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Zebra Fish: An Uncharted Behavior Genetic Model

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The zebra fish has been a preferred subject of genetic analysis. It produces a large number of offspring that can be kept in small aquaria, it can be easily mutagenized using chemical mutagens (e.g., ethyl nitrosourea [ENU]), and high-resolution genetic maps exist that aid identification of novel genes. Libraries containing large numbers of mutant fish have been generated, and the genetic mechanisms of the development of zebra fish, whose embryo is transparent, have been extensively studied. Given the extensive homology of its genome with that of other vertebrate species including our own and given the available genetic tools, zebra fish has become a popular model organism. Despite this popularity, however, surprisingly little is known about its behavior. It is argued that behavioral analysis is a powerful tool with which the function of the brain may be studied, and the zebra fish will represent an excellent subject of such analysis. The present paper is a proof of concept study that uses pharmacological manipulation (exposure to alcohol) to show that the zebra fish is amenable to the behavioral genetic analysis of aggression and thus may allow us to reveal molecular mechanisms of this behavioral phenomenon relevant to vertebrates.

KEY WORDS: Aggression; alcohol; mutagenesis; schooling; social behavior; zebra fish.

INTRODUCTION

Aggression may serve several important functions. For example, it allows the establishment of dominance hierarchy or maintenance of a territory or a harem. Briefly, it facilitates access to resources such as food, water, and mates. Thus aggression has an important role in propagating the alleles of the organism, that is, in evolutionary fitness. Not surprisingly, therefore, aggression is a nearly universal phenomenon in the animal kingdom. As is the case with other biological traits, understanding the biological mechanisms of aggression may be best achieved with the use of model organisms.

Zebra fish has been an important model organism in genetics and in developmental biology, including developmental neurobiology (Grunwald and Eisen, 2002). It is a small vertebrate that can be kept in captivity in large numbers easily (Westerfield, 2000). Its generation time is short and most importantly a single spawning can produce hundreds of offspring (Detrich *et al.,* 1999). Furthermore, zebra fish can be subjected to chemical mutagens and thus many mutants can be produced quickly (Granato and Nusslein-Volhard, 1996; Walker and Streisinger, 1993). Several genes discovered in this species are evolutionarily conserved and have homologs in mammals including our own species (Cerda *et al.,* 1998). These attributes make zebra fish an appropriate model organism for genetic studies (Eisen, 1996; Granato and Nusslein-Volhard, 1996; Grunwald, 1996). Indeed, the zebra fish has been successfully utilized in genetic analyses, and libraries containing hundreds of mutants have been established (Curie, 1996; Eisen, 1996; Grunwald, 1996; Holder and McMahon, 1996).

Although interesting discoveries have been made with regard to brain development of zebra fish (e.g., Guo *et al.,* 1999), surprisingly little is known about the behavior of this species (Gerlai *et al.,* 2000). This is unfortunate because behavioral analysis is perhaps the best way to investigate the function of the brain and should allow the analysis of mutation-induced alterations without restriction in terms of neuroanatomical locale or neurobiological function (Gerlai, 2001).

The present paper is a proof of concept study. Its goal is to show that the zebra fish possesses behavioral characteristics that make this species particularly amenable to the behavior genetic analysis of aggression. The behavioral tests employed utilize what is

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known about the ecology and ethology of zebra fish and are deliberately simplistic to facilitate future automation required for screening large number of mutagenized subjects. The studies presented here do not attempt to utilize the powerful genetic tools already available for this species, but instead use a pharmacological manipulation, exposure to alcohol, known to modulate levels of aggression, among other behavioral traits. Briefly, the results demonstrate that aggression may be modulated and different levels of aggression may be easily quantified in zebra fish.

