stimulus was placed behind the partition on one side of the test tank and a water filled empty tank was left on the other side. After the 2 minutes inter-test interval, the partitions were removed on both sides of the test tank and the predator stimulus as well as the empty tank was made visible to the test fish. The left–right positioning of the stimulus fish versus the empty tank was randomized.

Quantification of Behavior

Behavior of the experimental fish was quantified using the Etho Vision Color Pro (version 3.0) software (Noldus Info. Tech., Wageningen, The Netherlands), an automated videotracking method, as described by Blaser and Gerlai (2006). The software quantifies swim path characteristics of zebrafish precisely and without the need for the experimenter to view videotapes. A digital video-camera (Sony DCR-HC20; Sony Corporation, Japan) placed in front of the observation tank recorded the behavior of zebrafish. The video-recordings were converted to digital AVI files and were stored on an external hard drive. The Etho Vision software was configured to accept these AVI files. Before the test, a background image of the empty experimental tank was recorded. The software compared the incoming image (10 Hz image sampling rate) to this background image, a subtraction method allowing accurate subject detection. Detection threshold levels (the minimum number of pixel changes between the

background and live images) were set to minimize the effect of environmental noise (from debris, reflections, air bubbles, etc). An additional detection criterion, “minimum surface area of the subject” was also employed: it was defined as the number of adjacent pixels with differences above a set noise threshold (minimum of 25 pixels). The pixel cluster above this minimum was interpreted as the target subject, and the *X,Y* coordinates of the center of this subject were recorded. Tracks were recorded for the full 10 minutes of each recording session.

The following parameters were quantified.

Distance From Bottom—Zebrafish live and forages near the surface and escape from their predators by diving deeper and performing erratic movements on the bottom (Engeszer et al., 2007; Speedie and Gerlai, 2008). Thus, it has been suggested that distance from the bottom may be used as a measure of fear: the smaller the distance the higher the level of fear may be (Gerlai et al., 2000). In addition, zebrafish whose motor function is impaired may also spend more time on the bottom. Alcohol has known anxiolytic properties at intermediate doses (Becker and Anton, 1990) and motor impairing properties at higher doses (Nutt and Peters, 1994). The distance of the experimental zebrafish from the bottom of the tank was measured 10 times per second (10 Hz sampling rate) throughout the recording session and the recorded distance values were added (cumulative distance).

Distance From Stimulus—Experimental zebrafish are expected to prefer the sight of a group of conspecifics (shoaling) as well as their mirror image (aggression) and avoid their predator. Alcohol has been shown to alter group preference and aggression as well as antipredatory behavior in zebrafish (Gerlai et al., 2000). The distance of the experimental zebrafish from the glass wall of its test tank adjacent to the stimulus tank containing the stimulus (a group of conspecifics, a mirror, or the “predator”) was recorded similarly to the above distance measure (10 Hz sampling rate, cumulative distance).

Path Length—Alcohol has known activity increasing properties at intermediate and activity decreasing properties at higher doses. To quantify locomotor activity, the total distance moved by the experimental fish (swim path length) was recorded.

Turn Angle—Activity may manifest in multiple ways. We observed that at higher alcohol doses, zebrafish continued to move but their locomotion was often interrupted by small direction changes. To quantify this potential alcohol effect, we sampled the turn angle (the angular change in the direction of locomotion) 10 times per second (10 Hz) and added the values obtained for the entire session length to quantify Turn angle. Quantification of all measures was conducted after calibration of Etho Vision videotracking software by inputting the actual dimensions of the test tank and thus all distance values are expressed in cm.

Videotracking may not be able to quantify complex motor and posture patterns and some of these behavioral patterns have been shown not to correlate with swim path characteristics (Blaser and Gerlai, 2006). Thus, in addition to the videotracking measures, we also quantified a motor pattern using the Observer event recording software (Noldus Info. Tech.), which facilitates precise manual, i.e., observation-based quantification of behavior.

