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Short communication

Effects of nicotine and alcohol on zebrafish (*Danio rerio*) shoaling

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HIGHLIGHTS

- Zebrafish were acutely exposed to varying concentrations of alcohol and nicotine.
- Zebrafish shoaling behavior under each drug was explored using several measures.
- Both drugs of abuse disrupt shoaling, but in different ways.
- Alcohol primarily disrupts the polarization, the directional cohesion, of the group.
- Nicotine primarily disperses the group, increasing the distances between the fish.

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ABSTRACT

The zebrafish has been used in the analysis of the effects of drugs of abuse, including alcohol and nicotine. In the current study, we investigate the effects of these drugs on shoaling, group-forming behavior, in zebrafish, using a newly developed set of behavioral measures. We expose our fish acutely to 0.25, 0.50, 0.75 or 1.00% (% v/v) ethyl alcohol or 4 or 8 mg/L nicotine by immersing the fish in the corresponding solutions. The behavior of the exposed fish is compared to controls in a large (91 cm diameter) circular tank in which shoals of 8 subjects under the same treatment are allowed to swim freely. Several measures of shoaling are quantified including the nearest neighbour distance (NND), inter-individual distance (IID), swimming speed, polarization (a measure of the directional synchronization of the shoal), and the number and duration of excursions (departures from the shoal). Alcohol and nicotine were both found to exert significant effects on shoaling but impaired the behavior in different ways. For example, alcohol strongly disrupted polarization and only modestly reduced shoal cohesion, while nicotine had only a modest effect on polarization but robustly decreased shoal cohesion. Neither drug affected the number or the duration of excursions, but both reduced swimming speed. These results underscore the notion that using multiple measures of social behavior may allow one to characterize and distinguish different aspects of drug effects on behavior, which may facilitate discovery of novel drugs in drug screens and may also be utilized in the analysis of underlying mechanisms.

1. Introduction

The zebrafish (*Danio rerio*) has become a widely utilized model organism in pharmacological and toxicological research, particularly due to evidence that they may share with humans and other mammals some key receptors targeted by drugs of abuse (e.g., acetylcholine nicotinic and dopaminergic receptors [1]). It is thus feasible to suggest that the behavioral effects and mechanisms of operation of drugs such as nicotine and alcohol (ethyl alcohol or ethanol) can be usefully studied using zebrafish. A number of such studies have found that alcohol exposure affects zebrafish locomotion, aggression [2], stress [3], startle responses [4], and responses to a predator [5], all in a dose-dependent manner. Nicotine has also been shown to reduce stress in zebrafish [1,6] and improve learning (at low doses [7–9]). Other drugs, with affinities for other receptor classes, have also been found to disrupt zebrafish behavior in various ways [10–13].

Zebrafish spend the vast majority of their time in loose groups called shoals [14]. Shoaling behavior is affected by both environmental and internal conditions (such as the presence of predators or the level of hunger; [14,15]). Testing shoaling may serve as an effective assay for the effects of drugs on social preference and social behavior in general. Prior studies have demonstrated disruption of shoaling by, for example, LSD [16], MK-801 (an NMDA receptor antagonist [12]), and SKF38393 (a dopamine receptor agonist [12]). Buspirone (a 5-HT receptor antagonist) has been found to increase social preference [10]. Although promising, these pharmacological

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studies have been hampered by a lack of common behavioral and statistical methodology by which to characterize shoaling behavior. For example, several studies of the effects of alcohol on zebrafish shoaling, each using a different measure of social preference (but, interestingly, identical concentrations of alcohol), have concluded that alcohol exposure either reduces shoaling with increasing concentration [2,4], has an inverted-U-shaped dose-response effect (decreasing and then increasing shoaling; [5,17]), or has no effect at all on shoaling tendency [12].

