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# Context-dependent sensitization to ethanol in zebrafish (*Danio rerio*)

Rachel E. Blaser [a,*], Audrey Koid [b], Rebecca M. Poliner [c]

[a] Department of Psychological Sciences, University of San Diego, 5998 Alcala Park, San Diego, CA, 92110, United States
[b] Department of Psychology, Franklin & Marshall College, P. O. Box 3003, Lancaster, PA 17604, United States
[c] Department of Biology, Duke University, Box 90086 Flowers Drive, Duke University, Durham, NC 27708, United States

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ABSTRACT

One of the most robust and readily measurable effects of moderate doses of ethanol on zebrafish behavior is locomotor hyperactivity. Two experiments were designed to examine the effects of repeated exposures to ethanol on ethanol-induced locomotor hyperactivity, and to determine whether these effects are context-dependent. Adult, wild-type zebrafish were given repeated exposure to ethanol in the presence of one contextual stimulus (A), while exposed to water in the presence of a second contextual stimulus (B). Exposure to ethanol consistently induced locomotor hyperactivity. After repeated exposures, animals tested with ethanol in the ethanol-paired context (A) showed sensitization of locomotor activity. When tested with ethanol in the unpaired context (B), however, sensitization was not observed. When tested in the absence of ethanol, there were no differences in responding to the paired and unpaired stimuli. This is the first demonstration of ethanol-induced locomotor sensitization in zebrafish. Moreover, this sensitization was context-specific, indicating that learning can modify drug-induced behaviors in zebrafish.



## 1. Introduction

It is well established that long-term exposure to a drug of abuse often produces changes in drug response, both through non-associative adaptation and through associative learning. Tolerance (a shift to the right of the dose–response curve, or reduced responding) and sensitization (a shift to the left of the dose–response curve, or enhanced responding) are two such effects, commonly studied in rodents because they are thought to be involved in human drug abuse (for a review, see (Stewart and Badiani, 1993)). For example, tolerance may develop to the hedonic effects of addictive drugs, prompting dose increases that lead to excessive drug-taking (Solomon and Corbit, 1974), while locomotor sensitization has been postulated to relate to the motivational value of some drugs (Hunt and Lands, 1992; Robinson and Berridge, 1993; Wise and Bozarth, 1987). Although the precise relationship between tolerance, sensitization, and drug abuse is still being unraveled, virtually all drugs with abuse potential produce one or both effects, making them nonetheless a reasonable starting-point for understanding the effects of long-term drug use on the brain (Bradizza et al., 2009; Koob and Le Moal, 1997; Stewart and Badiani, 1993). The expression of tolerance or sensitization to ethanol depends on the species and strain, the dose, the schedule of administration, and the behavioral response being measured. For instance, tolerance is often observed to the sedative, ataxic and hypothermic effects of ethanol, and is typically induced using daily or multiple daily exposures, while sensitization is most commonly observed in locomotor activity, and is induced using longer intervals between ethanol exposures (e.g. (Crowell et al., 1981; Didone et al., 2008; Larson and Siegel, 1998; Lessov and Phillips, 1998; Stewart and Badiani, 1993)).

An additional characteristic effect of repeated drug exposure is associative learning, which plays an integral role in mammalian models of drug abuse (for reviews, see (Bradizza et al., 2009; Siegel, 1999; Tomie, 1995)). If predictive cues (administration cues or contextual cues) are repeatedly paired with a drug, the animal may associate the cues with the pharmacological effects of the drug. These cues, in turn, may contribute to tolerance or sensitization by eliciting anticipatory responses that may modulate behavior (Siegel, 1999). Tolerance to ethanol-induced hypothermia and ataxia is context-sensitive, indicating a role of conditioning (Crowell et al., 1981; Larson and Siegel, 1998). While ethanol-induced locomotor sensitization has not been consistently linked to context (Didone et al., 2008), context effects have been reported under some circumstances (Boehm et al., 2008; Cunningham and Noble, 1992; Meyer et al., 2005).

Zebrafish are an emerging animal model for behavioral pharmacology, due in part to several advantages over rodent models. Zebrafish are smaller, cheaper, and easier to house than rodents, and they reproduce prolifically, which is useful for forward genetic techniques (Fishman, 2001; Gerlai et al., 2000; Guo, 2004). Because of this, zebrafish are used widely for genetic research, and offer the potential for linking genetic, pharmacological, and behavioral pathways involved in brain disorders such as drug abuse (Darland and Dowling, 2001; Gerlai et al., 2000; Gerlai, 2003; Guo, 2004; Levin et al., 2007; Lopez-Patino et al., 2008; Ninkovic and

* Corresponding author. Tel.: +1 619 260 7736; fax: +1 619 260 4511.
*E-mail address:* rblaser@sandiego.edu (R.E. Blaser).

