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Dose dependent behavioral effects of acute alcohol administration in zebrafish fry

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ABSTRACT

The zebrafish is becoming increasingly utilized in behavioral neuroscience as it appears to strike a good compromise between practical simplicity and system complexity. Particularly in alcohol (ethanol) research, the zebrafish has been employed as a translationally relevant model organism. However, the majority of studies investigating the effects of alcohol on brain function and behavior has used adult zebrafish. In the current study, we utilize 6–8 post-fertilization day old larval zebrafish (fry) to investigate the effects of a 40 min-long, acute, immersion into the alcohol bath. We measure the behavioral responses of the fry during the immersion session in relatively large arenas, the petri dish, instead of the often employed 96 well plate, and report on significant modification of behavior induced by alcohol. For example, we found the intermediate dose of alcohol (0.5%, vol/vol) to exert a stimulant effect manifesting as slight elevation of swim speed, robust increase of turning, temporal variability of swim speed and turning, and diminished frequency of staying immobile. We also found the high dose of 1% alcohol to elicit an opposite response, a sedative effect. This biphasic dose response of alcohol mimics what has been found in mammals, including humans, and thus we conclude that a few day-old zebrafish fry may be a cost effective and efficient tool with which one can screen for small molecules or mutations with alcohol-effect modifying properties.

1. Introduction

Despite alcohol's known deleterious effects, its use and abuse continue to be a world-wide issue leading to major medical problems and substantial costs to society. The broad range of diseases resulting from alcohol consumption includes fetal alcohol spectrum disorders, cancer, cardiovascular diseases, liver diseases and a variety of psychological and psychiatric conditions in adults (May et al., 2009). The costs of these disorders to society combined with the loss of productivity are staggering (Bakoyiannis et al., 2014; May et al., 2009; Stade et al., 2009). Despite the prevalence and pervasiveness of alcohol abuse in the modern world, the underlying mechanisms mediating alcohol's deleterious effects still remain unclear due to the drug's complex pharmacology. For example, we still do not fully understand how alcohol exposure affects the development and functioning of the vertebrate brain.

Most animal models developed to address the above questions utilized rodents (Keane and Leonard, 1989), including the rat (Bell et al., 2017; Heyman, 2000) and the house mouse (Crabbe, 1989; Bennett et al., 2006), but the fruit fly has also been successfully employed (Devineni and Heberlein, 2013). The zebrafish has also been proposed for the analysis of alcohol effects on the brain (Gerlai et al., 2000; Tran and Gerlai, 2014; Tran et al., 2016a). This latter species is increasingly utilized in high throughput pharmacological assays due to the ease with which drugs, including alcohol, may be administered via immersing the fish into the drug solution. The advantage of its small size and relative ease of maintenance also makes the zebrafish an optimal compromise between systems complexity and practical simplicity. At 5 days post-fertilization, the zebrafish fry already possess major anatomical, physiological and some behavioral features typical of adults. Furthermore, for the first few days of its development, the zebrafish remains almost completely transparent, allowing observation of morphological changes under the microscope. The zebrafish is also excellent as a high-throughput screening tool. For example, randomly induced mutations, as well as compounds affecting numerous target phenotypes can be identified with phenotypical screens (Amsterdam and Hopkins, 2006; MacRae and Peterson, 2015). Last, the zebrafish has become one of the

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favoured study species of geneticists. Excellent genomic resources critical for mapping and cloning mutations are available for this species (Wolman and Granato, 2012). In summary, the zebrafish is becoming an excellent choice for neurobehavioral genetic research in general and may be a promising tool for alcohol research in particular.

The past 15 years have seen rapid development in the characterization of the behavioral repertoire of the zebrafish. Numerous sophisticated behavioral paradigms have been developed, mainly for adult zebrafish, including characterization of their swim path, motor patterns (Blaser and Gerlai, 2006), shoaling behavior (Miller and Gerlai, 2007), fear and anxiety responses (Gerlai, 2010) as well as simple (Sison and Gerlai, 2010) and complex spatial associative learning tasks (Karnik and Gerlai, 2012), to mention but a few tests. The behavioral repertoire of zebrafish fry has also started to be explored, and, for example, escape and avoidance behaviors (Colwill and Creton, 2011) and various forms of learning and memory (Gerlai, 2016) have been tested. Nevertheless, the number of studies employing behavioral analysis with zebrafish is still orders of magnitude smaller compared to the literature on laboratory rodents (Stewart et al., 2012; Wolman and Granato, 2012).

Given the sophisticated recombinant DNA tools, neurobiology methods and the rapidly developing behavioral tests, the zebrafish has also started to be utilized in alcohol research (Gerlai et al., 2000; Seguin and Gerlai, 2018; Tran et al., 2016a). Unlike in developmental biology that focussed on zebrafish of 0 to 5 days post-fertilization age, the first alcohol studies investigated the adult (Echevarria et al., 2011; Gerlai et al., 2000). The effects of both acute and chronic (Gerlai et al., 2006; Mathur and Guo, 2011; Tran et al., 2015) alcohol administration have been investigated, and numerous behavioral and neurobiological (including gene expression related) functional modifications have been identified (Araujo-Silva et al., 2018; Bartolini et al., 2015; Chatterjee et al., 2014; Lockwood et al., 2004; Pan et al., 2012; Tsang et al., 2018). Although less frequently than adults, larval zebrafish, i.e. zebrafish fry of a few days of age, have also been employed to test the effects of acute alcohol administration. However, the results of these studies were not always consistent and showed a complex interaction between alcohol, genotype and the manner in which behavior was measured.

For example, Lockwood et al. (2004) compared zebrafish of the AB and WIK strains and found that acute (20 min long) exposure to 1.5% (vol/vol) alcohol had a stimulant whereas exposure to 3% alcohol a depressant effect on larval (7 dpf old) zebrafish, effects that were genotype dependent with WIK being less responsive to the intermediate alcohol dose than AB. de Esch et al. (2012) observed a biphasic effect of alcohol with doses 0.5% to 2% increasing and a higher dose (4%) of alcohol decreasing responsivity to a sudden change in illumination in the 5–7 dpf old zebrafish larvae, as measured in the 96 well plate. Irons et al. (2010) also used light stimulation, alternating light/dark periods, but these authors found that 2% alcohol increased overall locomotor activity, 4% alcohol practically eliminated locomotor activity, while 1% alcohol only had a modest and often non-significant effect. Last, Ingebretson and Masino (2013) found 1% (vol/vol) alcohol to increase the number of swim episodes as well as the variability of movement in 7 dpf zebrafish larvae, but these authors also showed that the physical dimensions of the well in which the behavioral effects were tested make a significant difference on the performance of the zebrafish fry.

The current study is aimed to extend this small but growing body of literature by analysing the effects of acute alcohol administration on behavioral responses of zebrafish fry. We decided to analyze several parameters of the swim path of free-swimming zebrafish larvae using an automated video tracking system. Instead of the industry standard, the 96 well plate, employed for zebrafish fry (Farrell et al., 2011; Padilla et al., 2011; Selderslaghs et al., 2009), however, we opted for utilizing a larger arena, a 3.5 cm diameter petri dish. We argue that the larger open space allows one to observe and quantify a broader spectrum of behavioral responses in a manner that is less prone to artefacts and artificial space-constraint induced stress, a latter of which may be a critical issue for the 96 well plate (Ekker & Clark personal communication, also see Ingebretson and Masino, 2013). Furthermore, we decided to assess the behavior of single zebrafish at a time, unlike in Lockwood et al. (2004) for two reasons. One, technical limitations with most 2D tracking systems do not allow one to distinguish multiple subjects moving freely in the same arena, leading to erroneous behavior quantification. Two, although shoaling (group forming) behavior has been shown to become detectable only after 14 days post fertilization (dpf) in zebrafish, fish younger than this age placed together in the same arena may still influence behavioral responses of each other (Buske and Gerlai, 2011). Last, we decided not to employ light/dark or any other stimulation, because we were not interested in the potential effect of alcohol on perceptual mechanisms. Instead, we allowed the fish to swim freely and undisturbed in the novel open arena, a situation known to induce mild anxiety in juvenile (Colwill and Creton, 2011) as well as adult zebrafish (Stewart et al., 2012). Our main goal was to show that acute alcohol administration alters the behavior in as young as 7th dpf zebrafish and that these changes can be quantified by measuring the responses of single fish, a finding that would significantly increase the efficiency of zebrafish in the identification of efficacious drugs that modify or interact with alcohol's effects.