Recently, Miller and Gerlai [18] suggested a simple experimental paradigm and a set of defined behavioral measures to characterize zebrafish shoaling. Here, we use this analysis to explore how shoaling is affected by exposure to various concentrations of nicotine or alcohol. Groups of 8 zebrafish each were exposed to either alcohol or nicotine and were subsequently permitted to swim freely in a large circular tank. From the trajectories of the fish we extract a set of measures of collective behavior (see Ref. [18] for details) to characterize in detail how shoaling behavior may change under the effects of these drugs.

2. Methods

2.1. Subjects

Adult zebrafish (5–8 months old) of the AB wild type strain were bred in-house at the University of Toronto Mississauga (UTM). All fish to be tested together were housed together in 40 L holding tanks (51 cm × 25.2 cm × 30 cm) for at least 2 weeks before the start of testing. Groups consisted of approximately equal numbers of males and females. Fish were housed in the same room where behavioural testing was carried out. The holding tanks contained "system water" that was purified through reverse osmosis and mixed with sea salt (Instant Ocean Sea Salt, Aquarium Systems Inc., OH) such that the salinity of the water was between 300–450 μS (192–288 ppm). The water was filtered using hanging power filters (Penguin biowheel 100, Marineland, OH) and sponge filters (Seapora, Worldwide Pet Products Inc., ON) and was aerated and kept at a constant temperature of 25 ± 2 °C. Subjects were placed on a 14:10 light–dark cycle for the duration of the experiment. Fish were fed ad lib. on a mixture of Spirulina and flake food (Tetramin Tropical Flakes, Tetra, USA), approximately one hour before being tested. Fish were tested between 12:00 and 3:00 p.m. each testing day. After the test, fish were placed back in the holding tanks. Each fish was tested only once.

2.2. Apparatus

Tests took place in a white circular tank with a diameter of 91 cm filled to a depth of 10 cm with system water. Two fluorescent lights were placed on opposite sides of the tank and a blackout board was used in order to prevent any glare on the surface of the water during testing. A digital HD video camera (Sonly HDR-XR520V, Sony Corporation, Japan) was mounted 250 cm above the tank (distance from camera lens to the surface of the water), which allowed for the entire tank to be visible in the video frame.

2.3. Procedure

All testing sessions lasted for 30 min and were filmed at 1920 × 1080 pixels, 12 frames per second. Before each testing session, each group was exposed to their assigned drug in an exposure tank (30.5 cm × 15 cm × 20.5 cm) that contained 7.65 L of system water mixed with the appropriate drug concentration. A black garbage bag was used to black out the sides of the exposure tank so that no outside stimuli would affect the fish during exposure. After exposure to the drug, fish were netted and placed in a beaker containing approximately 500 mL of system water. Once all of the fish were in the beaker, they were gently poured into the centre of the testing arena, and the behavioral recording session was started. At the end of each session, all the fish were gently netted back into the beaker and returned to their home tanks.

2.3.1. Experiment 1: Alcohol

In this experiment fish were exposed to one of 5 alcohol concentrations: 0% (control), 0.25%, 0.50%, 0.75% and 1.00%, a between subject experimental design. There were 8 shoals in each condition and each shoal consisted of 8 fish (a total of 320 fish, $N = 40$ shoals). The order of testing was randomized each testing day. Before each session, each shoal was exposed to the selected concentration of alcohol (anhydrous ethanol) in the exposure tank for 60 min. This exposure length was chosen to match what was employed in previous publications [2,5,17] and also because blood–brain alcohol levels were found to take at least 40 min after the fish were immersed in the solution to reach a steady plateau [4], a time course that closely matches that of changes in neurochemical levels induced by acute alcohol exposure [19]

2.3.2. Experiment 2: Nicotine

In this experiment fish were exposed to one of 3 nicotine concentrations: 0 mg/L (control), 4 mg/L, and 8 mg/L. There were 9 shoals in each condition and each shoal consisted of 8 fish (a total of 216 fish, $N = 27$ shoals). Drug concentration order was randomized each testing day. Before each session, the fish were exposed to the appropriate amount of nicotine diatartrate (Fisher Scientific) solution for 3 min. This exposure length was selected based on prior studies [1,6–9,20]. One shoal had to be removed from the analysis due to technical problems with video-recording.

