ELSEVIER

Pharmacology, Biochemistry and Behavior 67 (2000) 773–782

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects

R. Gerlai*, M. Lahav, S. Guo, A. Rosenthal

Neuroscience Department, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA

Accepted 7 August 2000

Abstract

Zebra fish may be an ideal vertebrate model system for numerous human diseases with which the genetics and biological mechanisms of the disease may be studied. Zebra fish has been successfully used in developmental genetics, and recently, neurobiologists have also started to study this species. A potentially interesting target disease amenable for analysis with zebra fish is drug addiction, e.g. alcoholism. Although genetic tools to manipulate the genome of zebra fish are available, appropriate phenotypical testing methods are often lacking. In this paper, we describe basic behavioral tests to investigate the acute effects of alcohol on zebra fish. These behavioral paradigms will be useful for the genetic and biological analysis of acute and chronic drug effects as well as addiction. In addition to presenting findings for the acute effects of alcohol, we briefly describe our strategy for generating and screening mutants. We hope that our pilot work will facilitate the future development of behavioral tests and the use of zebra fish in the genetic analysis of the biological effects of drugs of abuse.

Keywords: Acute drug effect; Aggression; Alcohol; Anxiety; Behavior; *Brachydanio rerio*; Drugs of abuse; Fear; Habituation; Mutagenesis; Social behavior; Zebra fish

1. Introduction

Zebra fish is a small (3–4 cm long) freshwater teleost species that can be easily kept and bred in the laboratory [52]. A female can produce 200 eggs per spawning and the fry grow quickly and reach sexual maturity within 2–3 months [13]. Most genes discovered in this species are evolutionarily conserved and have homologs in mammals [7]. These characteristics and the experimenter's ability to generate a large number of mutant fish with ease using chemical mutagenesis [25,51] have made zebra fish popular in genetics and biomedical research [16,25,28]. By now libraries containing hundreds of mutants have been generated [12,16,28,30,33]. Research using such mutants has focussed on developmental aspects of zebra fish [15,16], including the embryogenesis of the nervous system [6,8,49], because the embryo is transparent and allows detailed anatomical characterization [13,18]. Only a few studies have attempted to investigate the genetics of behavior or brain function of zebra fish [17,25,26,41].

The behavioral repertoire of zebra fish is complex and should allow the development of a range of behavioral paradigms. Zebra fish is a cyprinid schooling fish [1,34,39]. Individuals exhibit social preference for their conspecifics so that they form a group [4,5], or school, in which individuals swim together in close proximity, a behavioral strategy shown to be effective against predators in several fish species [37]. Individuals swimming in such a group or alone exhibit a typical startle reaction and zigzagging in response to the sudden appearance of a predator or a large object (for alarm reactions see Refs. [14,31,32]). Based on the characteristics of its body and mouth, zebra fish is thought to be specialized for eating small insects from the water surface, and indeed is often found swimming and foraging near the surface of water [19]. Males exhibit territoriality, which includes an elaborate fin erecting display, dancing movements, and agonistic behavior [2]. Females have also been observed (personal observation) displaying to an individual opponent. Zebra fish is a diurnal

* Corresponding author. Lilly Research Laboratories, Lilly Corporate Center, Drop Code 0510, Indianapolis, IN 46285, USA. Tel.: +1-317-433-5244; fax: +1-317-276-7600.

E-mail address: gerlai_robert@lilly.com (R. Gerlai).

0091-3057/00/$ – see front matter
PII: S0091-3057(00)00422-6

species that prefers light to dark during the day but may hide in darker areas of its habitat in response to danger (Ref. [19]; and personal observation).

Based on the above characteristics, we decided to develop behavioral tests that may be used in the analysis of brain function and the screening of mutant fish. Our goal was to design simple paradigms with a potential use for testing the effects of drugs of abuse such as alcohol. Zebra fish may be especially suitable for this purpose because of the simplicity of alcohol delivery [46]. Alcohol mixed in the water of the fish tank is absorbed by the blood vessels of the gill and the skin of the fish so that blood alcohol levels reach equilibrium with the external alcohol concentration quickly [48] (also see Refs. [27,38]).

In this paper, we describe basic behavioral tests that reveal behavioral patterns characteristic of zebra fish and present evidence for acute alcohol treatment dependent behavioral alterations in these tests. Furthermore, we briefly present our strategy for mutagenesis.

2. Methods

In order to develop fast and simple tests, we investigated the behavioral characteristics of zebra fish and looked for consistent behavioral patterns or responses that could be evoked under certain circumstances and could perhaps be modified by alcohol without the need for extensive manipulation or training. In accordance with the exploratory nature of our work, measuring the behavioral responses of zebra fish was carried out using observational techniques in all tests. Nevertheless, the tests were designed so that automation, including videotracking or photocell-based motion detection, could be easily applied in the future.

2.1. General procedure

To determine characteristic behavioral patterns of zebra fish, 60 untreated naïve fish (3–4-months-old) were used for the development of the behavioral paradigms described below (data not presented). To investigate the effects of alcohol, another set of naïve, previously untested, fish (males and females pooled) were assigned to four groups (for sample sizes see figures): ETOH 0.00%, ETOH 0.25%, ETOH 0.50%, and ETOH 1.00%, where ETOH % represents the corresponding alcohol concentration (volume percentage) in the holding tank in which experimental fish were held for 1 h before the behavioral tests. Alcohol concentrations were chosen on the basis of data published previously for another cyprinid, the gold fish (*Carassius auratus*) [24,46,48] so that potential facilitatory (lower doses) as well as inhibitory (higher doses) effects of alcohol could be observed. All fish were exposed to the corresponding alcohol concentrations for 60 min prior to behavioral testing, a period that is expected to lead to a significant and stable blood alcohol level in the subjects (extrapolation from data presented in Ref. [47]). The alcohol-treated or control fish were tested subsequently in the behavioral paradigms in the order described below. During these tests, the alcohol concentration in the test tank was kept identical to that of the pretest holding tank. Fish were treated and tested in an order randomized across treatment groups and the group designations were unknown to the behavioral observer.