METHODS

The behavioral repertoire of the zebra fish is complex (personal observation) but remains mainly undescribed. Nevertheless, some natural behavioral responses of zebra fish have been utilized in the development of simple behavioral test screens (Gerlai, *et al.,* 2000). The present paper is built upon these preliminary test methods and reviews results based on them with a focus on behavioral traits relevant for aggression. In the design of the tests the following characteristics known of the natural behavior of this species were utilized. The zebra fish is a typical diurnal cyprinid schooling fish (Barber *et al.,* 1995; Kavaliers, 1989; McCann *et al.,* 1971) that exhibits social preference for its conspecifics in a group situation (Bloom and Perlmutter, 1977; Breder and Halpern, 1946). Individuals swim together in close proximity, and when a single fish (or a small group of fish) sees a larger group it tends to join it, a behavior termed social coherence. Male zebra fish can exhibit aggressive behaviors including fin erecting display, dancing or undulating movements, and attack (darting toward the opponent or biting the opponent) (Basquill and Grant, 1998). Females have also been observed (personal observation) displaying to an individual opponent. Aggressive zebra fish exhibit a visible color change: dominant or fighting fish or fish during spawning become darker and more colorful (Gerlai *et al.,* 2000). Aggression is mainly seen between pairs of fish but less so in large schools. Levels of aggression vs. social coherence could be modulated by exposure to alcohol, a substance whose delivery is simple in the case of zebra fish because the subjects absorb it easily from the water in which they swim (Gerlai, *et al.,* 2000; Ryback, 1970). Alcohol absorbed by the blood vessels of the gill and the skin of the fish results in blood alcohol levels that reach equilibrium with the external alcohol concentration quickly (Ryback *et al.,* 1969; also see Greizerstein, 1975; McCann *et al.,* 1971); thus dosing can be employed and the blood alcohol level can be maintained precisely. In the present study alcohol-induced behavioral changes are investigated in four simple tests that allow quantification of behavioral traits that are either associated with aggression or can influence performance in aggression tests.

General Procedure

To investigate the aggressive behavior of zebra fish naïve, previously untested male zebra fish were assigned to four groups (for sample sizes see figure legends) treated with different concentration of alcohol (ETOH 0.00%, ETOH 0.25%, ETOH 0.50%, and ETOH 1.00%, volume percentage). The corresponding alcohol concentration was administered by holding the fish in the alcohol-water solution for 1 h before the behavioral tests. The same alcohol concentration was employed during the behavioral tests. The alcohol concentrations were selected based on data published on another cyprinid, the gold fish (*Carassius auratus*) (Goodwin *et al.,* 1971; Ryback, 1970; Ryback *et al.,* 1969). The alcohol-treated and control fish were tested in the behavioral paradigms in the order described below. To minimize potential interference between behavioral paradigms (experience effects, McIlwain *et al.,* 2001) the interparadigm interval was maintained at 14 days. 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 observer.

Locomotor Activity in a Novel Place and After Habituation

Changes in motor activity can influence performance on numerous behavioral tasks. Alcohol is known to alter locomotory responses. To test this important performance characteristic the activity of zebra fish was monitored upon exposure to a novel place and after a 10-min habituation period. Fish were placed individually in a small experimental tank (30 × 15 × 10 cm length × height × width), and their behavior was video recorded 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 video recordings were later replayed and analyzed using the Noldus Observer event recording software (Noldus, Wageningen, The Netherlands) on a Macintosh laptop (PowerBook G3). Locomotion (swimming activity) was measured by placing a transparency grid in front of the TV monitor that divided the tank into four segments

that allowed the counting of the number of entries by the fish to each segment.

Aggression: The Inclined Mirror Task

The zebra fish is a social species and exhibits social coherence but also shows territoriality and aggression; therefore interaction between fish can be complex and 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 video-tracking or motion-detection photocell systems. Fish were individually netted into a small experimental tank (30 × 15 × 10 cm length × height × width). A mirror was placed inclined at 22.5 degrees 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 video recorded for 60 s after a 30-s short acclimation 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 indicated preference for proximity to the "opponent" and is quantified as the amount of time spent in that segment. In addition to the quantification of where the fish stayed, the length of time relative to the observation interval (%) for which the fish performed aggressive behaviors was also measured and analyzed. Fin erection display is defined as a posture during which the fish erects its dorsal, caudal, pectoral, and anal fins. Usually, fin erection display is associated with undulating body movements or small slaps carried out by the caudal fin. Attack behavior is also regarded as part of the aggressive behavioral repertoire and is a characteristic short bout of fast swimming directed toward the opponent. It is sometimes accompanied by opening the mouth and biting. Attack behavior often alternates with fin erection display and was quantified as "aggressive display" together with all other aggression-related behaviors.