Thrashing—Thrashing is a forceful back and forth swimming against the glass wall of the test tank. The fish performing this behavior are in physical contact with the glass wall. We quantified this behavior when it was performed towards the stimulus, i.e., when it was exhibited on the glass of the test tank adjacent to the stimulus tank containing the stimulus fish or the mirror. The duration relative to session length (percent of time) for which fish exhibited this behavior was calculated and was statistically analyzed.

Statistical Analysis

Analysis of data was conducted using SPSS (version 14 for the PC). Two factorial variance analyses, ANOVAs, were conducted with “Alcohol Dose” (4 levels) and “Population” (4 levels) as independent factors separately for each test paradigm. In case of significant main or interaction terms, the post hoc Tukey honestly significant difference (HSD) test was employed to investigate differences among treatment groups.

RESULTS

Analysis of responses to acute alcohol treatment revealed significant alcohol effects in several behaviors. Alcohol dose-independent population differences and alcohol dose by population interactions were also found. Briefly, the AB strain appeared to be most distinct among the 4 populations studied.

Novel Open Tank Test

ANOVA found a significant Population effect [$F(3,186) = 4.30, p < 0.01$] as well as Alcohol dose effect [$F(3, 186) = 7.39, p < 0.0001$] but no significant Population $\times$ Alcohol dose interaction [$F(9,186) = 1.22, p > 0.05$] for the behavioral measure “cumulative distance to bottom” (Fig. 1, Panel I). Perusal of this figure and post hoc Tukey HSD tests showed that fish from the AB strain swam least far from the bottom of the tank ($p < 0.05$) when compared with the other fish. Furthermore, zebrafish from strains LD, LFWT, and SFWT exhibited an inverted U-shaped dose–response curve suggesting that fish exposed to intermediate alcohol doses (0.25 to 0.50%) swam furthest from the bottom. However, the AB strain showed the opposite dose–response: fish exposed to intermediate alcohol doses swam closest to the bottom. Notably, ANOVA is known to be insensitive to detect interaction between main factors, e.g., the genotype and the environment (Wahlsten, 1990), and indeed, the above Population $\times$ Alcohol dose interaction could only be inferred from significant post hoc Tukey HSD test results. Analysis of path length revealed a Population effect that was bordering significance [ANOVA $F(3,186) = 2.58, p = 0.05$], which was due to the generally smaller path length values exhibited by fish from the AB strain (Fig. 1, Panel II). Although fish exposed to intermediate doses of alcohol appeared to show increased path lengths, the alcohol dose effect was found non-significant [$F(3,186) = 0.95, p > 0.05$], and the Population $\times$ Alcohol dose interaction was also nonsignificant [$F(9,186) = 0.82, p > 0.05$]. Analysis of the amount of turning (Fig. 1, Panel III), quantified as cumulative turn angle showed no significant differences among the 4 populations [ANOVA $F(3,186) = 0.82, p > 0.05$] but the alcohol dose effect was significant and consistent across the populations [ANOVA Alcohol dose $F(3,186) = 3.17, p < 0.05$; Population $\times$ Alcohol dose interaction $F(9,186) = 0.59, p > 0.05$].

Group Preference

Analysis of cumulative distance to bottom showed similar results (Fig. 2, Panel I) to those obtained in the Novel open tank test. ANOVA revealed a significant Population effect [$F(3,186) = 3.28, p < 0.05$], a significant Alcohol dose effect [$F(3,186) = 5.40, p < 0.001$], and a close to significant Population $\times$ Alcohol dose interaction [$F(9,186) = 1.75, p = 0.08$]. Tukey HSD post hoc tests confirmed these findings and showed that unlike the other 3 populations showing an inverted U-shaped dose–response curve, AB fish exhibited a U-shaped dose–response curve, i.e., swam nearest to the bottom of the tank when exposed to intermediate doses of alcohol. Analysis of path length (Fig. 2, Panel II) revealed again a significant Population [ANOVA $F(3, 186) = 5.16, p < 0.01$] effect but no significant Alcohol dose [$F(3, 186) = 0.77, p > 0.05$] or Population $\times$ Alcohol dose interaction effects [$F(9,186) = 0.72, p > 0.05$] and Tukey HSD showed that AB fish swam less ($p < 0.05$) compared with LD and SFWT. Cumulative turn angle (Fig. 2, Panel III) showed a general increase with increasing alcohol doses in all populations except the AB strain. ANOVA and Tukey HSD tests confirmed this