2. Materials and methods

2.1. Animals and housing

Seventy-four zebrafish fry (larvae) were used for behavioral testing. The fish were 7 ± 1 dpf old at the time of the behavioral test, an age variation that was found not to have any significant effect on behavior and on the effect of alcohol on behavior. Adult wildtype zebrafish of mixed sexes were obtained from a local pet store (PetSmart, Mississauga, Ontario, Canada), and were kept in our dedicated zebrafish facility (University of Toronto, Mississauga) for 6 months in 40 L housing tanks (50 cm × 30 cm × 25 cm) with chemical, biological, and mechanical filtration. We decided to utilize this genetically undefined population of fish because at the origin of this population (Singapore breeding facility) a large number of zebrafish are bred. As a result, genetic variability across individuals and high heterozygosity within each individual are expected, making this population more “prototypical” of the species and less unique than genetically well-defined and more inbred standard zebrafish strains. Water chemistry was checked daily, and parameters including conductivity (300 micro-Siemens), pH (6.5–7.5), temperature (26–28 °C), nitrate (0 ppm), nitrite (0 ppm), and ammonia (0 ppm) were maintained at optimal levels as required for zebrafish. The adults were kept on a 12-hour photoperiod via the Wattstopper lighting program (Legrand North America) that mimicked natural sunrise and sunset, with sunrise occurring between 06:30 to 07:00, and sunset between 20:30 to 21:00. The adults were fed a mixture of flake food (1:1 ratio of Tetramin and Omega One Tropical fish flakes) and were set up for breeding after 6 months in our facility.

200 zebrafish embryos were collected and kept in a 40 L tank with the water level lowered to 10 cm in height to increase oxygenation and survivability. Water parameters were kept identical to parental conditions. Zebrafish fry reached free swimming stage at their age of 5 days post-fertilization and were started to be fed API 100 (Zeigler®, Gardners, PA) fry food at that age.

2.2. Behavioral testing and alcohol exposure

7 ± 1 dpf-old zebrafish fry were used for behavioral testing. Premixed stock alcohol concentrations of 0%, 0.5% and 1% (v/v) in fish home water was made in advance. Although the highest dose (1%) of alcohol we employed is less than what others have used (e.g. 3, 4% alcohol), we decided to use this dose range as in our hand, and with this genetically heterogenous population, 1% alcohol induces robust effects both in larvae (unpublished) and in adults (e.g. Tran and Gerlai, 2013).

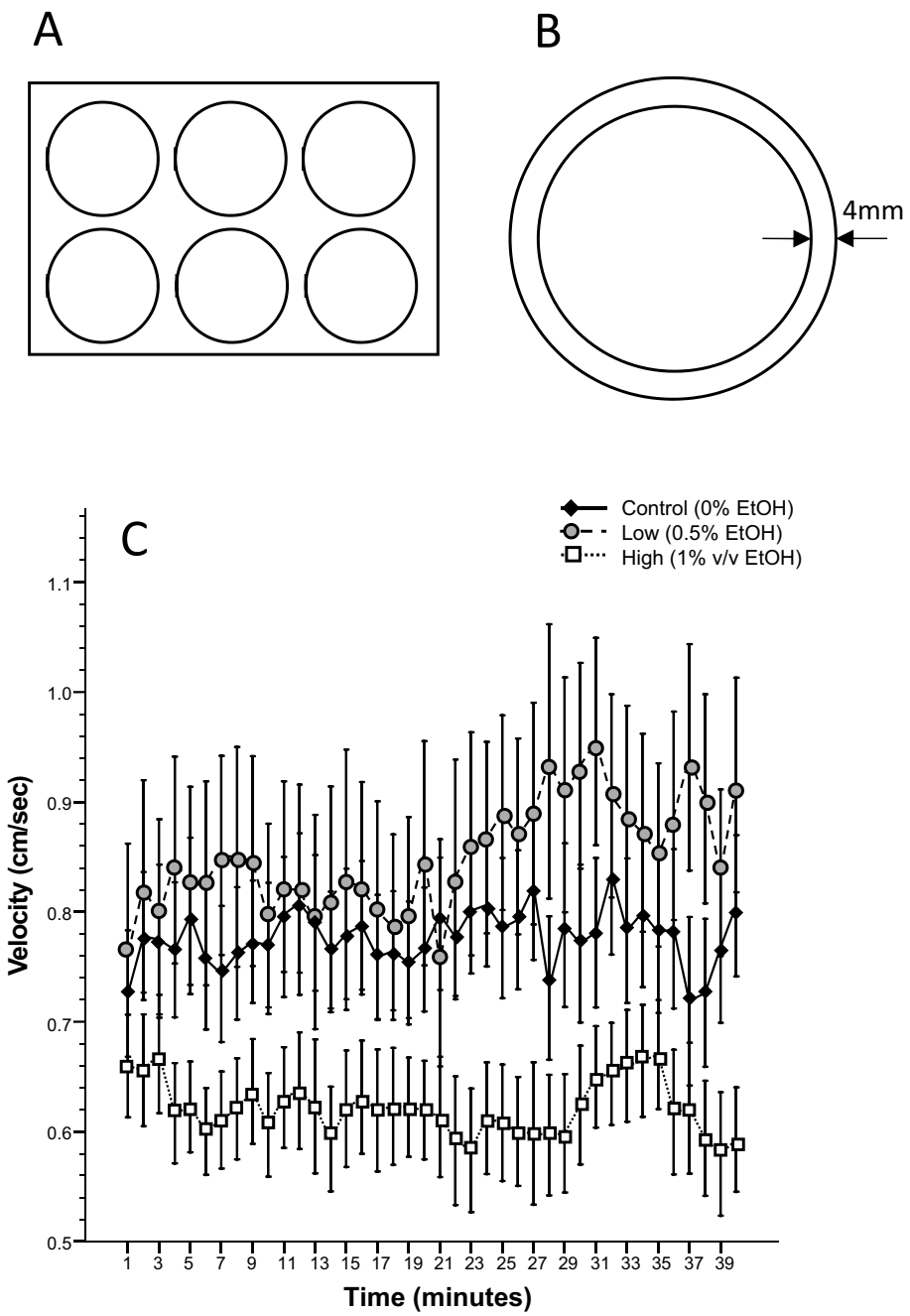

Fig. 1. The experimental set up consisted of 6 petri dishes (35 mm diameter) monitored by an overhead camera (panel A). Two zones were distinguished in the petri dish, the center and the thigmotactic area, the 4 mm thick perimeter annulus (panel B). Panel C shows Velocity (mean ± SEM) as a function of time (1-min intervals). Note the robust difference between the low (0.5%, vol/vol) and high (1.0%, vol/vol) alcohol groups. Also note that performance of control fish (receiving no alcohol) is in between that of the high and low alcohol concentration groups, indicating a biphasic dose response to acute alcohol exposure.

Zebrafish fry were singly pipetted into a single Petridish (3.5 cm diameter), each containing the corresponding concentration of alcohol. Each fish was tested with a single alcohol concentration, i.e. only once, a between subject experimental design. Experimental fish were kept in their immersion bath for 40-min, the immersion period which also served as the recording session. Six petri dishes were run at a time (Fig. 1A). These petri dishes were dimly illuminated from below using a Picker Light table to reduce glare and enhance video-tracking precision. The level of illumination measured from above was 1800 lx (iPhone 7, Lux Light Meter Pro v2 developed by Elena Polyanskaya). The behavior of the fish was recoded with a JVC GZ-MG330HV video camera placed 30 cm above the petri dish. The recordings were later replayed and analyzed (30 frames per *sec*) using the Ethovision XT 13 (Noldus Info Tech., Wageningen, The Netherlands) behavioral tracking program. Occasionally, i.e. in < 2% of recordings, we found tracking errors. These erroneous tracks were excluded from the analysis leading to small variation in the sample size (and the degrees of freedom reported in the Results section). The following behavioral parameters were quantified: velocity (swimming speed, cm/s), absolute angular velocity (the speed of turning irrespective of direction, °/s), duration of complete immobility (or freezing duration, sec, defined as the period during which < 20% of the pixels corresponding to the total visible surface area of the animal changed from one video-frame to the next), thigmotaxis (wall hugging, duration of time in sec the fish spent within

4 mm from the wall of the petri dish, Fig. 1B). In addition to the mean of the above behavioral measures, we also quantified the intra-individual variance (within-individual temporal variance, or inconsistency of performance) and/or the frequency of the above behaviors (number of times the behavior occurred) as described in the results section.