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Bally-Cuif, 2006). Although their behavior is not yet well characterized, behavioral research is proliferating rapidly; it is clear that zebrafish are capable of sophisticated behavioral output that can be used for pharmacological and genetic screens (Gerlai et al., 2000; Guo, 2004; Levin and Cerutti, 2008; Sison et al., 2006). Acute exposure to ethanol has been shown to produce a variety of behavioral changes in zebrafish, affecting social behavior (shoaling and aggression), locomotor activity, and light/dark preference (Dlugos and Rabin, 2003; Gerlai et al., 2000; Gerlai et al., 2006; Lockwood et al., 2004). One of the most widely reported effects of exposure to moderate doses of ethanol is locomotor hyperactivity. Hyperactivity to ethanol has been reported in larval zebrafish by the 7th minute of exposure, with peak activity at 1.5% ethanol, and hypoactivity at 4.0% ethanol (Lockwood et al., 2004). In adult zebrafish, peak hyperactivity is produced by 0.5% (Gerlai et al., 2000) or 1.0% ethanol (Gerlai et al., 2006), with significant effects appearing by about 15 min of exposure (Dlugos and Rabin, 2003). It appears that strain differences account for some variation in dose sensitivity, although a variety of other factors (individual differences, measure of activity, testing individuals vs. shoals) may also be involved (Dlugos and Rabin, 2003; Gerlai et al., 2008). If ethanol-induced hyperactivity in zebrafish shares similar pathways with the analogous behavior in rodents, zebrafish may be a particularly useful model for understanding the genetics of acute ethanol sensitivity.

Chronic exposure to ethanol (1–2 weeks of constant immersion) also has behavioral effects on zebrafish (Dlugos and Rabin, 2003; Gerlai et al., 2006). While acute exposure to 0.25% ethanol produced mild locomotor hyperactivity (increased swim distance) in adult subjects, chronic exposure to 0.25% ethanol prior to the acute challenge attenuated the hyperactivity response, suggesting tolerance (Gerlai et al., 2006). The effects of ethanol on shoaling behavior may also undergo tolerance with chronic exposure in some strains (Dlugos and Rabin, 2003). Additionally, chronic exposure to ethanol during development produces a variety of physiological and behavioral dysmorphologies, including deficient learning and shoaling (Bilotta et al., 2004); Bilotta et al., 2002; Carvan et al., 2004; Fernandes and Gerlai, 2009; Loucks and Carvan, 2004). It is reasonable, therefore, to predict that chronic exposure to ethanol has the capacity to alter one or more of the pathways involved in the acute response to ethanol, as is true of rodents. However, a more detailed characterization of the behavioral response to long-term ethanol exposure is required.

Information on associative learning in zebrafish is currently limited, although they clearly are capable of it (e.g. (Colwill et al., 2005; Darland and Dowling, 2001; Pather and Gerlai, 2009; Xu et al., 2007)). The degree to which associative learning might modify drug effects in zebrafish is therefore an important area of investigation if zebrafish are to be useful models of human drug disorders. Place preferences have been successfully conditioned to contexts associated with cocaine, amphetamine, and other drugs of abuse (Braida et al., 2007; Darland and Dowling, 2001; Lau et al., 2006; Swain et al., 2004), indicating that addictive drugs may support learning in fish as they do in rats. Whether learning actually modifies the behavioral effects of drugs, however, remains unknown. Therefore, the current experiments were designed not only to ask whether repeated, acute exposure to ethanol produces tolerance or sensitization of ethanol-induced hyperactivity, but also to determine the role of associative learning on the effect. In Experiment 1, the basic question is whether repeated exposures to moderate (0.5% and 1.0% v/v) doses of ethanol induce tolerance or sensitization of locomotor activity. Additionally, the question of associative learning was addressed first in Experiment 1, using a between-subjects design, and then again in Experiment 2, using a within-subjects design.

### 1.1. Experiment 1

In Experiment 1, three groups of animals received exposure to two different tanks, one of which was painted black, and the other painted white. For Group 1, one of the tanks was consistently paired with ethanol and the other unpaired, while for Group 2, there was no exposure to ethanol in training. Both groups were then given a single test with ethanol in order to determine whether the exposure of Group 1 to ethanol resulted in tolerance (less response than Group 2) or in sensitization (more response than Group 2). Group 3 received the same training as Group 1: one tank was paired with ethanol, and the other tank was unpaired. These animals also received a single ethanol test, but were tested with ethanol in the previously unpaired tank, and with water in the ethanol-paired tank. The purpose of this test was to determine whether the context in which ethanol is predicted, or received, influences locomotor activity (see Fig. 1 for an overview of the experimental design). Each of the three groups was subdivided into dose subgroups, with half of the animals receiving 0.5% ethanol, and the other half receiving 1.0%.

If repeated exposure to ethanol has no effect on acute ethanol-induced hyperactivity (no tolerance, no sensitization, no conditioning), all three groups are predicted to perform similarly in the test with ethanol. Higher levels of activity should be recorded in the presence than in the absence of ethanol, and ethanol-induced activity levels should be the same across all three groups. If repeated exposure has an effect (tolerance or sensitization), but there is no conditioning, Group 3 should be identical to Group 1 (they are trained and tested identically except for the context), but both should differ in the test from Group 2. Group 1 should respond less than Group 2 in the ethanol test if tolerance develops, and more if sensitization develops. Finally, if the repeated exposure produces conditioning to contextual stimuli, Group 3 should differ from Group 1 in the test, the direction of the difference indicating the nature of the conditioned response.