observation [ANOVA, Population $F(3,186) = 0.31, p > 0.05$; Alcohol dose $F(3,186) = 2.86, p < 0.05$; Population $\times$ Alcohol dose $F(9,186) = 0.81, p > 0.05$]. Despite the apparent trend, the effect of alcohol on the cumulative distance to the stimulus fish (Fig. 2, Panel IV) did not reach significance [ANOVA Alcohol dose $F(3,186) = 2.06, p > 0.05$]. The differences among the populations [$F(3,186) = 0.19, p > 0.05$] and the Population $\times$ Alcohol dose interaction [$F(9,186) = 0.50, p > 0.05$] was also nonsignificant. ANOVA found no significant population differences in Thrashing towards stimulus [$F(3,186) = 1.63, p > 0.05$] but the effect of Alcohol dose was significant [$F(3,186) = 5.94, p < 0.01$] and consistent across the populations [no significant Population $\times$ Alcohol dose interaction, $F(9,186) = 0.62, p > 0.05$; Fig. 2, Panel V]. Tukey HSD tests confirmed this finding: alcohol reduced the percent of time fish performed Thrashing towards the stimulus fish in all populations except in LD, where a similar, but nonsignificant, trend was seen.

Aggression Test

Analysis of the cumulative distance to bottom (Fig. 3, Panel I) in this task revealed results similar to those obtained in the other tasks: ANOVA found a Population effect with borderline significance [$F(3,186) = 2.18, p = 0.09$], a nonsignificant Alcohol dose effect [$F(3,186) = 0.61, p > 0.05$], but a significant Population $\times$ Alcohol dose interaction [$F(9,186) = 2.22, p < 0.05$]. Tukey HSD tests suggest that while AB exhibited a U-shaped dose–response curve, the curve was inverted U or flat (i.e., nonsignificant dose effects) for the other populations. ANOVA showed no significant effects for path length [Population $F(3,186) = 1.65, p > 0.05$; Alcohol dose $F(3,186) = 1.34, p > 0.05$, Population $\times$ Alcohol dose $F(9,186) = 1.02, p > 0.05$] (Fig. 3, Panel II). Cumulative turn angle (Fig. 3, Panel III) again showed an alcohol dose-dependent increase in all populations [Population $F(3,186) = 0.16, p > 0.05$; Alcohol dose $F(3,186) = 4.29, p < 0.01$, Population $\times$ Alcohol dose $F(9,186) = 0.57, p > 0.05$]. Analysis of cumulative distance to stimulus (Fig. 3, Panel IV) showed that the populations did not significantly differ from each other [ANOVA $F(3,186) = 1.76, p > 0.05$] but alcohol did have a significant effect [$F(3,186) = 9.65, p < 0.0001$]. Although the Population $\times$ Alcohol dose interaction was nonsignificant [$F(9,186) = 0.89, p > 0.05$], the dose–response curves of LD, LFWT, and SFWT fish and that of the AB strain appear different. While the former 3 are U-shaped with intermediate alcohol doses leading to somewhat diminished or unaltered distance values, AB fish show a dose-dependent increase of the distance values. The time fish spent thrashing at the mirror (Fig. 3, Panel V) was found significantly affected by alcohol dose [$F(3,186) = 5.99, p < 0.001$] as well as by the population origin of the fish [$F(3,186) = 3.54, p < 0.05$] but no significant interaction between these factors was found [$F(9,186) = 0.72, p > 0.05$]. Tukey HSD tests showed that AB fish spent significantly ($p < 0.05$) less time thrashing at the mirror compared with LFWT, and in general, intermediate alcohol doses increased while the highest dose decreased the response.