2.2. Locomotor activity

In this task, our goal was to describe where and how fast zebra fish swam upon exposure to a novel place and after having been habituated to this place. We wanted to determine if zebra fish exhibited particular swim patterns that could reveal behavioral patterns characteristic of anxiety and sensitive to the effects of alcohol. Fish were placed individually in a small experimental tank ($30 \times 15 \times 10$ cm length $\times$ height $\times$ width). Their behavior was videorecorded for 60 s twice: first, half a minute after having been placed in the tank (response to novelty) and a second time 10 min later (habituated state). The videorecordings were later replayed and analyzed using the Noldus Observer event recording software (Noldus, Wageningen, The Netherlands) on a Macintosh computer (PowerBook G3). Locomotion (swimming activity) was measured by placing a transparency in front of the TV monitor with vertical lines that divided the tank into four equal sections and counting the number of entries by the fish to each section. In addition to this general measure of activity, the approximate location of swimming was also recorded by placing a transparency in front of the TV monitor with horizontal lines that divided the tank into three layers: bottom, middle, and upper layer, and by measuring the duration of time the experimental fish spent in each of these layers.

2.3. Aggression

Interaction between two fish may involve aggressive responses or the opposite behavioral reaction, social preference (schooling), both of which may be influenced by alcohol. The goal of this paradigm was to quantify the responses of an individual experimental fish to its mirror image in a way that can be automated using videotracking or motion detection photocell systems. Fish were individually netted into a small experimental tank ($30 \times 15 \times 10$ cm length $\times$ height $\times$ width). A mirror was placed inclined at 22.5° to the back wall of the tank so that the left vertical edge of the mirror was touching the side of the tank and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank their mirror image appeared closer to them. Experimental fish were videorecorded for 60 s after a 30-s short habituation period and once again for 60 s after a 10-min habituation period. A transparency was placed in front of the TV monitor with vertical lines that divided the tank into four equal sections and allowed counting the number of entries by the fish to

each section. Entry to the left-most segment (SE1) indicated preference for proximity to the "opponent," whereas entry to the right-most segment (SE4) implied avoidance. The amount of time the experimental fish spent in each of the four segments was analyzed with the Observer event recorder program. In addition, the amount of time the fish spent with aggressive display, or attack behavior, was also measured and analyzed (aggression). Aggressive display is defined as a posture during which the fish erects its dorsal, caudal, pectoral, and anal fins. Usually, this fin erection display is associated with undulating body movements or small slaps carried out by the caudal fin. Attack behavior is a characteristic short bout of fast swimming directed towards the opponent and is sometimes accompanied by opening the mouth and biting. Attack behavior often alternates with fin erection display.

2.4. Group preference

Zebra fish is a schooling fish that exhibits preference to its conspecifics under neutral or mildly aversive conditions. In the previous task, in which a single experimental fish was faced with its mirror image, responses may mainly involve agonistic reactions. In the present task, response of a group of experimental fish to a school of conspecifics was tested, a behavior that is more associated with social preference than agonistic behavior. Fish were placed in groups of five in a small experimental tank ($30 \times 15 \times 10$ cm length $\times$ height $\times$ width). On one side of the experimental tank, an empty fish tank was placed and on the other side, a tank of identical size was holding 15 conspecifics. The experimental fish were allowed to habituate for a 30-s period after which their behavior was videorecorded. The first 10-s of this videorecording was analyzed as follows. A transparency was placed in front of the TV monitor with a vertical line that divided the tank into two equal sections. The amount of time (T2, T3, T4, and T5) during which two, three, four, or five fish swam on the side of the tank closer to the conspecific school was measured using the event recorder program (the smallest number of fish staying in this side of the tank was two, thus zero or one number of fish was not recorded and analyzed). The recorded values were weighted and added to obtain a preference score (P) as follows: $P = T5 \cdot 5 + T4 \cdot 3 + T3 \cdot 1 + T2 \cdot (-1)$. The weights were chosen so that they would weigh behavior with respect to baseline, i.e. random chance distribution, i.e. 2.5 fish on each side. Fish in this experimental tank were later tested for their responses to a moving predator model (see below).

2.5. Antipredatory behavior

Antipredatory behavior is a characteristic aspect of zebra fish, a response thought to be highly adaptive. Genetic predisposition is expected to lead to a consistent behavioral response when zebra fish is exposed to predatory fish or to stimuli that characterize such fish [9,20]. Alcohol may modify this behavioral response either by altering levels of anxiety, perceptual, or motor mechanisms. Fish, placed in a small experimental tank in groups of five, were first tested for their preference for conspecifics during a 10-s session as described above in the "group preference test." Immediately after this session, the fish were exposed to a predator model. Note that groups of five fish were tested because this mimics a natural situation and also allows the fish to habituate to the novel test environment more quickly. Fear responses to a predator model are markedly different from behaviors that characterize a school of undisturbed fish. Habituating the fish to the test environment first and testing a group of fish thus increased the ability of the experimenter to recognize and measure antipredatory responses. The predator model was made of a 5-ml falcon tube that was painted black and had two eye-like spots (diameter 5 mm, orange "iris," and black "pupil") placed at the conical end of the tube. This predator model while facing the experimental fish was moved in a standard manner (2 cm/s) as follows. First movement: vertical motion from bottom to top; second movement: vertical from top to bottom; third movement: horizontal from right to left; fourth movement: 'S'-shaped approach, first motion to the right parallel to the tank, then turn towards the tank then again moving parallel to the tank. Each movement was repeated twice and the number of fish responding with a jump was measured. Jump is defined as a fast leaping movement mostly with the use of the caudal fin.

2.6. Light versus dark preference

Zebra fish is active during the day and may rely significantly on its vision to detect predators and find food or conspecifics. Unlike nocturnal rodents, zebra fish is thus expected to prefer well-illuminated areas to dark places [10]. We tested fish individually in a light/dark preference test. Fish were netted in a fish tank ($50 \times 30 \times 25$ cm) that was divided into two compartments: one was illuminated with diffuse light from fluorescent light tubes and the other was covered with cardboard paper on all sides and the top. There was no physical barrier between the two compartments and the fish were allowed to swim freely in the entire fish tank. After a 30-s habituation period, the amount of time the fish spent in the dark compartment during a 60-s session was measured using the Observer event-recording program. The behavior of fish was recorded for another 60-s period 10 min later.