## 2. Methods

### 2.1. Subjects

Subjects were 69 adult (aged 6 months to 1.5 years) zebrafish of heterogeneous, wild-type phenotype, purchased from a local pet store (Aquatic Warehouse, San Diego, CA). About half were male and half female. Subjects were housed in an Aquaneering table-top housing rack, with a recirculating filtration system using mechanical, biological, and chemical filtration. The subjects were housed in groups of 20–25, in 9 L system tanks, before and after the data collection phase of the experiment. For a week prior to data collection, and during data collection, all subjects were pair-housed in 1.5 L system tanks to allow individual identification. The temperature of the tanks was held at 25 °C, and the room was maintained on a 14/10 light/dark cycle. Subjects were fed 1–2 times daily on a mixed diet of live brine shrimp and Tetra-Min flake food.

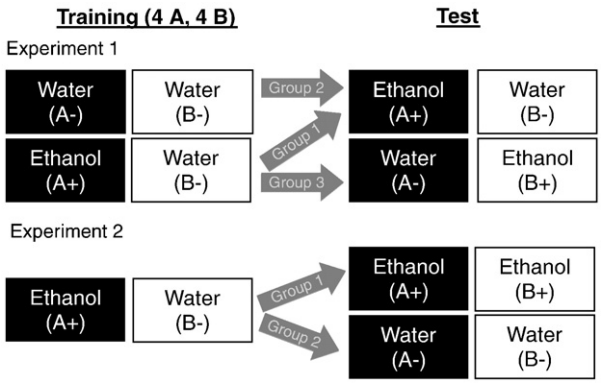


**Fig. 1.** Diagrammed in Fig. 1 are the designs of the two experiments. The black tank is in the role of A while the white tank is in the role of B; in the experiment, the role of each color as A or B was counterbalanced.

### 2.2. Apparatus

All behavioral tests used 2 L acrylic tanks, some of which were painted matte black, and others painted matte white. Homogeneous grey gravel covered the bottom of all tanks in order to ensure a consistent background for video-tracking across both tank color conditions. A video-camera suspended approximately 1 m above the test tanks was used to monitor the location and activity of the fish. The video-camera fed into a desktop computer using Noldus Ethovision® to track the swim-patterns of the fish at a rate of 10 samples/s. The video-tracking data were then used to determine relevant measures of behavior; path length (mean distance moved in cm/s) was the primary measure of interest.

### 2.3. Procedure

Subjects were randomly divided into three groups: Groups 1, 2, and 3. Each of these was further subdivided into two dose groups, one receiving 0.5% v/v ethanol, and the other receiving 1.0% v/v ethanol. This resulted in 6 groups of 10–12 subjects each: Group 1(0.5), Group 1(1.0), Group 2(0.5), Group 2(1.0), Group 3(0.5), and Group 3(1.0).

Each fish was observed individually in eight training trials and two tests. Following a 30-min habituation session on Day 1, each fish received a single 1-h trial each day for eight days (Days 2–9), and a single 1-h test on each of Days 10 and 11. The trials and tests were conducted at the same time each day. The full experimental procedure was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of San Diego.

### 2.4. Habituation

All subjects received a 15 min habituation trial in the black tank, and a 15 min habituation trial in the white tank, on the day before trials began. Tank order (black or white first) was balanced across fish. No ethanol was present during habituation. Data were recorded simply to compare baseline activity levels between the groups and between the black and white test tanks.

### 2.5. Trials

All subjects received four 1-h trials in a black tank, and four 1-h trials in a white tank; one trial was conducted each day for 8 days. For Groups 1(0.5) and 3(0.5), one of the colors always contained 0.5% v/v ethanol (A+ trials), and the other color contained only fresh system water (B− trials). For Groups 1(1.0) and 3(1.0), one of the colors always contained 1.0% v/v ethanol (A+ trials), and the other color contained only fresh system water (B− trials). Subjects in Group 2(0.5) and 2(1.0) were not exposed to ethanol during training, and both tanks contained only fresh system water (A− and B− trials). See Table 1 for a summary of the groups, and Fig. 1 for a graphic overview of the design. The colors (black or white) used as A and B were balanced across subjects. The trials were sequenced pseudorandomly, such that no more than two trials of the same type were on consecutive days, the color presented on the first and last trial being counterbalanced across subjects. Therefore, each fish received an ethanol trial on average once per 48 h, with a range of 24 to 72 h between doses. The purpose of this sequence was to prevent temporal cues from overshadowing the stimulus cues of interest.

### 2.6. Tests

All subjects received two 1-h tests, one in a black tank and one in a white tank. Subjects in Group 1 received ethanol (of the same dose used in training) in the tank previously paired with ethanol (A+ test), and water in the tank previously paired with water (B− test). Subjects in Group 2 received ethanol (either 0.5% or 1.0%) in the tank randomly assigned as A, and water in the other tank, B (A+ and B− tests). Subjects in Group 3 received ethanol (of the same dose used in training) in the tank previously paired with water (B+ test), and received water in the tank previously paired with ethanol (A− test). Half of the subjects received the ethanol test first, and half received the no-ethanol test first.