2.3. Statistical analysis

Statistical analyses were conducted using SPSS (version 24) written for the PC. We performed repeated measures Variance Analyses (ANOVAs) to test the effect of time interval (repeated measure factor with 40 levels, 1 min intervals), and alcohol concentration (the between subject factor with three levels, control and two alcohol concentrations). Because post hoc multiple comparison tests are not appropriate for repeated measures, when the results of ANOVA warranted, we compared the three alcohol groups during the first 3 min of the recording session and also during the last 3 min of the recording session. That is, we averaged the one-min interval data for the corresponding three minute periods and conducted a univariate one way ANOVA followed by post hoc Fisher's Least Significant Difference (LSD) multiple comparison test as warranted.

3. Results

Velocity (swimming speed) appeared to be affected by alcohol treatment, with the fish exposed to the highest (1%) alcohol concentration showing a robust reduction of activity and fish exposed to the intermediate dose (0.5%) showing a modest elevation of activity especially by the end of the 40 min long recording/exposure session (Fig. 1C). Repeated measures ANOVA found no significant time interval effect ($F(39, 2769) = 1.072$, $p > 0.35$), no significant time interval x alcohol concentration interaction ($F(78, 2769) = 1.155$, $p > 0.15$), but did detect a significant alcohol effect ($F(1, 71) = 3.188$, $p < 0.05$). The lack of significant time interval x alcohol concentration interaction effect found contradicts what is apparent on Fig. 1. Given that ANOVA is known to be insensitive to detect significance of interaction terms (Wahlsten, 1990), and given the relatively small p value obtained for this ANOVA term, we conducted a follow up analysis. We compared performance of fish of the three dose groups during the first three and during the last 3 min of the recording session. We argued that during the first 3 min alcohol is just starting to diffuse into the fish and thus may not have reached high levels in the brain, whereas by the end of the recording/exposure session it should be in the brain at maximum level (e.g. Tran and Gerlai, 2013). This analysis found no significant differences among the three alcohol concentration groups during the first 3 min of the session (ANOVA $F(2, 71) = 0.934$, $p > 0.35$), but did detect a significant alcohol concentration effect during the last 3 min of the session (ANOVA $F(2, 71) = 4.844$, $p = 0.011$). Subsequent multiple comparison analysis (LSD) showed that the 0.5% and 1.0% alcohol treated fish significantly differed from each other ($p < 0.01$), the difference between control (0% alcohol) and 1% alcohol treated fish approached but did not reach significance ($p = 0.086$) and the control and 0.05% alcohol treated fish did not significantly differ ($p > 0.05$).

Intra-individual temporal variance of velocity (a measure of how inconsistently fish swam with regard to speed) showed a robust difference among the three alcohol concentration groups (Fig. 2) with fish of the 1% alcohol group swimming less variably, while fish of the 0.5% alcohol group swimming more variably than control, especially during the last few minutes of the session. Repeated measures ANOVA confirmed these observations and found a significant time interval effect ($F(39, 2730) = 1.772$, $p < 0.01$), significant time interval x alcohol interaction ($F(78, 2730) = 1.781$, $p < 0.05$) as well as a significant alcohol concentration effect ($F(2, 70) = 7.061$, $p < 0.01$). Subsequently, as for velocity, we also analyzed potential differences among alcohol concentration groups during the first and during the last 3 min of the session. This analysis found the alcohol effect during the first 3 min not to reach significance (ANOVA $F(2, 70) = 2.981$, $p = 0.057$), whereas found the alcohol effect during the last 3 min highly significant (ANOVA $F(2, 70) = 8.093$, $p < 0.001$). Subsequent post-hoc multiple comparison test (LSD) revealed that the fish of the 1% alcohol group significantly ($p < 0.05$) differed from both the control and the 0.5% alcohol treated fish, and the latter also significantly ($p < 0.05$) differed from control.

Angular velocity also appeared to be robustly affected by acute alcohol administration. Fig. 3 shows that while fish of the 1% alcohol group exhibit a modest reduction of angular velocity compared to control, fish of the 0.5% alcohol group exhibit a dramatic increase but only after the first 14 min of the recording/exposure session. ANOVA confirmed these observations and found a significant time interval effect ($F(39, 2769) = 4.972$, $p < 0.001$), time interval × alcohol concentration interaction ($F(78, 2769) = 3.204$, $p < 0.001$) and also a significant alcohol concentration effect ($F(2, 71) = 10.328$, $p < 0.001$). Subsequent analysis comparing the three alcohol concentration groups during the first and last 3 min of the observation session showed that during the first 3 min alcohol had no significant effect ($F(2, 71) = 0.979$, $p > 0.35$), but during the last 3 min it had a highly significant effect ($F(2, 71) = 14.680$, $p < 0.001$). Post hoc multiple comparison (LSD) of the three alcohol groups during this latter period showed that fish of the 0.5% alcohol group differed significantly ($p < 0.05$) from both the control and the 1% alcohol exposed fish, but the latter two did not significantly ($p > 0.05$) differ from each other.

The intra-individual, temporal, variance of angular velocity (Fig. 4) also showed alcohol having an effect, but the pattern of results appeared slightly different compared to that of angular velocity itself. ANOVA found a significant time interval effect ($F(39, 2652) = 1.942$, $p < 0.001$), but the time interval x alcohol concentration interaction was found non-significant ($F(78, 2652) = 1.067$, $p > 0.30$). The effect of alcohol, however, was found highly significant ($F(2, 68) = 8.508$, $p < 0.001$). Analysis of behavioral performance during the first (ANOVA $F(2, 68) = 4.400$, $p < 0.05$) and last 3 min (ANOVA $F(2, 68) = 7.699$, $p < 0.001$) of the session showed that fish of the three alcohol groups significantly differed from each other during both periods. However, during the first 3 min this effect was attributable to fish of the 1% group being significantly ($p < 0.05$, LSD multiple comparison test) different from both the control and the 0.5% alcohol exposed fish, whereas during the last 3 min fish of the 0.5% alcohol group were found to significantly ($p < 0.05$, LSD multiple comparison test) differ from both the control and the 1% alcohol exposed fish, while other group differences were non-significant ($p > 0.05$).

Fish of all three alcohol concentration groups stayed immobile for only a short period of time (Fig. 5). This figure also suggest that fish immersed in 0.5% alcohol remained immobile longer, especially during the middle of the recording/exposure session. However, this observation was not supported by the results of statistical analysis, as ANOVA found no significant time interval effect ($F(39, 2730) = 0.957$, $p > 0.50$) or time interval × alcohol concentration interaction ($F(78, 2730) = 1.222$, $p > 0.05$). The apparent alcohol concentration related difference among the groups also did not reach the level of significance ($F(2, 70) = 2.791$, $p = 0.068$). Given the short duration of time of immobility observed and the above statistical results, we did not analyze the performance of the fish during the first and last 3 min of the session separately as we did for the other behaviors, and we also did not quantify the intra-individual variance of this behavior. Instead, we measured and analyzed the frequency, i.e., the number of times the fish stayed immobile.