Predator Test

Analysis of distance to bottom (Fig. 4, Panel I) again showed a U-shaped dose–response curve in AB fish and a mainly inverted U-shaped curve for the other populations [ANOVA Population $F(3,186) = 4.06, p < 0.01$; Alcohol dose $F(3,186) = 3.96, p < 0.01$, Population $\times$ Alcohol dose $F(9,186) = 3.48, p < 0.001$]. Path length (Fig. 4, Panel II) was not significantly affected by population origin [ANOVA $F(3,186) = 2.65, p = 0.05$], or alcohol dose [$F(3,186) = 1.20, p > 0.05$] and the Population $\times$ Alcohol dose interaction was also nonsignificant [$F(9,186) = 1.00, p > 0.05$]. Analysis of cumulative turn angle (Fig. 4, Panel III), however, showed an alcohol dose dependent [$F(3,186) = 2.96, p < 0.05$] but population independent change [Population $F(3,186) = 0.88, p > 0.05$; Alcohol dose Population $\times$ Alcohol dose $F(9,186) = 0.48, p > 0.05$] with the highest dose inducing significantly higher turn angle values in most populations. Analysis of the cumulative distance to stimulus (Fig. 4, Panel IV) revealed no significant population differences [ANOVA $F(3,186) = 1.59, p > 0.05$], alcohol dose effects [$F(3,186) =$

0.86, $p > 0.05$], or interaction between these factors [$F(9,186) = 0.95$, $p > 0.05$]. Last, analysis of the time spent thrashing near the stimulus (Fig. 4, Panel V) revealed no significant effects of either experimental factors [ANOVA Population $F(3,186) = 4.89$, $p < 0.01$; Alcohol dose $F(3,186) = 0.64$, $p > 0.05$, Population $\times$ Alcohol dose $F(9,186) = 0.72$, $p > 0.05$].

DISCUSSION

Briefly, acute alcohol treatment was found to significantly affect the behavior of adult zebrafish. Differences among zebrafish populations were also detected. However, these differences were not always independent of the effects of alcohol, i.e., Population $\times$ Alcohol dose interaction was found. Particularly interesting was how fish from the AB strain versus from the other 3 populations responded. For example, while LD, LFWT, and SFWT fish swam further from the bottom in response to intermediate doses of alcohol (inverted U-shaped dose–response curve) similarly to another outbred population tested before (Gerlai et al., 2000), AB fish showed the opposite, a U-shaped dose–response.

Zebrafish live near the surface of the water in nature (Engeszer et al., 2007) and has been observed performing erratic movement on the bottom in response to fear inducing stimuli in captivity (Speedie and Gerlai, 2008; Gerlai et al., 2000). Alcohol has known anxiolytic properties at intermediate doses in mammals (Becker and Anton, 1990). The increase of distance from bottom in response to intermediate alcohol doses has been interpreted as reduced fear, a response that was found independent of general locomotory activity (Gerlai et al., 2000). The reduced distance to bottom seen in fish exposed to the highest concentration of alcohol, on the other hand, was found to be associated with motor impairing properties of alcohol (Gerlai et al., 2000). Irrespective of the interpretation, the AB strain did not exhibit these characteristic responses. It is possible that AB zebrafish are less sensitive to alcohol (shift of dose–response trajectory to the left) and would require higher doses to exhibit responses characteristic of the other populations. Alternatively, the typical dose-dependent responses may not be possible to elicit in AB fish due to altered manifestation of alcohol effects, e.g., altered fear reactions or motor function, hypotheses that will be tested in the future.

Notably, the dose–response curves of any given population in the distance to bottom (as well as other measures) were remarkably similar across the 4 tests. By the end of the last recording session, the experimental zebrafish had been in their corresponding alcohol dose for over 100 minutes. Thus, we conclude that the effect of alcohol remained constant, i.e., no adaptation occurred during this period.