Group Preference

The zebra fish is a schooling fish that may exhibit preference for its conspecifics under certain circumstances. A pair of fish is expected to exhibit aggression, whereas aggression may be reduced in schools containing several fish. In the present task, response of a group of five experimental fish to a school of conspecifics was tested, a behavior expected to be more associated with social coherence than with aggression. The rationale behind using a group of five fish as subjects is that this social setting biases behavior toward schooling and is expected to reduce aggression. Fish were placed in groups of five in a small experimental tank (30 × 15 × 10 cm length × height × width). On one side of the experimental tank an empty fish tank was placed, and on the other side a tank of identical size held 15 zebra fish, the "stimulus fish." The experimental fish were allowed to acclimate to the experimental tank for a 30-s period, after which their behavior was video recorded. The first 10 s of this video recording 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 the five experimental fish spent on the side of the tank closer to the conspecific school was measured using the event recorder program.

Pigment Response

Zebra fish change their color in response to certain stimuli. 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 or attacking fish are darker and show more vivid colors. 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 × 15 × 10 cm length × height × width) that was illuminated by fluorescent light tubes. The background of the tank was uniformly light gray. Electronic photographs of the fish were taken, and the images were transferred to a computer (G3 Macintosh). 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 gray 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.

Fish Husbandry

Outbred zebra fish used for the behavioral studies were obtained from Scientific Hatchery (Huntington Beach, CA, USA). 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 Inc., Beverly, MA, USA) provided oxygenation. Fish were fed four times daily: twice with live brine shrimps (Artemia salina, San Francisco Bay Brand, San Francisco, CA, USA) and twice with dry food (Tetra-min, Tetra Co, Melle, Germany).

Statistical Analysis

Data handling and statistical analyses were carried out using Systat 5.1 for the 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.

RESULTS

Alcohol induced motor activity changes are known to be characterized by an inverse U-shaped dose response curve (reviewed in Draski and Deitrich, 1995). The results with zebra fish confirm these findings (Figure 1). Locomotion score was significantly affected by alcohol [$F(3,53) = 42.19$, $p < 0.0001$], and this effect was independent of whether locomotion was quantified at the first or the tenth minute of the test session [ANOVA time $F(1,53) = 0.213$, $p > 0.50$; alcohol × time interaction $F(3,53) = 0.60$, $p > 0.50$]. Comparison of the groups (post hoc Tukey's HSD test) showed that fish in the 0.25% and 0.50% alcohol treatment groups moved significantly ($p < 0.05$) more compared to the fish in the control group or in the group treated with 1.00% alcohol. Furthermore, fish treated with 1.00% alcohol were significantly ($p < 0.05$) hypoactive compared to fish in all the other three groups at the first minute of the session, a difference that diminished after 10 min.

Responses to the mirror image of an individual conspecific also significantly changed as a result of alcohol treatment. Time spent in the segment nearest to the mirror image (Figure 2) significantly differed among treatment groups [ANOVA $F(3,53) = 10.48$, $p < 0.0001$]. The ANOVA terms "time" [$F(1,53) = 0.01$, $p > 0.90$] and "time × treatment interaction" [$F(3,53) = 1.02$, $p > 0.40$] were found nonsignificant. However, Figure 2 suggests a clear interaction, an observation that is confirmed by post hoc Tukey's HSD test. Fish treated with 0.25% alcohol spent significantly ($p < 0.05$) more time nearest to the opponent compared with fish from the other groups during the first minute

