Social signals decrease the effectiveness of ethanol in zebrafish

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ABSTRACT

Pharmacological and toxicological studies involving aquatic species often expose organisms to compounds in isolation prior to physiological or behavioural testing. Recent evidence suggests that the presence of conspecifics during a stressful event can modulate behavioural outcomes (called 'social buffering') when testing occurs within the same context. It is unknown, however, whether the social environment during exposure interacts with the efficacy of anxiety-altering substances when subsequently tested in the absence of conspecifics. In this study, zebrafish were individually exposed to habitat water or ethanol (1.0% vol/vol) while untreated conspecifics were visually present or absent during dosing. Using the novel object approach test, a validated test of boldness behaviour, we observed significant effects of ethanol in isolated fish, but not in fish that had view of conspecifics during dosing. These results were not explained by locomotion during exposure and highlight the need to consider the social environment during exposure when conducting and interpreting behavioural research involving drug or toxicant exposure.

KEYWORDS: Social Buffering, Behavioural Mimicry, Ethanol, Zebrafish, Novel Object Approach Test, Boldness, Anxiety-like behaviour

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INTRODUCTION

Living in a social environment offers many evolutionary advantages. Belonging to a group facilitates reproduction, allows the earlier detection and evasion of predators, and improves food localization (Rubinstein, 1978). Social cues are commonly the mechanism that convey these messages between conspecifics and can guide responses in uncertain situations (Suboski et al., 1990). Another benefit of the presence of conspecifics is a resulting decrease in stress level that minimizes the impact of stressful situations (Kikusui, Winslow & Mori, 2006). This phenomenon, known as 'social buffering', has been experimentally demonstrated in many species including, cats (Masserman, 1943), goats (Liddell, 1949), rats (Davitz & Donald, 1955; Latané, 1969), humans (Hostinar, Johnson & Gunnar, 2015) and recently, zebrafish (Oliveira & Faustino, 2017; Faustino, Tacão-Monteiro & Oliveira, 2017).

The zebrafish has become a popular model organism for use in a variety of scientific disciplines including pharmacology. Behavioural neuroscience tests can be used to analyze a wide variety of cognitive processes in zebrafish including episodic-like memory (Hamilton et al., 2017), object recognition memory (May et al., 2016), classically conditioned memory (Sison & Gerlai, 2010), fear (Speedie & Gerlai, 2008), and anxiety-like behaviour (Maximino, de Brito & da Silva Batista, 2010). To test anxiety-like behaviour there are a variety of paradigms available, with the most common being the light/dark preference and novel tank diving tests (for a review see (Maximino, de Brito & da Silva Batista, 2010)). Due to the reliability of these tests and the practical simplicity in which psychopharmacological substances can be administered in zebrafish (Gerald, Lee & Blaser, 2006), adaptive behavioural responses can be easily manipulated with anxiolytic (anxiety-reducing) and anxiogenic (anxiety-enhancing) compounds (Collier & Echevarria, 2013). Recent evidence, however, suggests that the social environment in which anxiety-

87 altering compounds are administered and/or tested in may influence the behavioural effects of
88 these substances in zebrafish, which can complicate conclusions.

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89 Visual and olfactory conspecific cues have recently been found to protect zebrafish
90 against the effects of an anxiogenic compound when exposure and testing occurs within the same
91 environment. In a study that examined social buffering in zebrafish, the sight and/or smell of
92 conspecifics was found to lessen the anxiogenic effects of an alarm substance (Faustino, Tacão-
93 Monteiro & Oliveira, 2017). When this compound was administered in the same location where
94 behavioural testing took place, fish exposed to conspecific water and alarm substance while next
95 to a tank containing untreated conspecifics displayed significantly less freezing and erratic
96 movements than when the adjacent tank remained empty and no conspecific water was added
97 (Faustino, Tacão-Monteiro & Oliveira, 2017). When the effectiveness of each type of cue was
98 tested, visual cues were more effective than olfactory in reducing aversive behaviours in
99 zebrafish (Faustino, Tacão-Monteiro & Oliveira, 2017).
100 In the majority of acute pharmacological experiments in fish, substances are administered
101 while fish are isolated from conspecifics and the exposed fish are then transferred to a behav-
102 ioural arena for testing (Stewart et al., 2012). Few studies, however, specify whether conspecifics
103 are within or outside of view during dosing (Table. 1), and to the best of our knowledge, no
104 study has examined whether this may influence the efficacy of anxiety-altering substances when
105 subsequently tested in the absence of conspecifics. It is also unknown whether social buffering
106 may also act to alter the effects of anxiolytic substances. To test these questions, we exposed in-
107 dividual zebrafish to either habitat water or ethanol (1.0% vol/vol) while untreated conspecifics
108 were visually present or absent for the entire exposure period. Following exposure, the fish were
109 transferred to the novel object approach test which uses the exploration or avoidance of a novel

object to quantify anxiety-like behaviour and boldness (Dean et al., 2020; Krook et al., 2019; Leighton et al., 2018). Finally, we tested whether fish move at different rates and remain closer to conspecifics during the dosing period itself, in order to determine whether the social condition (*Isolated* vs. *In-view*) influences behaviour during exposure.