Alcohol-dependent changes in the distance of experimental zebrafish from their mirror image in the aggression test suggested that again AB fish differed from the other 3 populations. While fish from populations LD, LFWT, and SFWT swam either significantly closer to or at the same distance from the mirror when exposed to intermediate (0.25 to 0.50%) doses of alcohol, AB fish increased their distance with increasing alcohol doses. Previously, intermediate doses of alcohol were found to increase agonistic behaviors in zebra-fish in an outbred population manifesting as reduced distance from mirror image and increased aggressive displays (Gerlai et al., 2000). Thus again, AB fish differed from both this and the currently studied zebrafish populations.

Unexpectedly, no alcohol effects were found in the distance experimental fish maintained from the group of conspecifics (group preference test) and from the predator stimulus (predator task). These results are not in accordance with previous findings (Gerlai et al., 2000). In the latter study, groups of experimental fish were found to increase their distance from the stimulus conspecifics as a result of alcohol treatment. Here, however, experimental fish were tested singly and not in groups, a procedural change that simplified automated tracking but one which

may have altered shoaling tendencies of the experimental fish. It is also possible that cumulative distance is not a sensitive enough measure of shoaling tendencies. The latter is supported by the analysis of thrashing toward the stimulus. This behavior, which has been used to quantify shoaling tendencies (e.g., Saverino and Gerlai, 2008), was reduced by alcohol administration in all populations of zebrafish similarly to what has been found before (Gerlai et al., 2000). With regard to the lack of effect of alcohol on the responses to the predatory stimulus, it is notable that live predators have not been used before. The assumption that zebrafish would avoid the large black goldfish with enlarged eyes, a reaction that would be diminished by the anxiolytic properties of alcohol, was apparently incorrect. The results suggest that experimental zebrafish did not avoid the stimulus goldfish. Although the predator model used previously was black and had large eyes, like the current goldfish stimulus, it was moved in a manner resembling a predatory attack (Gerlai et al., 2000). Furthermore, recently zebrafish was shown to differentially respond to its sympatric predator (*Nandus nandus*) without any prior exposure to it (Bass and Gerlai, 2008). Thus, genetic predisposition facilitates appropriate antipredatory responses in zebrafish, and harmless fish or nonnative predators may not be avoided. Goldfish is not a predator, and the black moor variety does not exist in nature, thus it appears to have been an incorrect choice to induce avoidance responses.

Cumulative turn angle as a measure has not been used to quantify alcohol responses in adult zebrafish, but our current results suggest that it may be useful. We noticed that the bout length of straight locomotory swim paths decreased as fish got exposed to higher doses of alcohol. Locomotion of the fish was broken up by small movements, changes in direction, while the total amount of activity performed remained unaltered. This is properly reflected in cumulative turn angle, a measure that we now plan to use in future studies. The location of turning may also be important to note.

Thrashing toward the stimulus represents swim direction changes at the glass facing the stimulus. The videotracking system did not measure this behavior and instead we used the observation-based method. Alcohol dose-dependent changes in this behavior in the aggression task corresponded to what has been observed before (Gerlai et al., 2000): intermediate doses of alcohol facilitated and the highest dose diminished thrashing towards the mirror. This dose relationship was, however, not evident in the AB strain. It thus appears that AB differed from the other 3 populations tested here and also from another one tested previously in several behavioral parameters.

The differences between fish from the AB strain and from the other 3 populations of this study are unlikely to be due to environmental effects. All fish were bred, raised, and tested at the same time in the same room and under identical conditions. The differences thus likely have a genetic origin. Therefore, this study shows that heritable alterations in alcohol responses in adult zebrafish can be quantified using automated behavioral recording. This is noteworthy because automation is a prerequisite for high throughput screening in forward genetic applications, the ultimate goal of our work.