**Table 1**
Summarized in Table 1 are the designs of Experiments 1 and 2. The tank color (black or white) is designated by A and B, while X represents the presence of peripheral ethanol cues on trials when ethanol was present. The + and − indicate the presence or absence of ethanol, respectively.

| | Group | Training | Test | |
|---|---|---|---|---|
| Experiment 1 | Group 1 ($n=22$) | A(X)+, B− | A(X)+ | B− |
| | Group 2 ($n=23$) | A−, B− | A(X)+ | B− |
| | Group 3 ($n=24$) | A(X)+, B− | B(X)+ | A− |
| Experiment 2 | Group 1 ($n=8$) | A(X)+, B− | A(X)+ | B(X)+ |
| | Group 2 ($n=8$) | A(X)+, B− | A− | B− |

### 2.7. Ethanol exposure

Ethanol concentrations (0.5% or 1.0%) were measured by volume in 2 L of water at the beginning of each trial. Animals had no exposure to ethanol prior to the start of the trial. They were netted from their home tanks directly into the experimental tank, which already contained the relevant concentration of ethanol, and behavioral recording began within approximately 1 min. This procedure was used primarily so that exposure to the conditioned stimuli would precede the pharmacological effects of ethanol; an additional benefit, however, was that it provided a record of the change in locomotor activity as the ethanol entered the bloodstream and brain, allowing for the analysis of locomotor effects across time intervals. Although we did not wait for the potential effects of handling stress to dissipate before the onset of recording, this procedure does produce data throughout the trial so that all behavior (including any immediate effects of exposure to the experimental context) could be observed and analyzed.

### 2.8. Behavioral recording

Each trial and test was monitored using Noldus Ethovision ® videotracking software, which sampled the location of each fish at a rate of 10 samples/s, and calculated the total distance moved in each 5-min interval for the entire hour of each trial. The mean distance measures were calculated by dividing the distance moved in each 5-min interval by 300 (seconds in 5 min) to obtain a mean distance moved in cm/s.

## 3. Results

Results were analyzed using Repeated Measures Analysis of Variance (ANOVA). All of the trials were analyzed together, in order to compare the ethanol trials with the no-ethanol trials within-subjects as well as between-groups. The ethanol test and no-ethanol test were analyzed separately in order to simplify interpretation of the between-groups analysis. Post-hoc analyses were used when appropriate to further investigate the ANOVA results.

### 3.1. Trial results

As can be seen in Fig. 2 (left panel), ethanol exposure produced hyperactivity on all four trials. The 1.0% dose consistently elicited hyperactivity in both a within-subjects (compared to no-ethanol trials) and a between-groups (compared to the 0.0% group) comparison. The 0.5% dose produced milder hyperactivity, consistently elevated relative to no-ethanol trials, but only inconsistently relative to the 0.0% group. Additionally, as can be seen in Fig. 2 (right panel), activity level tended to

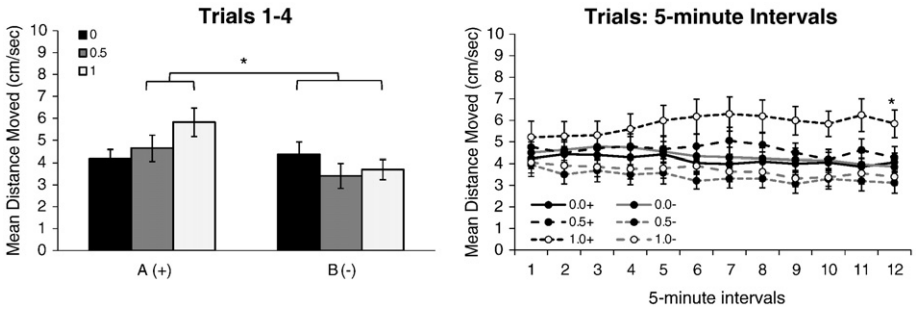


**Fig. 2.** Illustrated in Fig. 2 are the results from the eight training trials in Experiment 1. In the left panel, locomotor activity in trials with stimulus A (at 0.0%, 0.5% or 1.0%) is compared with that in trials with stimulus B (all at 0.0%). Animals that received ethanol during training were more active on A+ trials, and less active on B− trials, than those that did not receive ethanol during training, and activity was significantly greater on A+ than B- trials (main effect of Ethanol, $p<0.05$). In the right panel, activity levels are plotted as a function of the twelve 5-min intervals of the hour-long trials. All animals exhibited similar levels of activity in the beginning of the trials, with differences emerging by about 30 min (Concentration × Interval interaction, $p<0.05$).

decrease slightly across 5-min intervals on no-ethanol trials, while it tended to increase on ethanol trials.