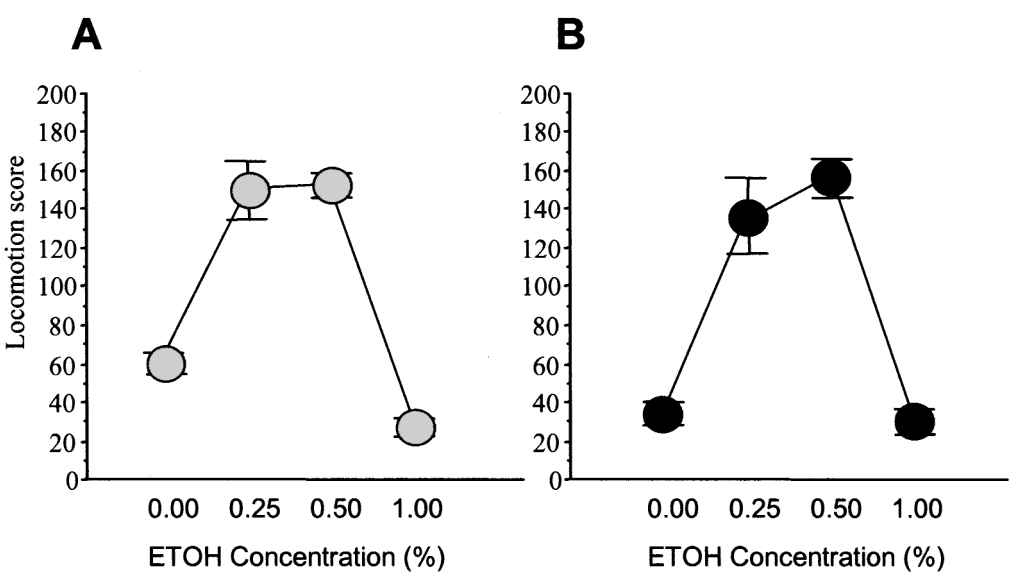

Fig. 1. Locomotion is increased by intermediate doses of alcohol in zebra fish. Locomotion score is calculated as the number of crossings between four segments of the observation tank during a 1-min observation session at the beginning of the test (panel A) and at the tenth minute of the test (panel B). Means ± SE are shown. Sample sizes were as follows: ETOH 0.00% $n = 13$, ETOH 0.25% $n = 15$, ETOH 0.50% $n = 13$, ETOH 1.00% $n = 16$.

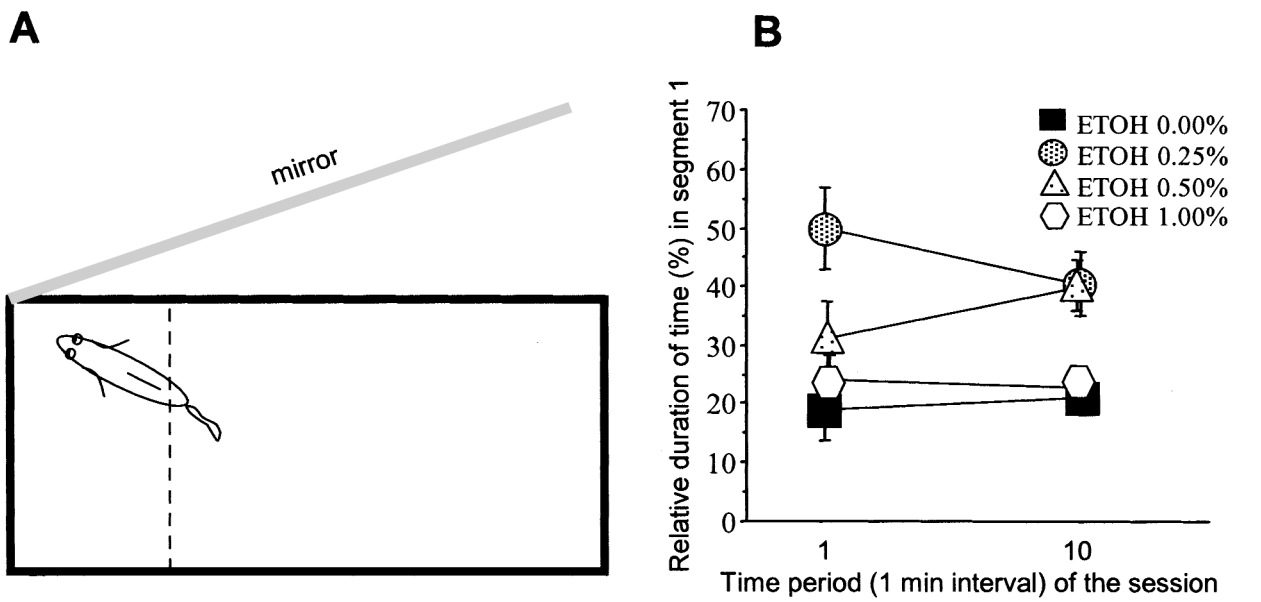

Fig. 2. Intermediate alcohol concentrations enhance preference for a conspecific in zebra fish. Panel A shows the experimental tank with the inclined mirror (for details see Methods). The imaginary border of the relevant segment is indicated by the dashed line. Panel B shows the percent of time spent by zebra fish in the segment from where the mirror image of the subject appeared nearest. Note that the location (percent of time spent in the relevant segment) of the experimental fish was quantified at the beginning (1st min) and at the end (10th min) of the recording session. Means ± SE are indicated. Sample sizes were as follows: ETOH 0.00% $n = 15$, ETOH 0.25% $n = 16$, ETOH 0.50% $n = 16$, ETOH 1.00% $n = 18$.