The number of times fish became immobile appeared to robustly differ among the fish of the three alcohol concentration groups (Fig. 6). ANOVA confirmed this observation and found a significant time interval effect ($F(39, 2769) = 1.451$, $p < 0.05$), a significant time interval x alcohol concentration interaction ($F(78, 2769) = 1.359$, $p < 0.05$) and a highly significant alcohol concentration effect ($F(2, 71) = 8.032$, $p < 0.001$). Analysis of the frequency of immobility

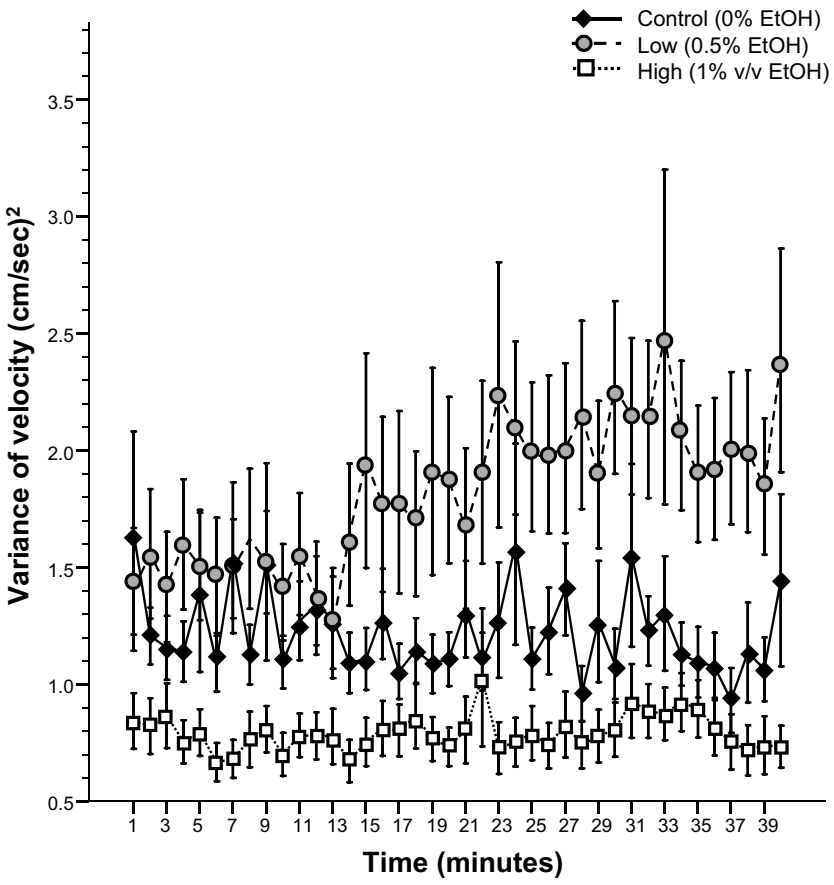

Fig. 2. Variance of velocity (mean ± SEM) as a function of time (1-min intervals). Note that this variance represents intra-individual variability, a measure of inconsistency of behavior of the individual. Observe the robust difference between the low (0.5%, vol/vol) and high (1.0%, vol/vol) alcohol groups. Also note that performance of control fish (receiving no alcohol) is in between that of the high and low alcohol concentration groups, indicating a biphasic dose response to acute alcohol exposure.

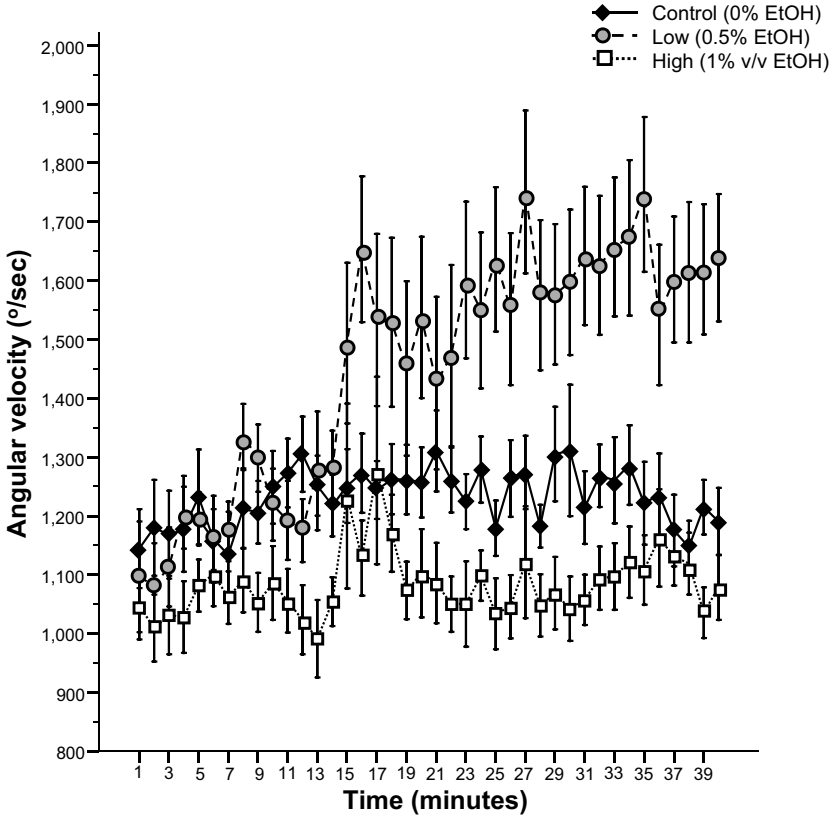

Fig. 3. Angular velocity (mean ± SEM), or speed of turning, as a function of time (1-min intervals). Note the robust difference between the low (0.5%, vol/vol) and high (1.0%, vol/vol) alcohol groups arising by the second half of the recording session. Also note that performance of control fish (receiving no alcohol) is in between that of the high and low alcohol concentration groups, indicating a biphasic dose response to acute alcohol exposure.

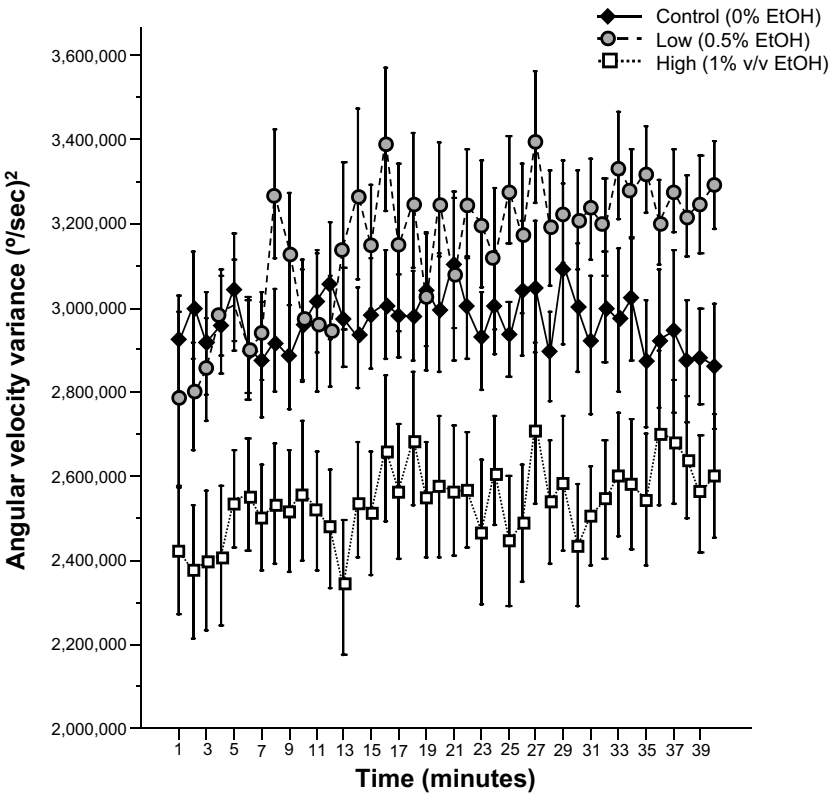

Fig. 4. Variance of angular velocity (mean ± SEM), variability of speed of turning, as a function of time (1-min intervals). Note that this variance represents intra-individual variability, a measure of inconsistency of behavior of the individual. Observe the difference between the low (0.5%, vol/vol) and high (1.0%, vol/vol) alcohol groups. Also note that performance of control fish (receiving no alcohol) is in between that of the high and low alcohol concentration groups, indicating a biphasic dose response to acute alcohol exposure.

during the first 3 min of the session showed a significant alcohol effect (ANOVA $F(2, 71) = 6.049$, $p < 0.01$) due to fish of the 1% alcohol group significantly differing from control and 0.5% alcohol exposed fish ($p < 0.05$, LSD post hoc multiple comparison test). A significant alcohol effect was also found for the last 3 min of the session (ANOVA $F(2, 71) = 3.435$, $p < 0.05$) a result that is explained by fish of the 1%