A five-way 2×3×4×3×12 (presence/absence of Ethanol × Concentration × Trial × Group × 5-min Interval) Repeated Measures ANOVA indicated a significant main effect of Ethanol (presence vs. absence: ($F(1, 64) = 40.78$, $p<0.05$)), confirming that animals were more active in the presence than in the absence of ethanol, and a significant main effect of Interval ($F(11, 704) = 4.66$, $p<0.05$), with activity decreasing, on average, across intervals. The ANOVA also yielded a significant Ethanol × Interval interaction ($F(11, 704) = 5.98$, $p<0.05$), as activity in the presence of ethanol tended to increase over time. Finally, there were significant Trial × Interval ($F(33, 2112) = 2.83$, $p<0.05$), Trial × Group ($F(3, 192) = 4.03$, $p<0.05$), Trial × Concentration × Ethanol ($F(3, 704) = 5.76$, $p<0.05$), and Ethanol × Concentration × Group interactions ($F(1, 192) = 4.36$, $p<0.05$). The failure to find a main effect of Group suggests that baseline group characteristics (overall activity level, response to handling stress and novelty) were similar. The Group interactions stem from differences between activity in Group 2, which did not receive ethanol in training, and Groups 1 and 3, which did. The Trial × Interval, and Trial × Concentration × Ethanol interactions appear to be due to a small increase in the magnitude of the ethanol effects across the trials. Although the five-way ANOVA makes the complex interactions difficult to interpret, the purpose of analyzing the trial data was primarily to ensure that the ethanol did induce hyperactivity, and to establish baseline response levels, so no further analyses were conducted.

Separate analyses indicated no baseline differences between activity in black and in white, no differences between animals receiving ethanol in black and those receiving ethanol in white, and no differences based on trial or test sequence (i.e. water first vs. ethanol first).

### 3.2. Test results

#### 3.2.1. Ethanol test

As is apparent in Fig. 3 (left panel), animals receiving 1.0% ethanol were more active than those receiving 0.5% ethanol, which in turn were more active than those in the no-ethanol test, an expected effect of dose. The mean activity levels of the three groups in the ethanol test are compared in Fig. 3 (center panel), from which it is clear that Group 1 was more active than Group 2, suggesting sensitization, and also more active than Group 3, suggesting that the sensitization is context-dependent. Data from the ethanol test were analyzed using a 3×2×12 (Group × Concentration × 5-min Interval) Repeated Measures ANOVA. Results indicate a significant main effect of Group ($F(2, 63) = 4.06$, $p<0.05$), a significant main effect of Interval ($F(11, 693) = 2.17$, $p<0.05$), but no significant main effect of Concentration ($F(1, 63) = 3.56$), $p>0.05$). Note that this lack of main effect indicates only a failure to find a difference between 0.5% and 1.0% levels, as the no-ethanol test data were analyzed separately. Additionally, results indicated a significant Group × Interval interaction ($F(22, 693) = 2.23$, $p<0.01$), and a significant Concentration × Interval interaction ($F(11, 693) = 4.33$, $p<0.01$), indicating that the difference between 0.5% and 1.0% emerged over time and that the difference between the groups decreased over time.

The significant main effect of Group was further analyzed using Dunnett's post-hoc test, which confirmed that both Groups 2 and 3 were significantly less active than Group 1 ($p<0.05$ for each). Taken together, the results from the ethanol test indicate that 1.0% ethanol induced a greater degree of hyperactivity than 0.5% ethanol. Additionally, the effect of repeated ethanol exposure to ethanol is locomotor sensitization (relative to Group 2), which is apparent in animals tested with ethanol in the context previously paired with ethanol (Group 1), but not in animals tested with ethanol in the unpaired context (Group 3).

#### 3.2.2. No ethanol test

Data from the no-ethanol test were analyzed using a 3×2×12 (Group × Concentration × 5-min Interval) Repeated Measures ANOVA. There were no significant main effects or interactions in the absence of ethanol, so further analysis was not done.

#### 3.2.3. Summary

Taken together, the results of Experiment 1 indicate that locomotor sensitization results from repeated, discrete exposures to ethanol in zebrafish. Because this sensitization was attenuated in Group 3, which was tested with ethanol in the presence of unpaired stimuli, conditioned cues may be a mediating factor. However, no conditioned hyperactivity to the ethanol-paired context was observed in Group 3, as might be predicted if the sensitization in Group 1 was due to conditioned responding to the context. Additionally, although animals in Group 3 exhibited ethanol-induced hyperactivity of a similar magnitude to animals in Group 2 (about 1.5 cm/s above no-ethanol response levels), their overall activity levels were somewhat lower. Even though there were no significant differences in baseline activity between the groups, Experiment 2 was designed to ask the same question using a more sensitive within-subjects design, in order to eliminate any possible effects of differing baseline activity.