of being exposed to the mirror image. After a 10-min habituation time, however, fish in both the 0.25% and 0.50% alcohol treatment groups spent significantly ($p < 0.05$) more time near their mirror image compared to fish from the other two groups (ETOH 0.00% and ETOH 1.00%).

It is possible that staying close to the mirror image is a sign of social cohesion and has nothing to do with aggression. However, quantification of time spent with aggressive behaviors suggested otherwise (Figure 3). ANOVA revealed a significant alcohol effect [$F(3,53) = 9.90$, $p < 0.0001$], but no significant time or alcohol × time interaction effects [$F(1,53) = 2.33$, $p > 0.10$; $F(3,53) = 0.91$, $p > 0.40$] were found. Post hoc Tukey's HSD showed 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 0.50% alcohol were more aggressive than those receiving 1.00% alcohol both at the first and at the tenth minute of the recording session.

Alcohol also altered schooling behavior (Figure 4); however, this alteration showed a different dose response curve compared to what was observed in the mirror test of aggression. Alcohol significantly reduced the preference for conspecifics in a dose-dependent manner [$F(3,53) = 2.85$, $p = 0.05$]. Treatment with alcohol led to a scattered, distributed spatial location of the experimental fish implying decreased preference for the school of stimulus conspecifics. Lastly, fish treated with alcohol (Figure 5) showed a dose-dependent and significant color change, darkening [ANOVA $F(3,53) = 131.32$, $p < 0.0001$].

DISCUSSION

In summary, the present results demonstrate that zebra fish interacts with its conspecifics and this interaction can have aggressive elements depending on the environmental circumstances. Furthermore, levels of aggression, along with other social behaviors, could be modulated by exposure to alcohol. Although genetic manipulation was not attempted in the present study, the latter findings suggest the existence of biological mechanisms in the brain of zebra fish that may be amenable to such manipulations.

Aggression has not been studied in zebra fish before. Medline search with words "aggression" or "agonistic behavior" or "fighting" or "territoriality" or "dominance" and "zebra fish" (or "zebrafish" or "Danio" or "rerio") returned no publications other than the original paper upon which this review is based (Gerlai *et al.,* 2000). Aggression has been studied in other fish species, particularly in the Siamese fighting fish (*Betta splendens*) (Bronstein, 1984) and species belonging to the family