2.7. Pigment response

Zebra fish may change their color in response to stimuli. Fish on a light background tend to be lighter than fish on a dark background. Fish that exhibit signs of fear, e.g. freezing or erratic movement, quickly loose their color and become pale, especially when the background is light. Aggressive, displaying fish are generally darker and exhibit

more vivid colors irrespective of the background of their environment. Alcohol may influence this pigment response by either directly altering the function of the chromophore cells or by influencing central neural mechanisms. Fish were exposed to the four alcohol concentrations as explained above. The fish were individually placed in a small holding tank ($30 \times 15 \times 10$ cm length $\times$ height $\times$ width) that was illuminated by fluorescent light tubes. The bottom of the tank as well as the wall behind the tank was painted light grey. Electronic photographs of the fish were taken and the images were transferred to a computer. The color reaction of the fish was rated visually by comparing the darkness of the experimental fish to three standard images of fish placed behind the same light grey background, a light (L), a medium (M), and a dark (D) fish. Scoring was done after the backgrounds of the experimental and standard fish images were adjusted to the same saturation level as follows: 0, lighter than L; 1, identical to L; 2, lighter than M but darker than L; 3, identical to M; 4, darker than M but lighter than D; 5, identical to D.

2.8. Fish husbandry

Outbred zebra fish used for the behavioral studies were obtained from Scientific Hatchery (Huntington Beach, CA). However, for mutagenesis, an inbred strain (e.g. AB) would need to be used because such a strain provides genetic homogeneity required for the proper identification of induced mutations. Fish were maintained in deionized water supplemented with 60 mg/l Instant Ocean Sea Salt (obtained from a local pet store). The water was filtered by canister filters containing a disinfecting ultraviolet light unit as well as through a biological filter tank in which aquarium gravel served as substrate for bacterial filtration. A water-dripping cage rack system (Marine Biotech, Beverly, MA) provided oxygenation. Fish were fed four times daily: twice with live brine shrimps (*Artemia salina*, San Francisco Bay Brand, San Francisco, CA) and twice with dry food (Tetra-min, Tetra, Melle, Germany).

2.9. Mutagenesis

The mutagen commonly used for mutagenesis is ethyl nitrosourea (ENU), which induces point mutations reliably and with high frequency [50]. Adult male fish are immersed in aqueous solution containing 3 mM ENU for three consecutive 1-h periods at weekly intervals. This exposure level is expected to induce one point mutation per genome on average [29].

Some behavioral mutations may be inherited as dominant or semidominant traits (e.g. Refs. [35,36]). A breeding scheme for dominant or semidominant mutations is simpler than one for recessive mutations and is as follows: mutagenized males are bred with wild-type females to produce the F1 generation. F1 fish are heterozygous for any induced mutation. Each F1 mutant may carry a mutation at a different locus. Mutants are identified by their abnormal behavioral patterns revealed by the paradigms described above. However, as behavioral traits are quantitative and are subject to variation of the environment, the mutant status of apparently abnormal fish must be ascertained by breeding the F1 individuals and testing their offspring. If the offspring exhibit an alteration similar to that of the parent, one can be certain that it is an inherited alteration due to a mutation. Thus, the suspected mutants (the founders) are bred to wild-type fish, and the resulting F2 offspring are raised to adulthood and tested for altered behavior. If the mutation is inherited in a Mendelian fashion, expected in case of a single nonlethal point mutation, approximately 50% of the F2 generation should exhibit abnormal behavioral patterns similar to the original F1 fish (heterozygotes).

In case the behavioral screen involves testing a school of fish, we suggest an alternative screening strategy. F2 offspring are generated from each F1 founder without behaviorally testing these founders and only the F2 families are tested. If the F1 founder carried a heritable mutation, 50% of the F2 offspring will be heterozygous for the same mutation. These mutants may significantly alter the behavioral scores obtained for the fish group making identification of the mutation possible.

To isolate recessive mutations that affect behavior, individual F1 founder males are bred with wild-type females to produce F2 generations. F2 families are raised to adulthood and then breedings are carried out within each F2 family. About 25% of the matings are expected to yield homozygous fish (F3) in which the recessive mutation may be observed at the phenotypical level. The probability of finding a mutation in an F2 family is $P = 1 - 0.75^n$, where n is the number of successful matings. Thus, with six matings ($n = 6$), for example, $P = 82\%$ for each F2 family, a reasonably high percentage. F3 progeny from each F2 matings are raised to adulthood for behavioral testing. If a phenotypical abnormality is observed, the F3 fish showing the abnormality are sibmated to produce the F4 generation, which is tested to confirm that the alteration is indeed heritable.

The probability of finding a mutation in a family is $P = (1 - 0.75^n)$, thus, one may want to analyze a minimum of 20 individuals, as the chance of finding at least one mutant out of 20 subjects is 99.6%. Since the time required to conduct the behavioral assays described above is short and multiple fish may be tested in an automated manner, testing 20 fish per family is not an unattainable goal. Once a mutation is isolated, candidate gene approach or positional cloning strategies can be employed to isolate the gene corresponding to the mutation [29].

2.10. Statistical analysis

Data matrix manipulation and statistical analyses were carried out with Systat 5.1 for Macintosh. Monovariate

repeated measure or multifactor variance analyses (ANOVA) were carried out. In case of significant main or interaction terms, post hoc tests such as the Tukey's Honestly Significant Difference (HSD) test, were conducted.

3. Results

The results of the test for locomotory activity are summarized in Figs. 1 and 2. Variance analysis of locomotion score revealed a significant alcohol effect [$F(3,45)=42.19$, $P<.0001$] but no significant time [$F(1,45)=0.213$, $P>.50$] or alcohol × time [$F(3,45)=0.60$, $P>.50$] interaction effects were detected. Post hoc comparison of the groups (Tukey's HSD) showed that 0.25% and 0.50% alcohol treatment led to a significant ($P<.05$) activity increase that was observable both at the first and tenth minute of testing in the novel experimental tank compared to the control group or to the group treated with 1.00% alcohol. Interestingly, the latter group was found significantly ($P<.05$) hypoactive not only compared to the other two alcohol-treated, and highly hyperactive, groups but also compared to the control group at the first minute of observation, a difference that diminished after 10 min (Fig. 1).