### 3.3. Experiment 2

The results from the between-subjects comparisons in Experiment 1 indicate that zebrafish exhibit ethanol-induced locomotor sensitization which is context-specific. Because this suggests a role of conditioning in the sensitization effect, it is then surprising that Group 3 did not show any conditioned changes in activity to the ethanol-paired context. One possibility is that the between-subjects design is not sensitive enough to detect such an effect, with the relatively high degree of individual variability in locomotor activity. Therefore, Experiment 2 was designed to test for conditioned effects using a within-subjects test. Animals in Experiment 2 were trained identically to those in Groups 1 and 3 of the

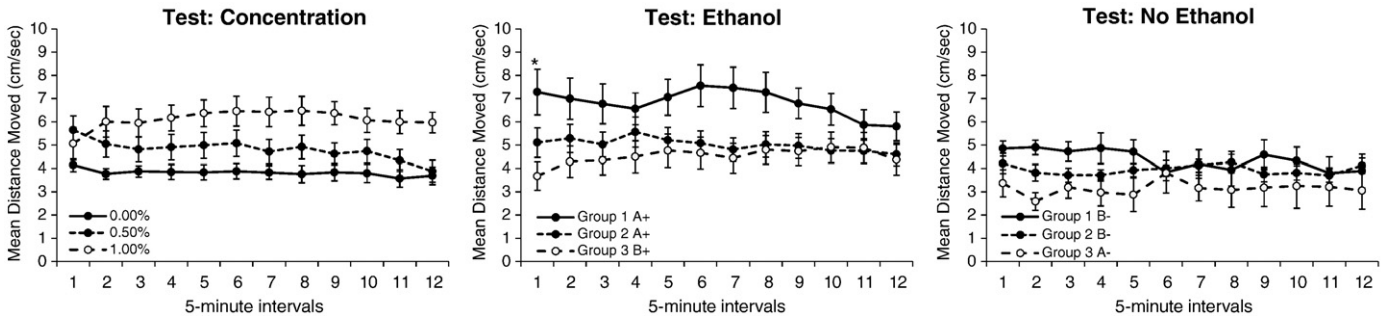


**Fig. 3.** Illustrated in Fig. 3 are the results from the two tests in Experiment 1. In the left panel, locomotor activity at each concentration of ethanol is plotted as a function of the twelve 5-min intervals of the test. Ethanol-induced hyperactivity is apparent in the test. In the center panel, the results of the ethanol test are illustrated for each of the three groups. More ethanol-induced hyperactivity can be observed in Group 1 than in Groups 2 and 3 (main effect of Group, $p<0.05$). In the right panel, the results of the no-ethanol test are illustrated for each of the three groups. There is little difference between the groups in the absence of ethanol.

first experiment (A+ and B− training). One tank (either black or white) was consistently paired with 1.0% ethanol, while the second tank was paired with plain water. The first group of animals received two tests with ethanol, one in the tank previously paired with ethanol and one in the unpaired tank (A+ vs B+), effectively collapsing the ethanol test comparison of Groups 1 and 3 in Experiment 1 into a single group. The other group received two tests with water, one in the tank previously paired with ethanol, and one in the unpaired tank (A− vs B−), effectively collapsing the no-ethanol test comparison of Groups 1 and 3 in Experiment 1 into a single group. The first group, therefore, provides a within-subjects test of ethanol-induced hyperactivity in ethanol-paired and unpaired contexts (context-dependent sensitization), while the second group provides a within-subjects test of conditioned responding to an ethanol-paired context in the absence of alcohol.

## 4. Methods

### 4.1. Subjects

Subjects were 16 adult zebrafish of heterogeneous wild-type phenotype, purchased from a local pet store (Aquatic Warehouse, San Diego, CA). Subjects were maintained under conditions identical to those described for Experiment 1. The procedure of Experiment 2 was approved by the University of San Diego IACUC.

### 4.2. Apparatus

Stimulus tanks and the video-tracking apparatus were identical to those used in Experiment 1.

### 4.3. Procedure

Subjects were randomly divided into two groups of 8 animals each, Group 1 and Group 2. Because the results in Experiment 1 suggested a more robust hyperactivity and sensitization response at the 1.0% dose, the current experiment did not subdivide the animals by dose. All animals received 1.0% ethanol in training.

Each fish was observed individually in eight training trials and two tests. Following a 30-min habituation session on Day 1, each fish received a single 1-h trial each day for eight days (Days 2–9), and a single 1-h test on each of Days 10 and 11. With the exception of the tests, ethanol exposure, habituation, and training were all identical to Groups 1 and 3 in Experiment 1 (see Fig. 1 for an overview of the experimental design).

### 4.4. Trials

Briefly, all subjects received four 1-h trials in a black tank, and four 1-h trials in a white tank, one trial each day for eight days. For both groups, one of the tanks contained 1.0% ethanol (A+), while the other tank contained water (B−). Colors assigned to A and B, and trial sequence, were again counterbalanced across subjects.

### 4.5. Tests

All subjects received two 1-h tests, one in a black tank and one in a white tank. Subjects in Group 1 received 1.0% ethanol in both tests, while subjects in Group 2 received only water in both tests. Test order was counterbalanced across subjects (some receiving the Ethanol-paired tank first, and some the unpaired tank first).