In order to determine the appropriate concentrations of nicotine to use, pilot tests were carried out in which the behaviors of zebrafish exposed to various concentrations of nicotine were observed. In previous studies of learning, memory, and anxiety, zebrafish were exposed to nicotine concentrations ranging from 50 mg/L [6,8] to 800 mg/L [7], i.e., between 1 and 2 orders of magnitude greater than the concentrations we use here. In our pilot tests, we observed that such high nicotine levels (dosages above about 12 mg/L) resulted in overt behavioral anomalies (twitching, lethargy, swimming in circles, and sometimes death). Since our interest in this paper was in the subtle behavioural effects of sub-lethal doses of nicotine on shoaling behaviour, lower dosages were used. The alcohol dosages we selected corresponded to those commonly employed with zebrafish in the literature [2,5,21].

2.4. Analysis

Trajectories of all the fish in each session were extracted from the videos using custom tracking software [18]. Since it is possible that the effects of the drugs might have subsided during our 30 min session, two 5 min-long segments of each session, at 5 and 15 min after the start of the session, were quantified. We performed 2 analyses on each segment: we extracted the excursions of fish away from the shoal, a measure of shoal cohesion [22], measuring both the number of excursions performed and their mean duration. Briefly, individuals or sub-groups of size n were considered to be on an excursion when their NNDn (i.e., the distance to their nth nearest neighbor) was greater than the mode of the distribution of all NNDn (see Ref. [22] for details). Excursion data were analyzed using a two-factor repeated-measures ANOVA (with drug concentration and session time as the factors). We also constructed the density distributions of four characteristics of the shoal: the Nearest Neighbor Distance (NND: the distance between each individual and its closest neighbor), the Inter-Individual Distance (IID: the mean distance from each individual to all the other fish), mean speed (the mean of the momentary speeds of all the fish), and polarization (a measure of the uniformity of the headings of the fish). Distributions were compared using 2-way Kolmogorov–Smirnov tests. All measures were extracted from the trajectories using *Mathematica* (*v.7.0*, *Wolfram Research*). Statistical analyses were conducted using SPSS (*v.17*, *SPSS Statistics*). A confidence level of 0.01 was used for all significance tests.

3. Results

3.1. Alcohol experiment

Fig. 1 shows the number (left) and mean duration (right) of excursions away from the shoal for each of the five alcohol concentrations, both early (5 min) and late (15 min) in the session. There was no significant effect of time in the session or of alcohol concentration on either number of excursions (ANOVA, effect of minute: $F(1,35) = 4.534$, $p = 0.04$; effect of alcohol concentration: $F(4,35) = 1.074$, $p = 0.384$) or excursion duration (effect of minute: $F(1,35) = 0.023$, $p = 0.88$; effect of alcohol concentration: $F(4,35) = 1.563$, $p = 0.206$). There were no significant interaction terms (both $p > 0.35$).

Fig. 2 shows the characteristic distributions of shoals under our 5 different concentrations of alcohol. Shoals exposed to 0.5% alcohol behaved similarly to control shoals (0% alcohol) on all measures (K–S test, all $D < 0.067$, all $p > 0.125$; complete test results are given in Table S1). The NND and IID distributions of shoals under the remaining three concentrations (0.25, 0.75 and 1%) were also not significantly different from each other (all $D < 0.04$, all $p > 0.715$) but the control and 0.5% alcohol shoals were significantly different from these three (all $D > 0.102$, all $p < 0.004$). The effects of alcohol on the speed and polarization distributions were more complex, but retained the pattern whereby control shoals were most similar to the 0.5% alcohol groups.