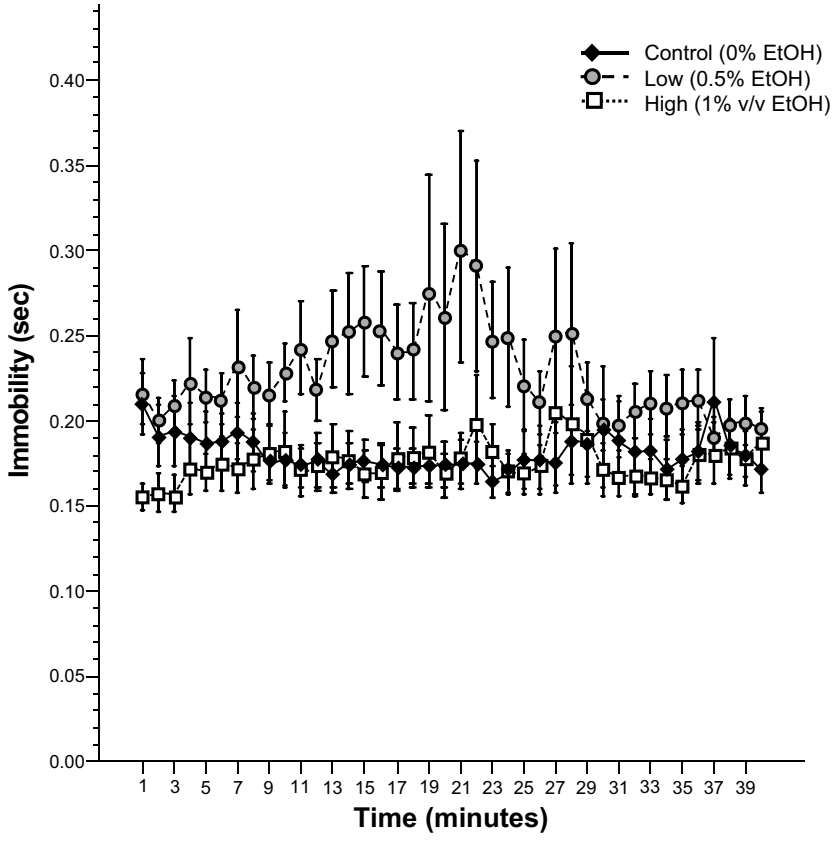

Fig. 5. Duration of immobility (mean ± SEM) as a function of time (1-min intervals). Note that this behavior occurred for only a short period of time. Although there is an apparent elevation of immobility induced by administration of low concentration of alcohol, the alcohol treatment groups were found not to differ significantly from each other.

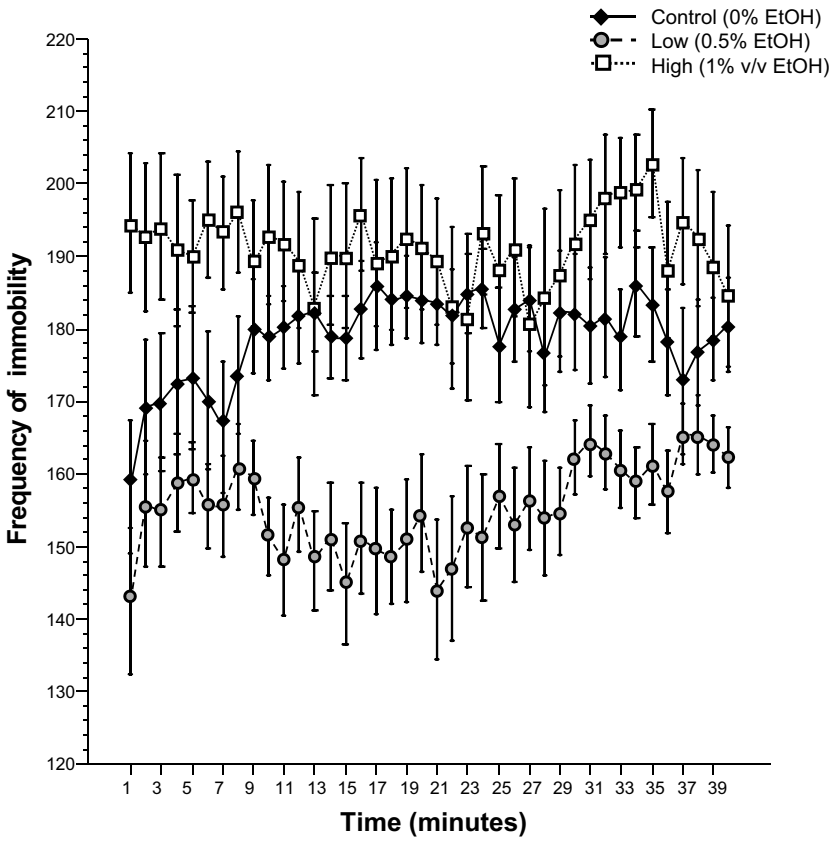

Fig. 6. Frequency of immobility (mean ± SEM), i.e. the number of times the individual stayed immobile, as a function of time (1-min intervals). Note the difference between the low (0.5%, vol/vol) and high (1.0%, vol/vol) alcohol groups and that the performance of control fish (receiving no alcohol) is in between the high and low dose alcohol treated fish.

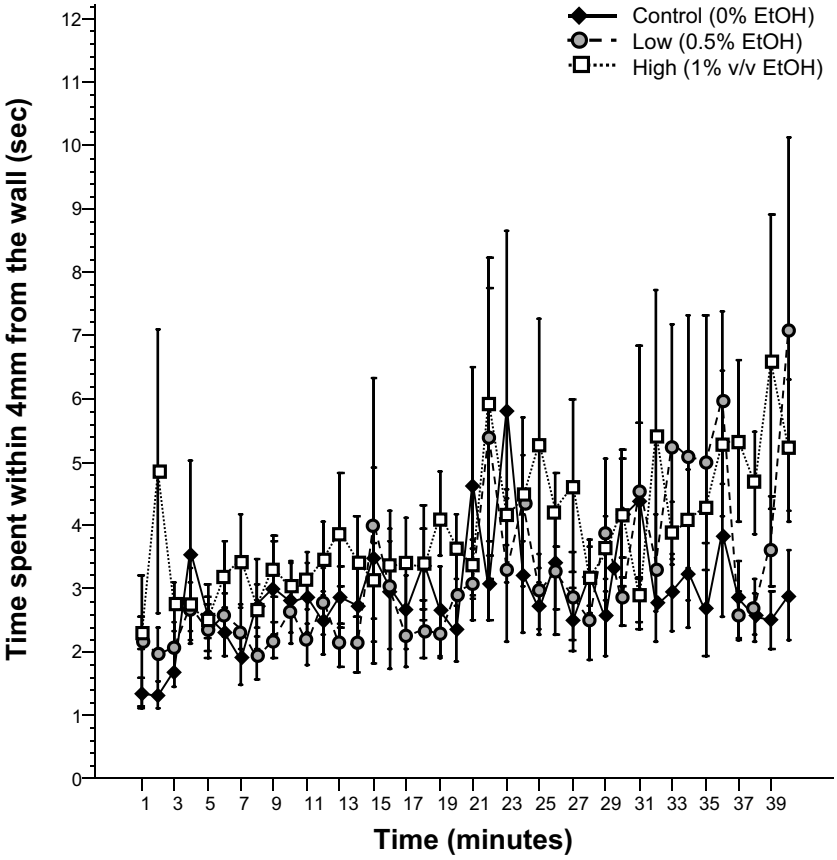

Fig. 7. Duration of time zebrafish spent in the 4 mm thick annulus near the wall of the petri dish, or thigmotaxis (mean ± SEM), as a function of time (1-min intervals). Note that alcohol treatment was found not to exert a significant effect.