## 5. Results

### 5.1. Trial results

As can be seen in Fig. 4 (left panel), ethanol exposure produced hyperactivity during training. A four-way 2×4×2×12 (presence/absence of Ethanol×Trial×Group×5-min Interval) Repeated Measures ANOVA indicated significant main effects of Ethanol (presence vs. absence: ($F(1, 14)=19.72$, $p<0.05$)) of Interval ($F(11, 154)=6.08$, $p<0.05$), and an Ethanol×Interval interaction ($F(11, 154)=4.21$, $p<0.05$); once again, activity decreased slightly across intervals when ethanol was not present, but increased when ethanol was present (see Fig. 4, center panel). Finally, ANOVA indicated a significant Ethanol×Trials×Group interaction ($F(3, 42)=3.64$) and a significant Trials×Interval interaction ($F(33, 463)=1.71$, $p<0.05$). As in Experiment 1, these interactions seem to be due to small changes in magnitude of the ethanol-induced response. In sum, statistical analysis indicates a replication of the results of Experiment 1. Ethanol produced hyperactivity on all four trials, the magnitude of which increased from the beginning to the end of each trial.

As in Experiment 1, separate analysis indicated no difference between overall activity in black and in white, no differences between animals receiving ethanol in black and those receiving ethanol in white, and no difference in those receiving ethanol first from those receiving water first. Once again, the two groups showed nearly identical levels of activity in the first 5 min of the trials, indicating no differential effects of handling stress on locomotor activity.

### 5.2. Test results

#### 5.2.1. Group 1 (ethanol test)

As is apparent in Fig. 4 (right panel), animals tested with ethanol exhibited considerably more activity in the tank that had been paired with ethanol (A+ test) than in the tank that had been paired with water (B+ test). This difference is largest in the beginning of the trial, and disappears around 45 minutes into the trial. Data from Group 1 were analyzed using a 2×12 (Test×5-min Interval) Repeated Measures ANOVA. Results indicate no significant main effect of Test ($F=1.8$, $p>0.05$) or of Interval ($F=1.79$, $p>0.05$), but a significant

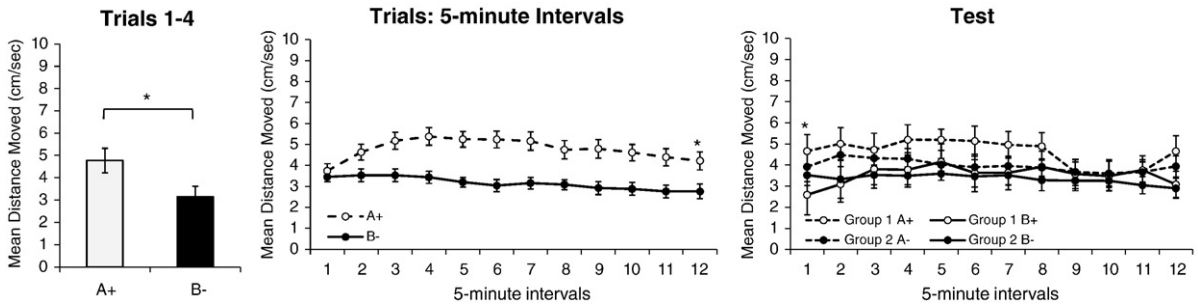


**Fig. 4.** Illustrated in Fig. 4 are the results from Experiment 2. In the left panel, locomotor activity in trials with stimulus A (1.0%) is compared to that in trials with stimulus B (0.0%). There was more activity in the presence of ethanol than in its absence (main effect of ethanol, $p<0.05$). In the center panel, activity levels are plotted as a function of the twelve 5-min intervals of the hour-long trials (Ethanol × Interval interaction, $p<0.05$). In the right panel, the results of the tests are plotted for all animals. Animals in Group 1 are more active in the A+ test than the B+ test (Interval × Test interaction, $p<0.05$). There is little difference between the A− test and the B− test in Group 2.

Test × Interval interaction ($F(11, 55) = 3.66$, $p<0.05$). These results are consistent with the large difference in activity observed early in the tests, which was reduced over the course of the hour.

*5.2.2. Group 2 (no ethanol test)*

It can be observed in Fig. 4 (right panel) that animals tested in the absence of ethanol showed a very slight increase in activity to the ethanol-paired context (A− test) relative to the unpaired context (B− test). Data were analyzed using a 2 × 12 (Test × 5-min Interval) Repeated Measures ANOVA, which indicated no significant main effects or interactions in animals tested in the absence of ethanol.

Although the test results in Experiment 2 are significant only in an interaction, they closely replicate the results of Experiment 1, using a different design. Once again, animals exhibited a reduction in ethanol-induced hyperactivity when tested in a context that had been paired with water, relative to testing in a context that predicted ethanol. This difference was once again largest in the beginning of the test, and decreased over time. And finally, animals once again exhibited no measurable change in response to the ethanol-paired context when tested in the absence of ethanol.