In summary, though the concentrations of alcohol to which we exposed our shoals did not dissolve the shoal or even significantly increase individual departures from the shoal (Fig. 1), both low and high concentrations of alcohol did increase the distance

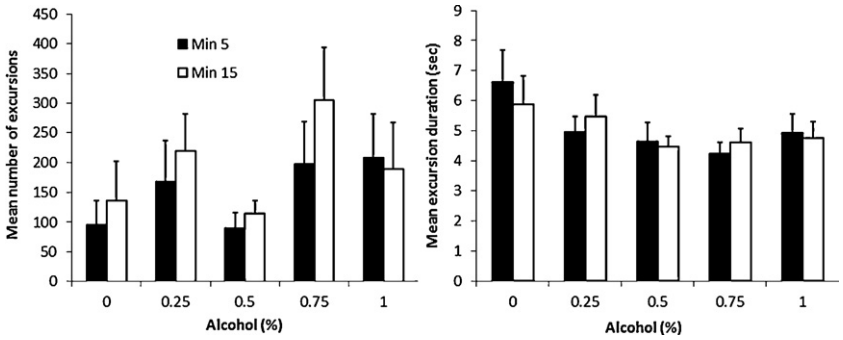

Fig. 1. The effect of alcohol on excursions. Mean number of excursions per session (left) and mean excursion duration (right) by time in the session (5 or 15 min after the start of the session) and by alcohol concentration (% v/v). Error bars represent ± SEM. None of the comparisons was significant (see main text for details).

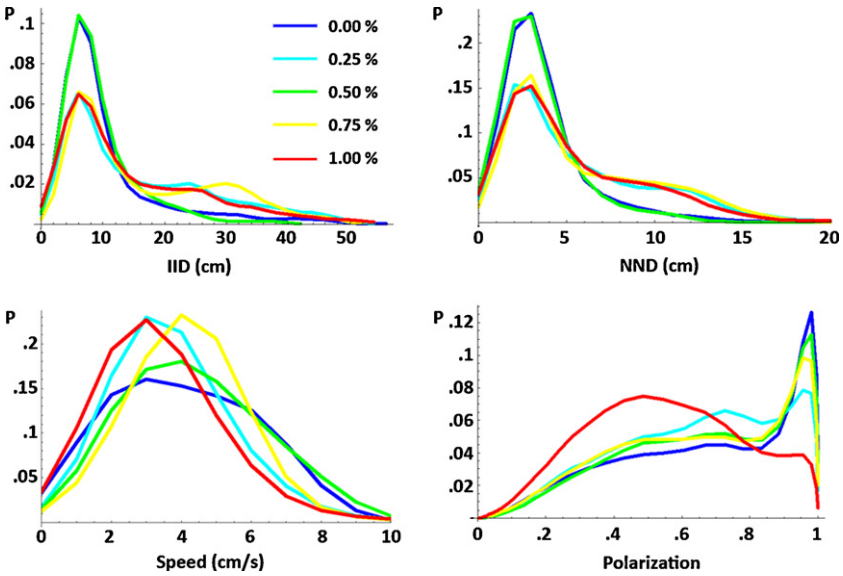

Fig. 2. Characteristic distributions of shoaling under alcohol. Density distributions of IID (top left), NND (top right), speed (bottom left), and polarization (bottom right) under five different alcohol concentrations. See text for statistical comparisons.

between shoal members and decreased their speed and polarization. Notably, the effects of alcohol appeared to follow an inverse U-shaped dose–response curve on most measures of shoaling.

3.2. Nicotine experiment

Fig. 3 shows the number and mean duration of excursions away from the shoal for each of the three nicotine concentrations, both early (5 min) and late (15 min) in the session. There was no significant effect of time in the session or of nicotine concentration on either number of excursions (effect of minute: $F(1,23) = 0.188$, $p = 0.668$; effect of nicotine: $F(2,23) = 0.068$, $p = 0.935$) or on the mean duration of excursions (effect of minute: $F(1,23) = 0.086$, $p = 0.772$; effect of nicotine: $F(2,23) = 1.933$, $p = 0.167$). There were no significant interaction terms (both $p > 0.31$).