The location of swimming in the novel tank was also significantly affected by alcohol treatment (Fig. 2). In the upper layer, the effects of alcohol [$F(3,50)=0.92$, $P>.40$] and time [$F(1,50)=0.01$, $P>.90$] were found nonsignificant but the alcohol × time interaction was significant [$F(3,50)=5.53$, $P<.01$], indicating a time-dependent alcohol effect. In the middle layer, ANOVA revealed a significant alcohol effect [$F(3,50)=4.97$, $P<.01$] but no significant time [$F(1,50)=0.30$, $P>.50$] or alcohol × time effect [$F(3,50)=0.61$, $P>.60$] was found. In the lower layer, the results were similar to those obtained for the upper layer. ANOVA showed no significant alcohol [$F(3,50)=0.99$, $P>.40$] or time [$F(1,50)=0.03$, $P>.85$] effects but revealed a significant alcohol × time interaction [$F(3,50)=5.77$, $P<.01$], again indicating a time-dependent alcohol effect. Tukey's HSD test demonstrated that during the first minute of testing, fish treated with 0.5% alcohol spent significantly ($P<.05$) more time in the upper layer of water than other fish, fish treated with 1.00% alcohol spent significantly ($P<.05$) less time in the middle layer compared to other fish, and fish treated with 0.00% alcohol or 1.00% alcohol remained in the lower layer of the tank for significantly ($P<.05$) longer time compared to those treated with 0.50% alcohol. After a 10-min habituation to the novel environment, these differences largely disappeared. The only difference detected was in the amount of time fish treated with 0.00–0.25% and with 1.00% alcohol spent in the middle layer of water. The former two remained in this portion of the tank for significantly ($P<.05$) longer periods of time than the group treated with 1.00% alcohol.

Responses to an individual conspecific are shown in Fig. 3A (first minute) and B (tenth minute). ANOVA revealed a significant alcohol effect for SEG1 [the segment of the tank

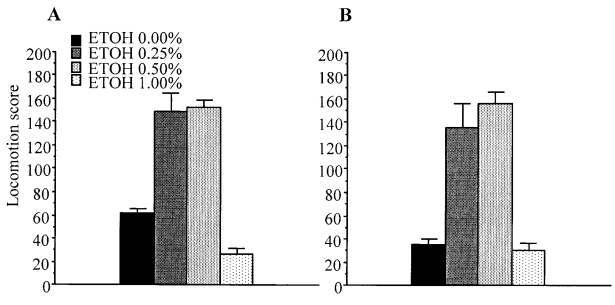

Fig. 1. Locomotory activity is increased by intermediate doses of alcohol in zebra fish. Locomotion score is calculated as the number of crossings between segments of the observation tank during a 1-min observation session at the beginning of the test (panel A) and at the tenth minute of the test (panel B). Means ± S.E. are shown. Sample sizes were as follows: ETOH 0.00% $n=13$, ETOH 0.25% $n=15$, ETOH 0.50% $n=13$, and ETOH 1.00% $n=16$. Note that ETOH 1.00% fish exhibited a dramatic depression of activity while the other two alcohol-treated groups showed a robust increase of activity.

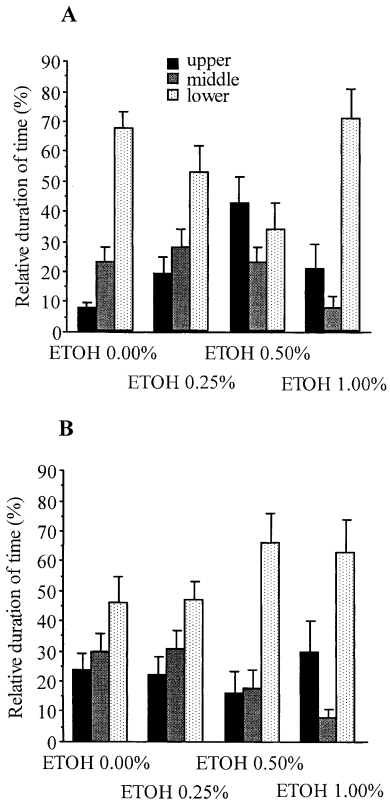

Fig. 2. Alcohol alters the location of swimming in zebra fish. Data represent the relative duration of time fish spent in three (upper, middle, and lower) horizontal layers of the fish tank. Means ± S.E. are indicated. Panel A: location during the first minute of the recording session. Panel B: location during the tenth minute of the recording session. Sample sizes are identical to those given in Fig. 1. Note that 0.50% alcohol treatment led to a significant increase of time spent by zebra fish in the upper layer of water and a decrease in the lower layer of water during the first minute of the recording session. Also note the U-shaped dose–response in these measures. The differences diminished by the tenth minute of the session.

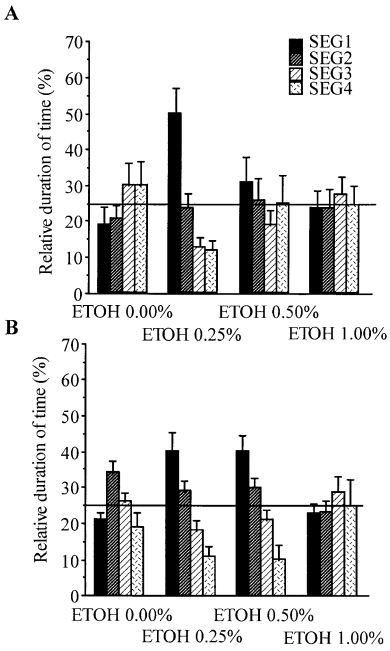

Fig. 3. Intermediate alcohol concentrations enhance preference for a conspecific opponent in zebra fish. Panel A shows the spatial distribution (time spent in four equal segments of the fish tank) of zebra fish during the first minute of the recording session, and panel B shows the results obtained in the tenth minute of the test. Note that an inclined mirror was placed behind the observation tank so that the mirror image of the experimental fish appeared the closest when viewed by the subject from segment 1 (SEG1) and furthest from SEG4. Means ± S.E. are indicated. Sample sizes were as follows: ETOH 0.00% $n=15$, ETOH 0.25% $n=16$, ETOH 0.50% $n=16$, and ETOH 1.00% $n=18$. The solid horizontal line represents chance level.