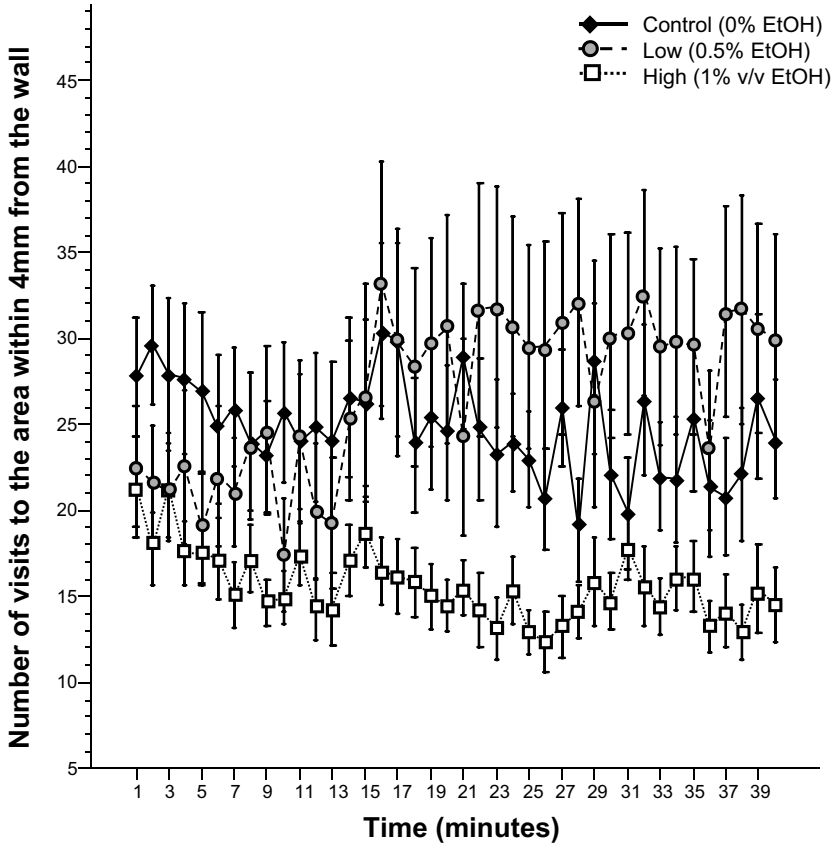

Fig. 8. Number of visits to the 1 mm annulus near the wall of the petri dish (mean ± SEM), frequency of thigmotactic responses, as a function of time (1-min intervals). Observe the difference between the low (0.5%, vol/vol) and high (1.0%, vol/vol) alcohol groups becoming apparent by the end of the recording session. Also note that performance of control fish (receiving no alcohol) is in between that of the high and low alcohol concentration groups, indicating a biphasic dose response to acute alcohol exposure.

and 0.5% alcohol groups significantly differing from each other ($p < 0.05$, post hoc LSD multiple comparison test).

The last behavior we analyzed was thigmotaxis, or wall-hugging. First, we quantified and analyzed the duration of time spent near the wall (within the 4 mm thick annulus) (Fig. 7). ANOVA found a significant time interval effect ($F(39, 2730) = 1.832$, $p < 0.01$), but the time interval x alcohol concentration interaction ($F(78, 2730) = 0.852$, $p > 0.80$), and the effect of alcohol concentration ($F(2, 70) = 1.296$, $p > 0.25$) were both non-significant. Analysis of behavioral performance during the first and last 3 min of the session also did not find significant alcohol treatment effects.

Subsequently, we investigated how many times fish entered the annulus considered being the thigmotaxis area (Fig. 8). ANOVA revealed a significant time interval effect ($F(39, 2769) = 1.476$, $p < 0.05$), and time interval x alcohol concentration interaction ($F(78, 2769) = 2.477$, $p < 0.001$). The main effect of alcohol concentration was found to border significance ($F(2, 71) = 3.070$, $p = 0.053$). Analysis of performance during the first 3 min of the session revealed no significant alcohol concentration effect (ANOVA $F(2, 71) = 2.265$, $p > 0.10$). However, alcohol was found to exert a significant effect during the last 3 min of the session (ANOVA $F(2, 71) = 3.889$, $p < 0.05$), and post hoc LSD multiple comparison test showed that the two alcohol concentration groups significantly ($p < 0.05$) differed from each other but not from control.

4. Discussion

We investigated the effect of acute administration of alcohol on the behavior of, 7 ± 1 day post-fertilization old, zebrafish fry. We found that administration of 0.5% alcohol had a stimulant effect on the zebrafish fry, as evidenced by a slight elevation of swim speed, robust and significant elevation of angular velocity, increased temporal variability of swim speed and of angular velocity, reduced frequency of immobility, and somewhat elevated frequency of visits to the wall of the petri dish. Briefly, fish exposed to 0.5% alcohol swam more irregularly and when they swam, they swam faster covering more areas of their arena, including the region near the wall. The effect of acute exposure to 1% alcohol had the opposite effect. These fish swam more slowly, less variably, and more in a straight line (reduced angular velocity).

These findings replicate those obtained with adult zebrafish. For example, numerous authors have demonstrated that alcohol increases locomotor activity in adult zebrafish at doses below 1% vol/vol (Nowicki et al., 2015; Tran et al., 2017a, 2017b). On the other hand, 1% alcohol administered acutely have been reported to reduce absolute turn angle (turning behavior), variance of distance to bottom (vertical exploration) consistent with motor-impairing effects (Tran et al., 2017a, 2017b). Similar biphasic effects with stimulant properties at low to moderate doses and sedative effects at higher doses exerting motor impairing effects of alcohol have been demonstrated in a variety of species including rodents and non-human primates (Brabant et al., 2014), as well as in humans (King et al., 2002). Our results with 7 ± 1 day-old zebrafish fry replicated these findings and thus imply that the mechanisms of acute alcohol-induced functional changes in the vertebrate brain can be tested efficiently with zebrafish. Whether the mechanisms mediating alcohol's biphasic effects in zebrafish fry are the same as in adult zebrafish or in mammals, is an empirical question that will be addressed in the future.

Although the results presented in the current study are promising, we also have to point out a few inconsistencies with them. First, the biphasic (high dose sedative, low dose stimulant) effect of alcohol we found in the current study replicates results in principle obtained with a variety of species. Nevertheless, the actual alcohol concentration at which these effects were induced is not fully consistent across all reports. For example, Guo et al. (2015) report no effect of 1% (vol/vol) ethanol on activity levels of 5–7 dpf zebrafish of the AB strain during

the light phase of the photoperiod, but found 2% alcohol to induce hyperactivity. MacPhail et al. (2009) on the other hand found acute alcohol administration to result in increased activity at 1 and 2% alcohol concentrations and severely decreased activity at 4% in 6 dpf old zebrafish of undisclosed genetic background and strain origin. Lockwood et al. (2004) found acute administration of 1.5% alcohol to have a stimulant whereas 3% alcohol to have depressant effect in 7 dpf old zebrafish of AB strain, but not in age-matched zebrafish of the WIK strain, the latter being less responsive to the stimulant effects of alcohol. On the other hand, Ali et al. (2011) report on motor depressant effects of alcohol of much lower doses when administered to zebrafish of undisclosed origin during earlier developmental stages and measured at 5 dpf age, and no effect when administered at later stages of development and measured at 5dpf. Irons et al. (2010) found 1% alcohol to have no or only modest effects on locomotor activity while 2% ethanol had stimulating and 4% ethanol depressant effects.

There may be several explanations for these discrepancies. Perhaps the most important to note among them is the strain origin, i.e. the genetic background, of the fish used. Lockwood et al. (2004) demonstrated such a strain-dependent effect of alcohol in larval zebrafish, and others have also uncovered such strain x alcohol interaction in adult zebrafish in behavior (Gerlai et al., 2008, 2009), neurochemistry (Chatterjee et al., 2014; Gerlai et al., 2009), and gene expression (Pan et al., 2012). Although well appreciated in the mouse literature, such genotype effects are often difficult to trace in zebrafish research, as several zebrafish studies do not disclose the strain origin or source of their subjects. Another likely source of variation is the physical dimensions of the test environment. A majority of experimenters using zebrafish fry, including authors of some of the above cited studies, opt for employing the 96 well plate, a convenient method that allows screening a large number of subjects efficiently. However, the 96 well plate may not be an ideal setup for behavioral analysis, as the small volume of the wells represent significant problems. The fish may experience stress (Ekker and Clark personal communication) due to space constraint, oxygen depletion, or even due to rapidly changing temperature in these wells. Ingebretson and Masino (2013) already demonstrated that the physical dimensions of the test environment significantly affect the behavior of larval zebrafish. Others have found similar results with adult zebrafish suggesting that standard small-sized housing tanks crowded with zebrafish represent a stressful environment (Shams et al., 2017). Although the discrepancies surrounding which doses of alcohol may be exerting stimulant or depressant effects on zebrafish fry remain unresolved, the similarity of alcohol's biphasic effect found across several vertebrate species suggests evolutionary conservation of some mechanisms mediating this dose-response trajectory. What these mechanisms may be, will be addressed by future empirical studies.