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METHODS

Subjects and housing

Short-fin wild-type zebrafish (n = 90) were acquired from Aquatic Imports (Calgary, AB) at a minimum age of 9-months. Fish were experimentally naïve and comprised of mixed males and females (~50/50 ratio). Following a month-long quarantine period, the fish were held in either 3 or 10L polypropylene tanks within a three-shelf bench top system (Aquatic Habitats, Aquatic Ecosystems, Inc. Apopka, FL, USA) which was controlled for filtration and aeration. No fish was ever housed in isolation and tank capacities never exceeded five fish per liter. Temperature and pH remained between 26 - 30°C and 6.0 – 8.0, respectively. Lights were kept on a 12-hour light/dark cycle with lights on at 8AM and off at 8PM. Fish were fed dry brine shrimp (Omega One Freeze Dried Mysis Shrimp nutri-treat, OmegaSea Ltd., Germany) once per day after experimentation. All experiments were approved by the MacEwan University Animal Research Ethics Board (AREB) under protocol number 05-12-13 in compliance with the Canadian Council for Animal Care (CCAC) guidelines for the care and use of experimental animals.

Experimental design

The study used a 2 x 2 factorial design. The between-subject experimental variables

included visual access of conspecifics (*Isolated or In-view*) and the type of substance the fish were exposed to (habitat water (CTL) or ethanol) while in the dosing containers. Prior to experimentation, fish were randomly assigned to one of four groups: *Isolated-CTL*, *Isolated-Ethanol*, *In-view-CTL*, and *In-view-Ethanol.* Following exposure, anxiety-like behaviours were tested in the novel object approach test to examine whether the social environment during exposure influences the efficacy of this anxiolytic substance.

Isolated vs. conspecifics in-view

Fish assigned to one of the two *Isolated* conditions (*Isolated-CTL* (n = 15), or *Isolated-Ethanol* (n = 15)) were carried in their habitat tanks into the experimental room prior to feeding and were given at least 10 minutes to acclimatize to this new environment. A white corrugated plastic barrier was set up surrounding habitat tanks to limit external stimuli. Following the habituation period, fish were individually netted from their habitat tanks and placed into one of two experimental dosing containers (600 mL). Each dosing container contained 500 mL of solution and was also surrounded by white corrugated plastic barriers (Fig. 1A). Two dosing containers were used rather than one to increase efficiency and allow two fish to be dosed simultaneously. Once in the dosing container, a square piece of the same plastic was placed on top to prevent evaporation of the solution and to ensure fish remained inside (Cachat et al., 2010; Holcombe et al., 2013). Fish assigned to the *In-view* conditions (*In-view-CTL* (n = 15) or *In-view-Ethanol* (n =15)) underwent the same procedure, with the exception that a second tank containing 12 untreated conspecifics was placed to the right of the experimental dosing containers. The dosing containers used in the *In-view* conditions were positioned in front of each other to ensure fish in both dosing containers had equal view of their conspecifics. The same group of conspecifics were used for

each *In-view* condition and were selected from the aquatic habitat. A white corrugated plastic barrier covered the remaining two sides of the conspecific tank (Fig. 1B) and water temperatures were maintained between 26 and 30°C by seedling heat mats (Hydrofarm Horticultural Products, Petaluma CA). Fish in the *Isolated* or *In-view* conditions remained in the dosing containers for 30 minutes. At the end of the 30-minute dosing, the solution (including the fish) was carefully poured into a net, with a second dosing container collecting the solution. Once in the net, the fish was placed into the adjacent behavioural arena for testing.

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Exposure to ethanol

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Fish exposed to control water (*Isolated-CTL* (n = 15) or *In-view-CTL* (n = 15)), were placed into 600 mL glass dosing containers that only contained habitat water (500 mL). Fish in the ethanol groups (*Isolated-Ethanol* (n = 15) or *In-view-Ethanol* (n = 15)) were placed into dosing containers with 1.0% ethanol. Solutions for each compound were made fresh each day by mixing 5.26 mL of non-denatured, 95% ethanol into 495 mL of habitat water in the respective dosing containers. The selected concentration and duration of ethanol exposure was based on previous experiments in zebrafish (Johnson & Hamilton, 2017).