from where the mirror image of the experimental fish appeared closest; $F(3,58)=10.48$, $P<.0001$] but showed no significant time or alcohol × time interaction effects [$F(1,58)=0.01$, $P>.90$; $F(3,58)=1.02$, $P>.40$]. The results for the other segments were found nonsignificant. Perusal of Fig. 3A as well as the results of post hoc Tukey's HSD test suggest that fish treated with 0.25% alcohol spent significantly ($P<.05$) more time nearest to the opponent (SEG1) compared with fish from the other groups during the first minute of being exposed to the mirror image. After a 10-min habituation time (Fig. 3B), fish treated with 0.25% or 0.50% alcohol became statistically indistinguishable, and both spent significantly ($P<.05$) more time near their mirror image (SEG1) compared to fish from the other two groups (ETOH 0.00% and ETOH 1.00%). The preference for SEG1 may be due to either agonistic or schooling behavior. Our results confirmed the former possibility (Fig. 4). Variance analysis of aggressive display showed a significant alcohol effect [$F(3,61)=9.90$, $P<.0001$] and no significant time or alcohol × time interaction effects [$F(1,61)=2.33$, $P>.10$; $F(3,61)=0.91$, $P>.40$]. Post hoc Tukey's HSD revealed that fish treated with 0.25% alcohol spent significantly more time exhibiting aggressive behavioral responses compared to all other fish and that fish treated with 0.5% alcohol were

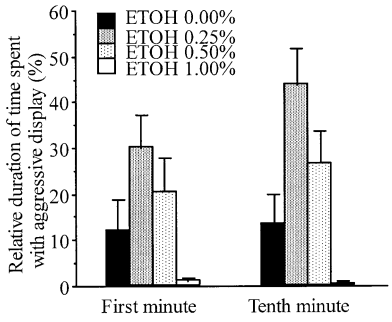

Fig. 4. Intermediate alcohol concentrations enhance aggressive behavioral responses elicited by the sight of an opponent in zebra fish. Means ± S.E. are shown. Note that an inclined mirror was placed behind the observation tank and the behavioral responses were elicited by the mirror image of the experimental fish. Sample sizes were as indicated in Fig. 3.

more aggressive than those receiving 1.00% alcohol both at the first and at the tenth minute of the recording session.

Alcohol was also found to influence schooling behavior. Fig. 5 shows the raw data, which were transformed to obtain a single preference score for each group of five fish as explained in the Methods section. Analysis of these scores revealed that treatment with alcohol significantly reduced the preference for conspecifics in a dose-dependent manner [$F(3,35)=2.85$, $P=.05$]. Treatment with higher alcohol concentrations led to a more scattered, distributed, spatial location of the experimental fish, implying decreased preference for the school of stimulus conspecifics.

Antipredator behavior was elicited with a moving predator model (Fig. 6). Analysis of the total number of jumps exhibited by groups of five fish during the repeated presentation of a predator model revealed significant alcohol effects [$F(3,32)=19.78$, $P<.0001$]. Post hoc Tukey's HSD test showed that all groups were significantly ($P<.05$) different from each other with fish treated with 0.25% alcohol exhibiting the strongest response.

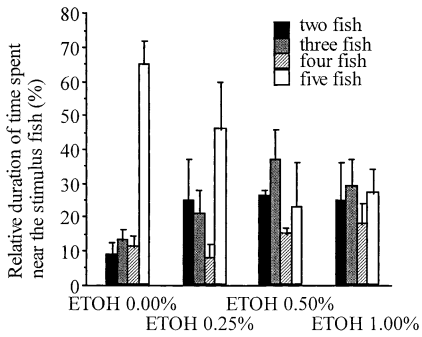

Fig. 5. Preference for a school of zebra fish is diminished by high doses of alcohol. The response of groups of five fish is tested. Sample sizes (n) representing the number of these groups were as follows: ETOH 0.00% $n=15$, ETOH 0.25% $n=9$, ETOH 0.50% $n=6$, and ETOH 1.00% $n=9$. Error bars show S.E. Note the dose-dependent decrease of the duration of time during which five fish occupied the side of the tank adjacent to the stimulus fish. Also note that the more evenly distributed values in fish groups treated with higher alcohol concentrations represent decreased preference for the conspecific stimulus fish.

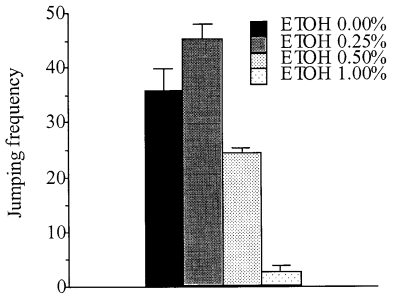

Fig. 6. Antipredator behavior, measured as the total number of jumps in response to a predator model, is facilitated by a low dose of alcohol and impaired by higher doses. Sample sizes represent the number of such fish groups (five individuals per group) and were as follows: ETOH 0.00% $n=15$, ETOH 0.25% $n=9$, ETOH 0.50% $n=6$, and ETOH 1.00% $n=9$. Note the U-shaped dose–response and also that each group is significantly different from the other.