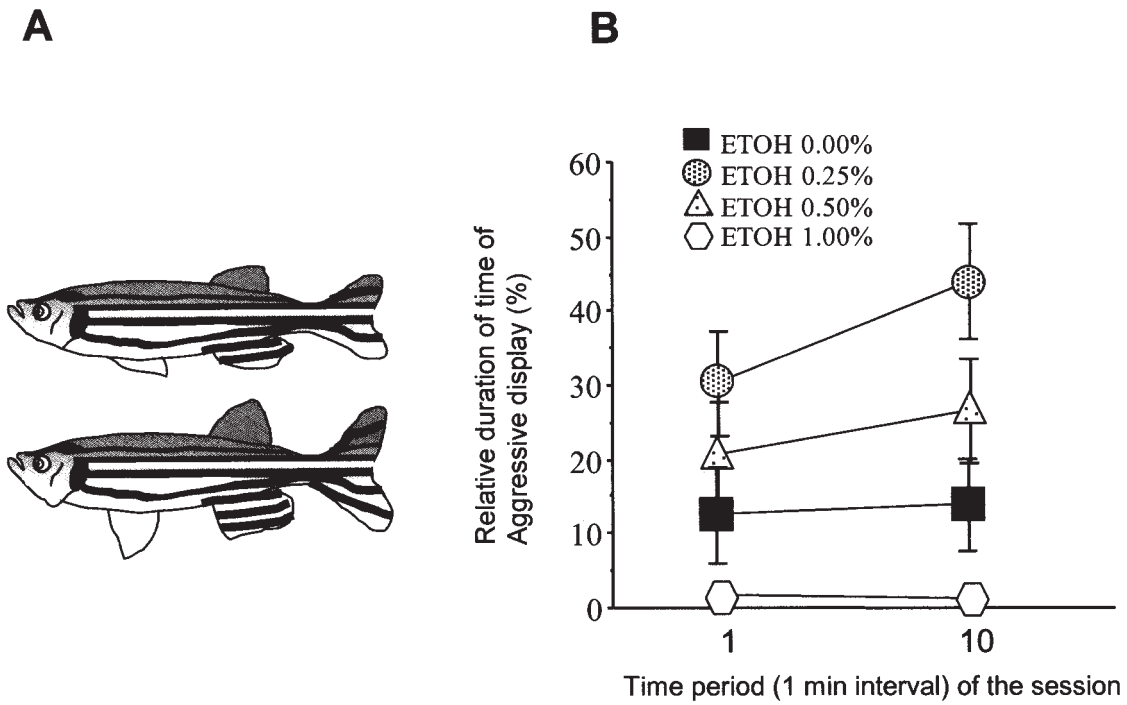

Fig. 3. Intermediate alcohol concentrations enhance aggressive behavioral responses elicited by the sight of an opponent in zebra fish. Panel A shows an example, fin erection display, of an aggressive behavior (bottom fish is exhibiting fin erection display, top fish is not). Note that the quantification of behavior is based on the same experiment as in Figure 2, but here a side view of the fish is replayed and different elements of aggressive behavior (see Methods) are quantified. Note that the behavior of the experimental fish was quantified at the beginning (1st min) and at the end (10th min) of the recording session. Means ± SE are shown. Sample sizes were as indicated in Figure 2.

Cichlidae, for example, the convict cichlid (*Cichlasoma nigrofasciatum*) (Peeke *et al.,* 1981). However, these species do not offer the advantages of having powerful genetic tools already developed for zebra fish. Therefore characterization of the zebra fish and development of proper quantification techniques for its behavior relevant to aggression are very important. The present paper suggests that zebra fish indeed possesses

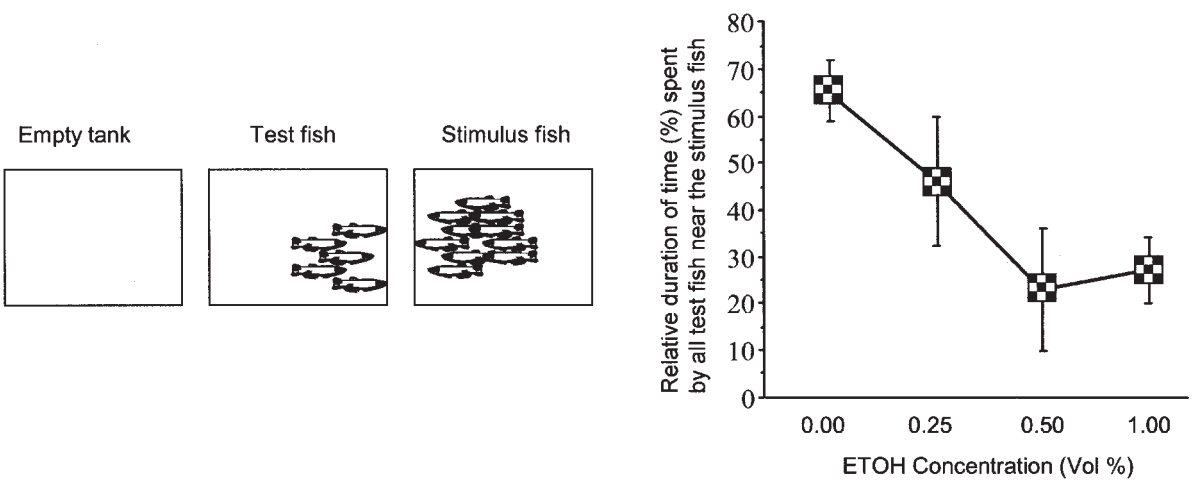

Fig. 4. Preference for a school of zebra fish is diminished by high doses of alcohol. The response of groups of five fish is tested. Sample sizes (n) representing the number of these groups were as follows: ETOH 0.00% $n = 15$, ETOH 0.25% $n = 9$, ETOH 0.50% $n = 6$, ETOH 1.00% $n = 9$. Means and SE are shown. 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.