## 6. Discussion

The results of Experiment 1 provide the first clear evidence for locomotor sensitization following repeated ethanol exposure in zebrafish. Previous reports of tolerance to the effects of chronic exposure to ethanol in fish are based on 1–2 weeks of constant immersion, and may therefore be comparable to frequent-exposure techniques that generally produce tolerance in rodents (Dlugos and Rabin, 2003; Gerlai et al., 2006). The current results, based on spaced, discrete exposures to ethanol, are more similar to procedures that reliably produce locomotor sensitization in mice (Cunningham and Noble, 1992; Didone et al., 2008; Frye and Breese, 1981; Lessov and Phillips, 1998; Masur et al., 1986). Of course, a number of other variables differed between this and the previous fish experiments: for example, the behavioral measure (locomotor activity vs. anti-predator activity or shoaling), the cumulative time spent in ethanol, the genetic background of the fish, and the presence of discriminative cues, to name only a few. Further experimentation will be necessary to isolate the circumstances under which tolerance and sensitization are observed in zebrafish. It is, however, notable that these results appear to parallel those observed with rodents (Stewart and Badiani, 1993).

The results also provide evidence that the observed sensitization is mediated by the context in which the drug is received. In both Experiments 1 and 2, subjects exhibited a heightened hyperactivity response to ethanol in the context that had previously predicted it (A), relative to the context that had previously predicted water (B). In both cases, the difference between activity in contexts A and B was greatest in the first 5 min of the test, and decreased in magnitude over the course of the test. This is consistent with a conditioning interpretation of the ethanol-induced locomotor sensitization, since the principal conditioned effects might be expected to occur immediately upon presentation of the conditioned stimulus. It is unlikely that the early effects are due to handling stress, since no such effects appeared in the trials when stress effects should have been comparable. There are two straightforward explanations for these results, based on a simple elemental processing account of conditioning (e.g. (Rescorla and Wagner, 1972)). The first possibility is that stimulus A elicits conditioned hyperactivity, which sums with the pharmacological effects of the ethanol itself to produce sensitization. When ethanol is presented in the absence of the conditioned response, the sensitization effect is attenuated. The second possibility is that context B elicits a suppression of activity which sums with the pharmacological effects of the ethanol in the test. In this case, the presence of stimulus B in the test would cause a reduction in activity. Although the current experiment was designed simply to determine whether any effects of conditioning exist—not to unravel the nature of these effects—the trial data in Experiment 1 offer some support for the latter explanation. Those subjects receiving A+/B− training (Groups 1 and 3) were less active on negative (water) trials than those receiving A−/B− training (Group 2), suggesting that stimuli predicting the absence of ethanol may produce a suppression of activity relative to those animals that do not receive ethanol.

While there are two simple explanations for the results of the ethanol tests, neither is easily reconciled with the results of the no-ethanol tests. Both explanations predict that animals should show more activity to context A than to context B in the absence of alcohol (either through hyperactivity to A, or suppression to B), which was not found in either experiment. One difficulty with the procedure, and possibly a clue to interpretation, is the fact that the immediate perceptual properties of ethanol (chemosensory cues, tactile cues, etc.) are certainly present along with the color stimuli, immediately upon exposure to the tank, and prior to the pharmacological effects. These cues (call them X) could overshadow the color stimuli, or at least compound with them to create a distinctive context. A white tank that 'tastes' of ethanol (compound AX), for example, may be rather distinct from a white tank that does not (A alone). The results of the ethanol tests clearly indicate that something was learned about the context, so it is apparent that perceptual ethanol cues did not entirely overshadow the contextual stimuli. It is nonetheless possible that stimulus A, when presented without the perceptual ethanol cues X, suffers a generalization decrement sufficient to disrupt conditioned responding. In other words, the animals may show a robust conditioned hyperactivity response to the familiar compound stimulus AX, but no observable response to stimulus A alone.

In conclusion, the parameters used in this experiment produced locomotor sensitization to ethanol, which was context-specific. The modification of the sensitization effect by test context makes it clear that there was some associative learning to the tank color. However, the precise nature of the learning effect is not clear from these data. In

tests with ethanol, animals respond more in a tank that previously predicted ethanol than in a tank that predicted the absence of ethanol. This might be due to conditioned hyperactivity to A, or conditioned suppression of activity to B. However, the absence of any effect when A and B are compared in the absence of ethanol is difficult to reconcile, and suggests that responding to the color cues may be modified by the presence of the peripheral alcohol cues (e.g. taste or other chemosensory elements). Although this study provided the first evidence that learning influences drug-induced behavior in zebrafish, further research will be needed to delineate which of the many possible effects of learning are present. For example, future studies in which animals are trained with A(X)+ and B−, then tested in novel context C, would be useful to determine the relative roles of stimuli A and B in training. Additionally, a procedure in which the perceptual ethanol cues and contextual cues may be presented separately will be needed to determine how the presence of these cues modifies learning about other stimuli. Nonetheless, the results of the current experiments provide a framework for future studies investigating the neurobiological basis of sensitization. This may prove particularly fruitful in zebrafish given their wide use in genetic research, providing another link between the genetic, pharmacological, and behavioral pathways involved in brain disorders such as drug abuse.

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