The results obtained in the light/dark preference test are summarized in Fig. 7. ANOVA revealed a significant alcohol effect [$F(3,62)=3.02$, $P<.05$], a significant time effect [$F(1,62)=25.72$, $P<.0001$], and a significant alcohol × time interaction [$F(3,62)=4.53$, $P<.01$]. Post hoc Tukey's HSD showed no significant differences between groups of fish during the first minute of the observation: all fish avoided the dark compartment. However, after 10 min in the tank, fish treated with 0.00% or 0.25% alcohol showed a dramatic habituation and exhibited no apparent preference for or avoidance of any compartment, whereas fish treated with 0.50% or 1.00% alcohol continued to avoid the dark part of the tank (Tukey's HSD, $P<.05$). The color response of fish treated with different concentrations of alcohol (Fig. 8) was also found significantly to depend on alcohol treatment [ANOVA $F(3,61)=131.32$, $P<.0001$].

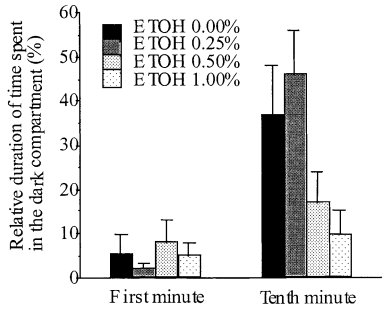

Fig. 7. Higher doses of alcohol decrease habituation of zebra fish to a dark compartment in a light/dark choice paradigm. Amount of time zebra fish spent in the dark in an experimental tank that was divided into a well illuminated and dark compartment of identical size is shown for the first minute and the tenth minute of the recording session. Error bars represent S.E. Sample sizes were as follows: ETOH 0.00% $n=15$, ETOH 0.25% $n=16$, ETOH 0.50% $n=15$, and ETOH 1.00% $n=20$. Note that initially all fish exhibited a robust avoidance of the dark compartment but by the tenth minute the control group and the group treated with the lowest alcohol dose (ETOH 0.25%) showed a significant habituation to the dark compartment whereas fish treated with higher alcohol doses continued to avoid it.

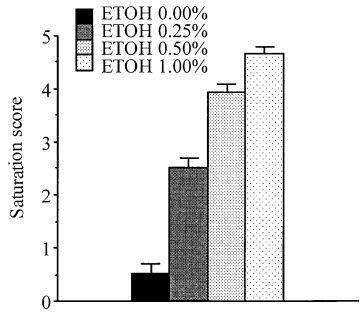

Fig. 8. Alcohol enhances the color of zebra fish. Higher saturation scores represent darker (more vivid) colors. Means ± S.E. are indicated. Sample sizes were as follows: ETOH 0.00% $n=15$, ETOH 0.25% $n=16$, ETOH 0.50% $n=16$, and ETOH 1.00% $n=18$. Note the near linear increase of saturation score in response to increasing alcohol doses.

All groups were found to be significantly different from each other (Tukey's HSD test, $P<.05$)

4. Discussion

Acute alcohol treatment elicited characteristic behavioral changes that could be measured in simple and fast behavioral tasks. The tests were designed to be automatable to allow screening a large number of fish, a necessary requirement in a mutagenesis study. The simplicity of alcohol delivery, the well established methods of mutagenesis developed for this species, and our present behavioral results imply that zebra fish will be a potentially successful model system in the genetic analysis of the effects of alcohol on behavior and brain function.

The effects of alcohol could be observed in multiple behavioral paradigms. For example, alcohol significantly affected the activity of zebra fish in a novel situation. At lower concentrations (e.g. 0.25% and 0.50%), it increased locomotion but at a higher dose (1.00%), it depressed activity even below the level of control fish during the first minute of the test. The passivity of control (0.00% alcohol) fish appeared to be associated with novelty-induced fear, including freezing (decreased activity) and erratic movement (temporarily increased bouts of activity), behavioral responses associated with anxiety (e.g. Refs. [21,22]). However, the reduced activity levels of 1.00% alcohol-treated fish were due to anesthetic-like sedative effects of alcohol associated with general slowness and impaired coordination and swimming. Although the forms of passivity (fear versus slowness) are difficult to differentiate using automated techniques, the amount of activity increase or decrease could be measured using motion detection devices. As such changes were detected quickly (during a 60-s session in the present study), we conclude that analysis of swimming activity offers a simple and fast method of measuring acute alcohol effects.

Alcohol also affected where zebra fish swam. Control fish swam mostly near the bottom of the observation tank, a

response that habituated over time. As zebra fish is normally found swimming near the surface of the water and its natural predators include birds, this response may be interpreted as an antipredatory behavior associated with fear or anxiety [9]. The effects of alcohol on the location of fish in this tank were characterized by a U-shaped dose–response curve. Alcohol can be anxiolytic [3] and our results, as well as the observation that the differences between treatment groups diminished after 10 min, are consistent with a suggestion that alcohol ameliorated fear-induced behavioral responses in zebra fish. The anxiolytic effect of alcohol may also explain the findings we obtained in the group preference test in which increasing amounts of alcohol led to decreasing preference for a group of conspecifics in a stimulus tank placed adjacent to the experimental tank. Group cohesion, and preference for conspecifics, have been found to correlate with predator or novelty-induced fear [1,5].

Alcohol has also been demonstrated to influence aggression in fish [43,44] as well as in rodents and monkeys [40]. Our results revealed an inverted U-shaped dose–response curve with the alcohol doses applied, a finding similar to what has been described in other fish [44] or mammalian species [40]. Fish receiving 0.25% alcohol treatment exhibited the strongest preference for the segment of the tank (SEG1) from where their mirror image appeared the closest, control fish slightly avoided SEG1, and fish treated with 1.00% alcohol swam randomly. The preference for being close to a conspecific may be a result of aggressive behavior but it could also be due to schooling tendencies. Aggression is a strong motivating force, e.g. access to a conspecific opponent is rewarding and can sustain performance in instrumental conditioning in fish [23]. Our results suggested that, indeed, the preference for SEG1 is due to aggressive behavior. Fish showed elevated aggressive display and attack behavior when exposed to their own mirror image. Notably, although aggressive display and attack behavior are difficult to quantify with methods other than direct observation, using the inclined mirror and measuring the spatial location of fish makes this paradigm amenable to analysis with automated motion detection devices.