The complex mode of action through which alcohol alters behavior is not completely understood, but the effect of this drug is known to be mediated, e.g., by numerous processes underlying synaptic plasticity (McCool, 2011). The dose-dependent stimulant and depressant effects of alcohol likely result from a combinatorial effect of alcohol on a large number of molecular targets, including ion channels and other mechanisms affecting particular brain areas differently. A recent review of some of these neuroanatomical locale-dependent processes in mammals is provided by Harrison et al. (2017). Among the several processes altered by acute alcohol administration we only mention but a few examples, including the GABAergic system, which is implicated in alcohol's sedative effects (Naito et al., 2014), the glutamatergic system, including the NMDA-Receptor, and the dopaminergic system (Harrison et al., 2017) as well as the hypothalamic-pituitary-adrenal axis, vasopressin receptors, and opioid receptors, which are considered underlying alcohol's stimulant properties, among other psychopharmacological features of alcohol, in mammals (Zhou and Kreek, 2014).

A similar level of complexity is expected with regard to the mechanisms of acute alcohol-induced behavioral changes in zebrafish. For example, acute alcohol exposure has been found to alter the functioning of all neurotransmitter systems studied in zebrafish (e.g. Chatterjee et al., 2014; Tran et al., 2016b, 2015b). Furthermore, changes in the expression of numerous genes have also been found in response to acute alcohol in zebrafish (Rico et al., 2007; Rosemberg et al., 2010). Furthermore, acute exposure to alcohol in adult zebrafish has been shown to dose-dependently alter the activity of high and low-affinity alcohol metabolising enzymes, isoforms of alcohol dehydrogenase, while dose-independently activating aldehyde dehydrogenase in the zebrafish liver (Tran et al., 2015).

The last point we discuss concerns how generalizable our results may be. We have decided to conduct this study using a genetically undefined zebrafish population in which we expect high genetic variability among the individuals and high ratio of heterozygosity within each individual. The reason for our decision was that we were not interested in discovering idiosyncratic, i.e., strain specific features, but rather wanted to explore how “prototypical” zebrafish may respond. A common issue raised against pre-clinical studies for drug development is that the subjects, mostly mice or rats, are inbred (reduced genetic variance), but humans are not (showing high level of total phenotypical variance). As a result, drug candidates that showed efficacy in preclinical research, often fail in human clinical trials (Lowenstein and Castro, 2009). Whether the results we obtained with our current zebrafish population are indeed species typical of zebrafish, and how well they can be generalized to other species including our own, are empirical questions that will be tested in the future.

Despite the pioneering aspects of our research, the biphasic effects of alcohol shown previously in mammals and demonstrated in the current study in zebrafish fry suggest common underlying mechanisms. This assumption is likely correct given the evolutionary conservation found between zebrafish and mammals at several levels of the biological organization of these species (Gerlai, 2011a, 2011b, 2012). Thus, the few day-old zebrafish fry may become an appropriate translational model system with which compounds and mutations with alcohol-effect altering properties may be identified in a fast, space- and cost-effective manner.

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References

Ali, S., Van Mil, H.G., Richardson, M.K., 2011. Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing. PLoS One 6 (6), e21076.
Amsterdam, A., Hopkins, N., 2006. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. Trends Genet. 22 (9), 473–478.
Araujo-Silva, H., Pinheiro-da-Silva, J., Silva, P.F., Luchiari, A.C., 2018. Individual differences in response to alcohol exposure in zebrafish (Danio rerio). PLoS One 13 (6), e0198856. https://doi.org/10.1371/journal.pone.0198856.
Bakoyiannis, I., Gkioka, E., Pergialiotis, V., Mastroleon, I., Prodromidou, A., Vlachos, G.D., Perrea, D., 2014. Fetal alcohol spectrum disorders and cognitive functions of young children. Rev. Neurosci. 25 (5), 631–639.
Bartolini, T., Mwaffo, V., Butail, S., Porfiri, M., 2015. Effect of acute ethanol administration on zebrafish tail-beat motion. Alcohol 49 (7), 721–725. https://doi.org/10.1016/j.alcohol.2015.06.004.
Bell, R.L., Hauser, S.R., Liang, T., Sari, Y., Maldonado-Devincci, A., Rodd, Z.A., 2017. Rat animal models for screening medications to treat alcohol use disorders. Neuropharmacology 122, 201–243.
Bennett, B., Downing, C., Parker, C., Johnson, T.E., 2006. Mouse genetic models in alcohol research. Trends Genet. 22 (7), 367–374.
Blaser, R., Gerlai, R., 2006. Behavioral phenotyping in Zebrafish: comparison of three behavioral quantification methods. Behav. Res. Methods 38, 456–469.
Brabant, C., Guarnieri, D.J., Quertemont, E., 2014. Stimulant and motivational effects of alcohol: lessons from rodent and primate models. Pharmacol. Biochem. Behav. 122, 37–52.
Buske, C., Gerlai, R., 2011. Shoaling develops with age in zebrafish (*Danio rerio*). Prog. Neuro-Psychopharmacol. Biol. Psychiatry 35, 1409–1415.
Chatterjee, D., Shams, S., Gerlai, R., 2014. Chronic and acute alcohol administration induced neurochemical changes in the brain: comparison of distinct zebrafish populations. Amino Acids 46 (4), 921–930.
Colwill, R.M., Creton, R., 2011. Locomotor behaviors in zebrafish (*Danio rerio*) larvae.