The demonstrated genetic differences have implications for future studies. First, they open the possibility of employing quantitative genetic approaches with which one can utilize naturally existing genetic variability. Artificial selection for particular alcohol responses, quantitative trait locus analysis, and estimation of parameters of the genetic architecture using cross systems appears now feasible for alcohol related traits in the adult zebrafish. Second, for random mutagenesis, one will need to consider the choice of host strain (the strain that carries the randomly induced mutation) and the mapping strain (to which the host is crossed for linkage analysis based mapping). AB is often used as a host strain that is crossed to mapping strains (for references see in Guryev et al., 2006 and Guo, 2004). If genetic differences exist between the AB and the mapping strains in alcohol responses, however, mapping will be complicated

by segregation of alleles at naturally variable loci. That is, a set of mapping strains for which genetic markers are available may have to be characterized. One will need to analyze the behavior of not only these strains, but also their F1 and F2 hybrids. Significant behavioral differences between the parental strains would indicate that linkage analysis-based positional cloning of the mutant gene is problematic. But even if the parental strains show no differences, increased phenotypical variability in the F2 generation would lead to the same conclusion: it would be an indication of genetic variability due to segregation of alleles at loci that differed between the parental strains. If genetic differences among the parental strains are thus revealed, instead of ethyl nitrosourea (ENU) chemical mutagenesis, viral vector mediated insertional mutagenesis will need to be performed. The latter allows the identification of the locus of the mutation using the unique viral DNA tag left behind. Although less efficient and technically more challenging than ENU mutagenesis, insertional mutagenesis has been successfully employed with zebrafish (Amsterdam and Hopkins, 1999).

In summary, finding genetic differences among the 4 zebrafish populations using automated behavioral quantification methods shows the general feasibility of behavioral screening but also demonstrates the need to systematically characterize zebrafish strains and their crosses before the optimal mutagenesis strategy could be chosen for the analysis of the genetic factors underlying alcohol's effects.

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Fig. 1.

Behavioral responses of 4 zebrafish populations (AB, LD, LFWT, and SFWT) to different doses of alcohol (0.00, 0.25, 0.50, and 1.00%) in the novel open tank. Means $\pm$ SEM are shown. Sample sizes are indicated in the Methods section. Panels I, II, and III show different behavioral measures. Note that cumulative distance to stimulus or thrashing towards stimulus measures are not shown because no stimulus was employed in this test. The small letters above the bars indicate the results of post hoc Tukey HSD multiple comparison tests. Bars that share at least 1 letter designation within a bar-graph belong to a nonsignificant ($p > 0.05$) range. Note that bars are not labeled by letters if ANOVA found no significant alcohol effect or Alcohol $\times$ Population interaction.

Fig. 2.

Behavioral responses of 4 zebrafish populations (AB, LD, LFWT, and SFWT) to different doses of alcohol (0.00, 0.25, 0.50, and 1.00%) in the group preference task. Means $\pm$ SEM are shown. Sample sizes are indicated in the Methods section. Panels I to V show different behavioral measures. The small letters above the bars indicate the results of post hoc Tukey HSD multiple comparison tests. Bars that share at least 1 letter designation within a bar-graph belong to a nonsignificant ($p > 0.05$) range. Note that bars are not labeled by letters if ANOVA found no significant alcohol effect or Alcohol $\times$ Population interaction.

Fig. 3. Behavioral responses of 4 zebrafish populations (AB, LD, LFWT, and SFWT) to different doses of alcohol (0.00, 0.25, 0.50, and 1.00%) in the mirror (aggression) task. Means $\pm$ SEM are shown. Sample sizes are indicated in the Methods section. Panels I to V show different behavioral measures. The small letters above the bars indicate the results of post hoc Tukey HSD multiple comparison tests. Bars that share at least 1 letter designation within a bar-graph belong to a nonsignificant ($p > 0.05$) range. Note that bars are not labeled by letters if ANOVA found no significant alcohol effect or Alcohol $\times$ Population interaction.

Fig. 4. Behavioral responses of 4 zebrafish populations (AB, LD, LFWT, and SFWT) to different doses of alcohol (0.00, 0.25, 0.50, and 1.00%) in the “predator” task. Means $\pm$ SEM are shown. Sample sizes are indicated in the Methods section. Panels I to V show different behavioral measures. The small letters above the bars indicate the results of post hoc Tukey HSD multiple comparison tests. Bars that share at least 1 letter designation within a bar-graph belong to a nonsignificant ($p > 0.05$) range. Note that bars are not labeled by letters if ANOVA found no significant alcohol effect or Alcohol $\times$ Population interaction.