In most of the above paradigms, alcohol at the highest dose (1.00%) suppressed activity and reduced response levels. This may be due to a generalized sedative action [42] affecting basic performance factors such as motor function and perception. Although the former action appears obvious as fish treated with 1.00% alcohol exhibited clear signs of abnormal motor control, the latter possibility may not be supported by our findings. For example, in the light/dark preference test, all fish initially exhibited a profound avoidance of the dark compartment. This suggests that even fish treated with 1% alcohol can respond to visual stimuli, so the differential responses found in our other behavioral tests may not be explained by altered visual perception per se. Furthermore, the significant habituation to the dark compartment after 10 min in control and 0.25% alcohol-treated fish but not in 0.50% and 1.00% alcohol-treated fish suggests that alcohol was likely to affect central neural mechanisms rather than perception. Finally, the light/dark preference test can be conducted using standard shuttle box hardware and software in an automated manner and therefore could be a useful test for the analysis of the effects of alcohol.

The predator model containing species-specific key stimuli [20] used in the present study elicited consistent jumping. Alcohol treatment modified this response, and the dose–response curve was found to be inverted U-shaped. Importantly, the effect of alcohol on antipredator response and on general activity did not correlate. For example, activity was increased in both the ETOH 0.50% and ETOH 0.25% groups whereas jumping frequency was increased in the latter and was decreased in the former. The stimulus presentation as well as the recording of behavioral responses of the experimental fish in this task can be automated. However, it is notable that identifying a mutation based on the behavior of a group of fish, as conducted in the social preference or antipredator behavior screen, may require additional steps of breeding (see Methods section).

Our results indicated a U-shaped dose–response curve for several behavioral measures recorded. The dose–response curves of alcohol-induced changes suggested that, in general, alcohol has a facilitatory effect at lower and an inhibitory effect at higher doses. Although this trend was fairly consistent across the tests, idiosyncratic characteristics of individual tests were clearly found. One notable example was the color reaction: increasing doses of alcohol elicited increasingly dark (more vivid) colors in the fish. Although it is tempting to speculate that the more vivid colors imply a more ‘‘hedonistic’’ state in zebra fish, one cannot rule out the possibility of a direct effect of alcohol on the chromophore cells, a question that needs to be investigated in the future. The idiosyncratic characteristics of different tests may allow one to identify molecular and neurobiological characteristics specific for certain alcohol-induced changes. Furthermore, the behavioral tests were used to analyze acute effects of alcohol but the tests are appropriate for the analysis of chronic as well as withdrawal effects of alcohol. Zebra fish may also be an appropriate subject for the analysis of alcohol preference. Mutants exhibiting alterations in such preference could be identified using the ‘‘place preference’’ paradigm (for recent application in rodents see Refs. [11,45]).

In summary, behavioral tests offer a useful tool with which the responses of zebra fish to alcohol may be analyzed. We believe that with the application of multidisciplinary research involving behavioral, neurobiological, and genetic analyses, zebra fish will be a useful model system in the study of the biological mechanisms of alcohol effects. The behavioral tests described in the present paper and those that will be developed in the future will facilitate the identification of genes involved in such mechanisms.

Acknowledgments

We thank Robert Griedel for his technical help and suggestions on the manuscript.