Behav. Process. 86 (2), 222–229.
Crabbe, J.C., 1989 Feb. Genetic animal models in the study of alcoholism. Alcohol. Clin. Exp. Res. 13 (1), 120–127.
de Esch, C., Slieker, R., Wolterbeek, A., Woutersen, R., de Groot, D., 2012. Zebrafish as potential model for developmental neurotoxicity testing: a mini review. Neurotoxicol. Teratol. 34 (6), 545–553.
Devineni, A.V., Heberlein, U., 2013. The evolution of Drosophila melanogaster as a model for alcohol research. Annu. Rev. Neurosci. 36, 121–138.
Echevarria, D.J., Toms, C.N., Jouandot, D.J., 2011. Alcohol-induced behavior change in zebrafish models. Rev. Neurosci. 22 (1), 85–93.
Farrell, T.C., Cario, C.L., Milanese, C., Vogt, A., Jeong, J.H., Burton, E.A., 2011. Evaluation of spontaneous propulsive movement as a screening tool to detect rescue of Parkinsonism phenotypes in zebrafish models. Neurobiol. Dis. 44 (1), 9–18.
Gerlai, R., 2010. Zebrafish antipredatory responses: a future for translational research? Behav. Brain Res. 207, 223–231.
Gerlai, R., 2011a. Using zebrafish to unravel the genetics of complex brain disorders. In: Behavioral Neurogenetics. Springer, Berlin, Heidelberg, pp. 3–24.
Gerlai, R., 2011b. A small fish with a big future: zebrafish in behavioral neuroscience. Rev. Neurosci. 22 (1), 3–4.
Gerlai, R., 2012. Using zebrafish to unravel the genetics of complex brain disorders. Curr. Top. Behav. Neurosci. 12, 3–24.
Gerlai, R., 2016. Learning and memory in zebrafish (*Danio rerio*). Methods Cell Biol. 134, 551–586.
Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67 (4), 773–782.
Gerlai, R., Lee, V., Blaser, R., 2006. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). Pharmacol. Biochem. Behav. 85, 752–761.
Gerlai, R., Ahmad, F., Prajapati, S., 2008. Differences in acute alcohol-induced behavioral responses among zebrafish populations. Alcohol. Clin. Exp. Res. 32 (10), 1763–1773.
Gerlai, R., Chatterjee, D., Pereira, T., Sawashima, T., Krishnannair, R., 2009. Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav. 8 (6), 586–599.
Guo, N., Lin, J., Peng, X., Chen, H., Zhang, Y., Liu, X., Li, Q., 2015. Influences of acute ethanol exposure on locomotor activities of zebrafish larvae under different illumination. Alcohol 49 (7), 727–737.
Harrison, N.L., Skelly, M.J., Grosserode, E.K., Lowes, D.C., Zeric, T., Phister, S., Sailing, M.C., 2017. Neuropharmacology 122 (SI), 36–45.
Heyman, G.M., 2000. An economic approach to animal models of alcoholism. Alcohol Res. Health 24 (2), 132–139.
Ingebretson, J.J., Masino, M.A., 2013. Quantification of locomotor activity in larval zebrafish: considerations for the design of high-throughput behavioral studies. Front. Neural Circuits 7, 109.
Irons, T.D., MacPhail, R.C., Hunter, D.L., Padilla, S., 2010. Acute neuroactive drug exposures alter locomotor activity in larval zebrafish. Neurotoxicol. Teratol. 32 (1), 84–90.
Karnik, I., Gerlai, R., 2012. Can zebrafish learn spatial tasks? An empirical analysis of place and single CS-US associative learning. Behav. Brain Res. 233, 415–421.
Keane, B., Leonard, B.E., 1989. Rodent models of alcoholism: a review. Alcohol Alcohol. 24 (4), 299–309.
King, A.C., Houle, T., De Wit, H., Holdstock, L., Schuster, A., 2002. Biphasic alcohol response differs in heavy versus light drinkers. Alcohol. Clin. Exp. Res. 26 (6), 827–835.
Lockwood, B., Bjerke, S., Kobayashi, K., Guo, S., 2004. Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. Pharmacol. Biochem. Behav. 77 (3), 647–654.
Lowenstein, P.R., Castro, M.G., 2009. Uncertainty in the translation of preclinical experiments to clinical trials. Why do most phase III clinical trials fail? Curr. Gene Ther. 9 (5), 368–374.
MacPhail, R.C., Brooks, J., Hunter, D.L., Padnos, B., Irons, T.D., Padilla, S., 2009. Locomotion in larval zebrafish: influence of time of day, lighting and ethanol. Neurotoxicology 30 (1), 52–58.
MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 14 (10), 721.
Mathur, P., Guo, S., 2011. Differences of acute versus chronic ethanol exposure on anxiety-like behavioral responses in zebrafish. Behav. Brain Res. 219 (2), 234–239.
May, P.A., Gossage, J.P., Kalberg, W.O., Robinson, L.K., Buckley, D., Manning, M., Hoyme, H.E., 2009. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. Dev. Disabil. Res. Rev. 15 (3), 176–192.
McCool, B.A., 2011. Ethanol modulation of synaptic plasticity. Neuropharmacology 61 (7), 1097–1108.
Miller, N., Gerlai, R., 2007. Quantification of Shoaling Behaviour in Zebrafish (*Danio rerio*). Behav. Brain Res. 184, 157–166.
Naito, A., Muchhala, K.H., Asatryan, L., et al., 2014. Glycine and GABA(A) ultra-sensitive ethanol receptors as novel tools for alcohol and brain research. Mol. Pharmacol. 86 (6), 635–646.
Nowicki, M., Tran, S., Muraleetharan, A., Chatterjee, D., Gerlai, R., 2015. Inhibition of phosphorylated tyrosine hydroxylase attenuates ethanol-induced hyperactivity in adult zebrafish (Danio rerio). Pharmacol. Biochem. Behav. 138, 32–39.
Padilla, S., Hunter, D.L., Padnos, B., Frady, S., MacPhail, R.C., 2011. Assessing locomotor activity in larval zebrafish: influence of extrinsic and intrinsic variables. Neurotoxicol. Teratol. 33 (6), 624–630.
Pan, Y., Chatterjee, D., Gerlai, R., 2012. Strain dependent gene expression and neurochemical levels in the brain of zebrafish: focus on a few alcohol related targets. Physiol. Behav. 107 (5), 773–780.
Rico, E.P., Rosemberg, D.B., Dias, R.D., et al., 2007. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol. Lett. 174 (1-3), 25–30.
Rosemberg, D.B., Da Rocha, R.F., Rico, E.P., et al., 2010. Taurine prevents enhancement of acetylcholinesterase activity induced by ethanol exposure and decreases the level of markers of oxidative stress in zebrafish brain. Neuroscience 171 (3), 683–692.
Seguin, D., Gerlai, R., 2018. Fetal alcohol spectrum disorders: zebrafish in the analysis of the milder and more prevalent form of the disease. Behav. Brain Res. 352, 125–132.
Selderslaghs, I.W., Van Rompay, A.R., De Coen, W., Witters, H.E., 2009. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod. Toxicol. 28 (3), 308–320.
Shams, S., Seguin, D., Facciol, A., Chatterjee, D., Gerlai, R., 2017. Chronic social isolation affects anxiety-related behaviors, cortisol, and monoamines in adult zebrafish. Behav. Neurosci. 131, 492–504.
Sison, M., Gerlai, R., 2010. Associative learning in zebrafish (*Danio rerio*) in the plus maze. Behav. Brain Res. 207, 99–104.
Stade, B., Ali, A., Bennett, D., Campbell, D., Johnston, M., Lens, C., ... Koren, G., 2009. The burden of prenatal exposure to alcohol: revised measurement of cost. Can. J. Clin. Pharmacol. 16 (1), e91–e102.
Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., Kalueff, A.V., 2012. Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacology 62 (1), 135–143.
Tran, S., Gerlai, R., 2013. Time-course of behavioural changes induced by ethanol in zebrafish (Danio rerio). Behav. Brain Res. 252, 204–213.
Tran, S., Gerlai, R., 2014. Recent advances with a novel model organism: alcohol tolerance and sensitization in zebrafish (Danio rerio). Prog. Neuro-Psychopharmacol. Biol. Psychiatry 55, 87–93.
Tran, S., Nowicki, M., Chatterjee, D., Gerlai, R., 2015. Acute and chronic ethanol exposure differentially alters alcohol dehydrogenase and aldehyde dehydrogenase activity in the zebrafish liver. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 56, 221–226.
Tran, S., Nowicki, M., Muraleetharan, A., Chatterjee, D., Gerlai, R., 2015b. Differential effects of acute administration of SCH-23390, a D 1 receptor antagonist, and of ethanol on swimming activity, anxiety-related responses, and neurochemistry of zebrafish. Psychopharmacology 232 (20), 3709–3718.
Tran, S., Facciol, A., & Gerlai, R. (2016a). The zebrafish, a novel model organism for screening compounds affecting acute and chronic ethanol-induced effects. In International review of neurobiology (Vol. 126, pp. 467–484). Academic Press.
Tran, S., Nowicki, M., Fulcher, N., Chatterjee, D., Gerlai, R., 2016b. Interaction between handling induced stress and anxiolytic effects of ethanol in zebrafish: a behavioral and neurochemical analysis. Behav. Brain Res. 298, 278–285.
Tran, S., Chow, H., Tsang, B., Facciol, A., Gandhi, P., Desai, P., Gerlai, R., 2017a. Zebrafish are able to detect ethanol in their environment. Zebrafish 14 (2), 126–132.
Tran, S., Fulcher, N., Nowicki, M., Desai, P., Tsang, B., Facciol, A., Gerlai, R., 2017b. Time-dependent interacting effects of caffeine, diazepam, and ethanol on zebrafish behaviour. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 75, 16–27.
Tsang, B., Ansari, R., Azhar, A., Gerlai, R., 2018. Drinking in the morning versus evening: time-dependent differential effects of acute alcohol administration on the behavior of zebrafish. Pharmacol. Biochem. Behav. 175, 174–185.
Wahlsten, D., 1990. Insensivity of the analysis of variance to heredity-environment interaction. Behav. Brain Sci. 13 (1), 109.
Wolman, M., Granato, M., 2012. Behavioral genetics in larval zebrafish: learning from the young. Dev. Neurobiol. 72 (3), 366–372.
Zhou, Y., Kreek, M.J., 2014. Alcohol: a stimulant activating brain stress responsive systems with persistent neuroadaptation. Neuropharmacology 87, 51–58.