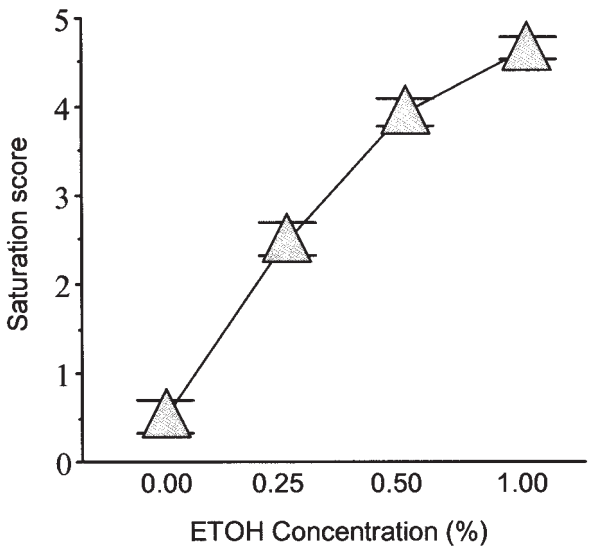

Fig. 5. Alcohol enhances the color of zebra fish. Higher saturation scores represent darker (more vivid) colors. Means ± SE are indicated. Sample sizes were as follows: ETOH 0.00% $n = 15$, ETOH 0.25% $n = 16$, ETOH 0.50% $n = 16$, ETOH 1.00% $n = 18$. Note the near linear increase of saturation score in response to increasing alcohol doses.

characteristics that make this species useful for the analysis of aggression, and the results also demonstrate that traits relevant for aggression can be easily quantified, a necessary requirement in large-scale mutagenesis screening studies.

Alcohol proved to be an appropriate tool with which aspects of behavior relevant for aggression could be modulated. However, it also induced hyperactivity at intermediate doses (0.25% and 0.50%) and led to hypoactivity at the highest dose (1.00%), a typical inverse U-shaped dose response curve. Alteration of locomotion may influence behavioral performance in other test paradigms as well, including tests of interaction between conspecifics, and thus may hinder one's ability to study intraspecific behaviors, including aggression. However, the increased locomotory activity observed in fish treated with 0.25% or 0.50% alcohol concentrations in unlikely to explain why these fish spent more time in the proximity of their mirror image. The latter finding may be due to enhanced social preference (schooling) or aggressive tendencies. Analysis of the behavior of zebra fish supported the latter notion. It showed enhanced aggression (aggressive displays, attack, and biting) in fish treated with the intermediate doses of alcohol. It is notable that the dose response characteristic of the effect of alcohol on measures of aggressive behavior is also the typical inverse U shape, because the highest dose (1.00%) reduced aggressive tendencies. Finally, it is also notable that this dose response relationship did not hold in the test of group preference. In this test, all fish treated with alcohol reduced their preference for the group and the reduction (scattering as opposed to social cohesion) was dose dependent. The different dose response curves obtained in the tests of aggression and group preference suggest that different biological mechanisms may underlie these effects, and perhaps aggression and social preference are under the control of separate biochemical pathways and sets of genes. Finally, the effect of alcohol on the color of the fish was also significant, with a dose response curve almost linearly dependent on the concentration. The question whether the enhanced color was the result of the direct effect of alcohol on the chromophore cells or an indirect effect mediated by central CNS mechanisms could not be addressed in the present study. Nevertheless, the correlation between the behavioral findings and the color change in zebra fish, as well as the known role of color in dominance, aggression, or sexual arousal in fish and the known role of alcohol in these behaviors in other species is suggestive of a possible centrally mediated mechanism.

The present proof of concept study suggests that the zebra fish will be an appropriate model organism for the genetic analysis of aggression in vertebrates. Detailed description of the natural behavioral repertoire of zebra fish will aid the development of simple and automatable behavioral paradigms. Such paradigms will be crucial in screening of mutants generated by chemical mutagenesis and thus will significantly facilitate the discovery of novel genes and biochemical pathways involved in aggression. It is hoped that such discoveries will allow us to better understand these mechanisms in our own species.

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