References

[1] Barber I, Huntingford FA, Cropton DWT. The effect of hunger and cestode parasitism on the shoaling decisions of small freshwater fish. J Fish Biol 1995;47:524–36.
[2] Basquill SP, Grant JWA. An increase in habitat complexity reduces aggression and monopolization of food by zebra fish (*Danio rerio*). Can J Zool 1998;76:770–2.
[3] Becker HC, Anton RF. Valporate potentiates and picrotoxin antagonizes the anxiolytic action of ethanol in a nonshock conflict task. Neuropharmacology 1990;29:837–43.
[4] Bloom HD, Perlmutter A. A sexual aggregating pheromone system in the zebrafish, *Brachydanio rerio*. J Exp Zool 1977;199:215–26.
[5] Breder CM, Halpern F. Innate and acquired behavior affecting the aggregation of fishes. Physiol Zool 1946;199:19154–90.
[6] Canger AK, Passini MA, Asch WS, Leake D, Zafonte BT, Glasgow E, Schechter N. Restricted expression of the neuronal intermediate filament protein plasticin during zebrafish development. J Comp Neurol 1998;399:561–72.
[7] Cerda J, Conrad M, Markl J, Brand M, Herrmann H. Zebrafish vimentin: molecular characterisation, assembly properties and developmental expression. Eur J Cell Biol 1998;77:175–87.
[8] Concha ML, Adams RJ. Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: a time-lapse analysis. Development 1998;125:983–94.
[9] Csányi V. Ethological analysis of predator avoidance by the paradise fish (*Macropodus opercularis* L.): II. Key stimuli in avoidance learning. Anim Learn Behav 1986;14:101–9.
[10] Csányi V, Gervai J. Behavior genetic analysis of the paradise fish, *Macropodus opercularis*: II. Passive avoidance learning in inbred strains. Behav Genet 1986;16:553–7.
[11] Cunningham CL. Localization of genes influencing ethanol-induced conditioned place preference and locomotor activity in BXD recombinant inbred mice. Psychopharmacology 1995;120:28–41.
[12] Currie PD. Zebrafish genetics: mutant cornucopia. Curr Biol 1996;6:1548–52.
[13] Detrich WH, Westerfield M, Zon LI, editors. The zebrafish: biology. Methods in cell biology, vols. 59–60. San Diego, CA: Academic Press, 1999.
[14] Dill LM. The escape response of the zebra danio (*Brachydanio rerio*): I. The stimulus for escape. Anim Behav 1974;22:711–22.
[15] Eisen JS. Motoneuronal development in the embryonic zebrafish. Development 1991;2:141–7.
[16] Eisen JS. Zebrafish make a big splash. Cell 1996;87:969–77.
[17] Fetcho JR, Liu KS. Zebrafish as a model system for studying neuronal circuits and behavior. Ann NY Acad Sci 1998;860:333–45.
[18] Fetcho JR, O'Malley DM. Visualization of active neural circuitry in the spinal cord of intact zebrafish. J Neurophysiol 1995;73:399–406.
[19] Frey H. Das Aquarium von A bis Z. Radebeul: Neuman Verlag, 1966.
[20] Gerlai R. Can paradise fish (*Macropodus opercularis*) recognize its natural predator? An ethological analysis. Ethology 1993;94:127–36.
[21] Gerlai R, Csányi V. Artificial bidirectional selection for a species-specific behavioural element, staccato movement, in paradise fish (*Macropodus opercularis*). Anim Behav 1994;48:1293–300.
[22] Gerlai R, Csányi V. Genotype-environment interaction and the correlation structure of behavioral elements in paradise fish (*Macropodus opercularis*). Physiol Behav 1990;47:343–56.
[23] Gerlai R, Hogan JA. Learning to find the opponent: an ethological analysis of the behavior of paradise fish (*Macropodus opercularis*, Anabantidae) in intra- and inter-specific encounters. J Comp Psychol 1992;106:306–15.
[24] Goodwin DW, Dowd CP, Guze SB. Biphasic and reversible adaptation of goldfish to alcohol. Nature 1971;232:654–5.
[25] Granato M, Nusslein-Volhard C. Fishing for genes controlling development. Curr Opin Genet Dev 1996;6:461–8.
[26] Granato M, Van Eeden FJM, Schach U, Trowe T, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard C. Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. Development 1996;123:399–413.
[27] Greizerstein HB. Effect of rate of absorption and dose on the development of tolerance to ethanol. Int Union Pharmacol, Proceedings of the International Congress of Pharmacology, 6th Abstract, Helsinki, Finland. Oxford, England: Pergamon, 1975 (632 pp.).
[28] Grunwald DJ. A fin-de-siècle achievement: charting new waters in vertebrate biology. Science 1996;274:1634–5.
[29] Guo S, Brush J, Teraoka H, Goddard A, Wilson SW, Mullins MC, Rosenthal A. Development of noradrenergic neurons in the zebrafish hindbrain requires BMP, FGF8, and the homeodomain protein soulless/Phox2a. Neuron 1999;24:555–66.
[30] Haffter P, Nussleinvolhard C. Large-scale genetics in a small vertebrate, the zebrafish. Int J Dev Biol 1996;40:221–7.
[31] Hall D, Suboski MD. Sensory preconditioning and second-order conditioning of alarm reactions in zebra danio fish (*Brachydanio rerio*). J Comp Psychol 1995;109:76–84.
[32] Hall D, Suboski MD. Visual and olfactory stimuli in learned release of alarm reactions by zebra danio fish (*Brachydanio rerio*). Neurobiol Learn Mem 1995;63:229–40.
[33] Holder N, McMahon A. Genes from zebrafish screens. Nature 1996;384:515–6.
[34] Kavaliers M. Day–night rhythms of shoaling behavior in goldfish: opioid and pineal involvement. Physiol Behav 1989;46:167–72.
[35] King DP, Takahashi JS. Forward genetic approaches to circadian clocks in mice. Cold Spring Harbor Symp Quant Biol 1996;61:295–302.
[36] Li L, Dowling JE. Disruption of the olfactoretinal centrifugal pathway may relate to the visual system defect in night blindness b mutant zebrafish. J Neurosci 2000;20:1883–92.
[37] Lim TM. Effects of schooling prey on the hunting behavior of a fish predator. In: Noakes DLG, Ward JA, editors. Developments in environmental biology of fishes, vol. 1. Ecology and ethology of fishes: proceedings of the 2nd biennial symposium, Normal, IL, USA. The Hague, Netherlands: O.W. Junk Publishers, 1979. p. 142.
[38] Macovschi O. Permeability of living branchial membrane of *Carassius auratus gibelio* for cocaine hydrochloride. Trav Mus Hist Nat Grigore Antipa 1968;8:415–21.
[39] McCann LI, Koehn DJ, Kline NJ. The effects of body size and body markings on nonpolarized schooling behavior of zebra fish (*Brachydanio rerio*). J Psychol 1971;79:71–5.
[40] Miczek KA, Weerts EM, DeBold JF. Alcohol, benzodiazepine-GABAA receptor complex and aggression: ethological analysis of individual differences in rodents and primates. J Stud Alcohol 1993;11:170–9.
[41] Neuhauss SCF, Biehlmaier O, Seeliger MW, Das T, Kohler K, Harris WA, Baier H. Genetic disorders of vision revealed by a behavioral screen of 400 essential loci in zebrafish. J Neurosci 1999;19:8603–15.
[42] Nutt DJ, Peters TJ. Alcohol: the drug. Br Med Bull 1994;50:5–17.
[43] Peeke HV, Figler MH. Modulation of aggressive behavior in fish by alcohol and congeners. Pharmacol, Biochem Behav 1981;1:79–84.
[44] Peeke HV, Peeke SC, Avis HH, Ellman G. Alcohol, habituation and the patterning of aggressive responses in a cichlid fish. Pharmacol, Biochem Behav 1975;3:1031–6.
[45] Risinger FO, Oakes RA. Dose- and conditioning trial-dependent ethanol-induced conditioned place preference in Swiss–Webster mice. Pharmacol, Biochem Behav 1996;55(1):117–23.

[46] Ryback RS. The use of fish, especially goldfish, in alcohol research. Q J Stud Alcohol 1970;31:162–6.
[47] Ryback RS. A method to study short-term memory (STM) in the goldfish. Pharmacol, Biochem Behav 1976;4:489–91.
[48] Ryback R, Percarpio B, Vitale J. Equilibration and metabolism of ethanol in the goldfish. Nature 1969;222:1068–70.
[49] Schier AF. Genetics of neural development in zebrafish. Curr Opin Neurobiol 1997;7:119–26.
[50] Solnica-Krezel L, Schier AF, Driever W. Efficient recovery of ENU-induced mutations from the zebrafish germline. Genetics 1994;136: 1401–20.
[51] Walker C, Streisinger G. Induction of mutations by gamma-rays in pregonial germ cells of zebrafish embryos. Genetics 1993;103: 125–36.
[52] Westerfield M. The zebrafish book. Eugene, OR: University of Oregon Press, 1993.