Fig. 4 shows the characteristic distributions of shoals under our 3 different concentrations of nicotine. In this case, the control groups were significantly different from both drug groups on most measures (with one exception, 0 mg/L was not different from 8 mg/L in polarization, $D = 0.099$, $p = 0.022$; complete test results are given in

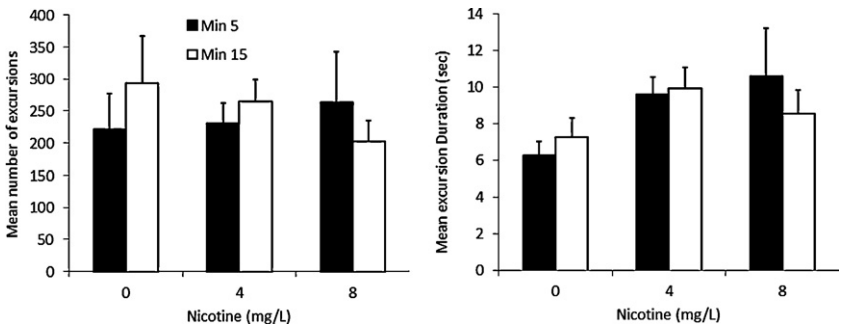

Fig. 3. The effect of nicotine on excursions. Mean number of excursions per session (left) and mean excursion duration (right) by time in the session (5 or 15 min after the start of the session) and by nicotine amount (mg/L). Error bars represent ± SEM. None of the comparisons was significant (see main text for details).

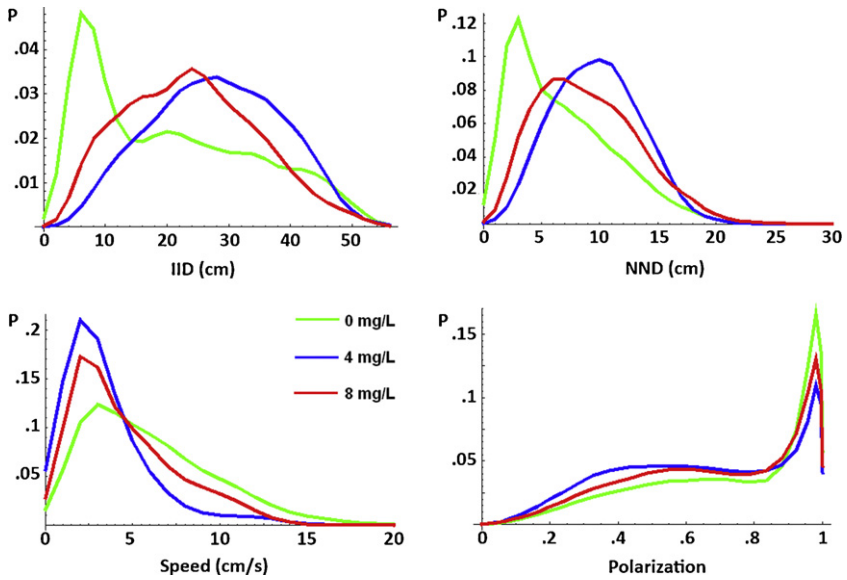

Fig. 4. Characteristic distributions of shoaling under nicotine. Density distributions of IID (top left), NND (top right), speed (bottom left), and polarization (bottom right) under three different nicotine concentrations. See text for statistical comparisons.

Table S2). The two drug concentrations were different from each other only on IID and speed (both $D > 0.117$, both $p < 0.004$).

Thus, similarly to alcohol, the concentrations of nicotine that we used did not dissolve the shoals but did increase the spacing between their members, decrease their speeds, and slightly disrupt their polarization. Sample frames from videos of each drug condition are shown in Fig. S1.

4. Discussion

This study examined the effects of ethanol on the shoaling behaviour of zebrafish and is the first of its kind to perform a similar analysis for acute nicotine exposure. Previous studies have shown that zebrafish respond to alcohol exposure with significant changes in social behaviour [2,5]. Alcohol is absorbed from the water in which the fish swim and within 20–40 min reaches the zebrafish brain [4] leading to a consistent and measurable effect on many dimensions of zebrafish behavior. Here, we show that nicotine has similarly significant, but qualitatively not identical, effects on shoaling behaviour to those of alcohol.

The effects of exposure to alcohol depended on the concentration used. Low (0.25%) and high (0.75%, 1%) concentrations both affected the polarization, speed, and internal spacing of shoals (Fig. 2). Intermediate (0.5%) doses, however, produced behavior very similar to that of the control group. These results partially replicate the pattern of effects reported by Gerlai et al. [5], who noted that the spacing between zebrafish in a shoal increased with increasing alcohol concentration. Alcohol has been shown to be anxiolytic in zebrafish [3] and this may, at least partly, explain why it disrupts shoaling. Previous studies have shown that less fearful or less stressed shoals of zebrafish are less densely spaced [15,23]. This does not, however, explain the U-shaped dose response we found. At this point, we can only speculate as to why such a U-shaped dose–response was found. Alcohol's anxiolytic effects might lead to reduced shoal cohesion at low doses. In addition, shoal cohesion could also be affected by the motor impairing effects of higher alcohol doses. Furthermore, alcohol has been shown to affect aggression in zebrafish [2] in a U-shaped dose dependent manner (intermediate doses enhance and high doses reduce the level of agonistic behaviors), which might serve to increase shoal cohesion. Whatever the behavioural mechanisms may be, it is clear that detailed quantification of behaviour is necessary to uncover complex drug effects such as those of alcohol [12].

Exposure to nicotine also affected multiple measures of shoaling. Contrary to some existing data [1], nicotine reduced the speed of swimming of zebrafish when in freely moving shoals, at both concentrations employed (Fig. 4, bottom left panel). This reduction in speed could have been due to the fact that the testing arena we used was large and circular as opposed to the small rectangular [7] or bowl-shaped [4] arenas used in previous studies. It is also possible that the lower concentrations of drug that we used caused the difference in effect. Previous studies have used concentrations 1–2 orders of magnitude greater than the current study. Similarly to alcohol, nicotine has also been shown to be anxiolytic in zebrafish [1,6], which may underlie its effects on shoaling.

Our data do not show a change in the number or duration of excursions (i.e., fish leaving the shoal) in shoals that are acutely exposed to either nicotine or alcohol. However, both drugs had noticeable effects on several of the other measures used to determine shoal cohesiveness. Thus, although neither drug caused the shoal to dissolve, both drugs significantly loosened the shoal. Interestingly, the main effects of each drug were apparent in different measures. Alcohol at both low and high (but not intermediate) concentrations severely disrupted the polarization of the shoal (Fig. 2, bottom right panel) but had only a slight effect on the distances between the fish (Fig. 2, top panels). Nicotine, on the other hand, greatly increased the distances between individuals at both concentrations (Fig. 4, top panels) whilst having a far smaller effect on the polarization of the group (Fig. 4, bottom right panel). This suggests that, even though both drugs affect shoaling, their precise mechanisms of action are sufficiently different to be detected by our behavioral measures, for instance by showing that the modes of the NND and IID distributions are changed by administration of alcohol but not nicotine (compare Figs. 2 and 4, top panels) whilst the effects of nicotine on polarization are more drastic than those of alcohol (compare Figs. 2 and 4, bottom right panels). This set of results underscores the notion that using a range of measures is warranted when attempting to characterize the effects of drugs on brain function and behavior. Our data also suggest that further work on the precise mechanisms by which alcohol and nicotine affect social behavior is in order. Detailed behavioral characterization

will be particularly powerful when combined with selective drugs that only bind a specific, or a small number of, receptor subtypes (e.g. [10]) thereby allowing a detailed mechanistic dissection of the complex effects of such drugs of abuse as alcohol and nicotine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2012.11.033.

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