Experimental apparatus and behavioural testing

Fish were individually tested in the novel object approach test following the 30-minute exposure period. The behavioural arena used in this experiment was circular and made from white opaque plastic (Ø = 34 cm; depth = 15 cm; Fig.1C). The arena was placed on top of a heat mat to maintain habitat water temperatures and was surrounded by a three-sided white corrugated plastic enclosure to limit external stimuli during testing. Habitat water was added to the arena up to a max-

imum height of 5 cm and was replaced with fresh habitat water every four hours. An equal amount of heated habitat water was also exchanged whenever temperatures fell below 26°C. The object used in this study was a 2 cm x 4.25 cm Lego figurine which was multi-coloured to rule out possible colour preferences (Fig. 1D; Dean et al., 2020; Hamilton et al., 2017; Johnson & Hamilton, 2017) and was adhered using velcro to the bottom of the arena's center. Prior to testing, three virtual zones representing thigmotaxis, transition and inner zones were defined using EthoVision XT motion tracking software (Fig. 1E; version 11.0, Noldus, VA, USA). All experimental procedures occurred between 9AM and 6PM prior to feeding. The time, in seconds, fish spent in each zone (thigmotaxis, transition, inner) was recorded to assess exploratory preferences and anxiety-like behaviour, and locomotion was assessed by tracking the distance moved(cm) and immobility(s). Fish were tested individually for a period of 10 minutes following dosing and recording began as soon as the fish was placed into the transition zone facing the object.

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Distance moved and side preference during exposure

To determine if the social condition during exposure affected the distance fish moved while in the dosing container, the activity of a new group of fish (n = 30) was assessed. We also explored whether fish that were able to view conspecifics would have a preference for the side of the dosing container closest to their conspecifics (conspecific side). To isolate the effect of social condition, these measures were compared between separate groups of *Isolated* and *In-view* control groups (*Isolated-Dosing* (n = 15) and *In-view-Dosing* (n = 15)). Following a 10-minute habituation period, one fish was individually netted from their habitat tank and placed into a 600 mL dosing container with habitat water (500 mL). A rectangular piece of white corrugated plastic was placed beneath the dosing container to assist with motion tracking. As in the novel object

approach test, a three-sided enclosure was set up during behavioural tracking and seedling heat mats maintained water temperatures. To ensure these fish received the same treatment as fish in the *Isolated-CTL* and *In-view-CTL* conditions, a white piece of corrugated plastic was also set up across the front of the three-sided enclosure (Fig. 2A). For fish in both the *Isolated-Dosing* and *In-view-Dosing* conditions, EthoVision was set up to record the distance (cm) each fish moved throughout the 30-minute exposure period. For fish in the *In-view-Dosing* condition, a habitat tank containing the same conspecifics (n =12) that were used in the other In-view conditions was positioned to the right of the beaker. Using EthovVision, the beaker was then vertically split into two equal-sized virtual sections to compare the amount of time, in seconds, fish explored the side of the beaker closest to conspecifics (conspecific side) and the side farthest from conspecifics (empty side; Fig.2B). To rule out external variables potentially contributing to a side preference, the habitat tank was placed to the left of the beaker for the final three trials. No differences were observed in the time spent exploring either side of the beaker regardless of whether the habitat tank was on the right or left side of the beaker (Mann-Whitney; $p = 0.2549$; $p = 0.2945$) so these were combined for analysis.

Statistical analysis

All data was analyzed using GraphPad Prism (Version 6; CA, USA). Normality was assessed using D'Agostino & Pearson omnibus normality tests. To analyze the effect of social condition and/or ethanol on anxiety levels, parametric data was analyzed using ordinary Two-Way ANOVAs. As we were unaware of a non-parametric equivalent of a Two-Way ANOVA, Kruskal-Wallis tests were used for analyzing the non-parametric data. Differences between experimental groups were analyzed using Tukey's and Dunn's post-hoc tests for the parametric and non-

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parametric data, respectively. To assess differences in distance moved and side preferences for *Isolated-Dosing* and *In-view-Dosing* fish during exposure, unpaired *t*-tests were used. Significance across all tests was determined using alpha levels of 0.05 and 95% confidence intervals.

RESULTS

Effect of social context

The social context did not have a significant effect on fish behaviour. No significant differences were found between control groups in the time spent in either the thigmotaxis ($p = 0.1646$; Fig. 3A), transition ($p = 0.1879$; Fig. 3B) or inner ($p = 0.0738$; Fig. 3C) zones, the distance fish moved ($p > 0.9999$; Fig. 3D), or time spent immobile ($p > 0.9999$; Fig. 3E).

Effect of ethanol following isolated vs. in-view exposure

The time fish spent in the thigmotaxis, transition, and inner zones was significantly affected by ethanol. Fish in the *Isolated-Ethanol* condition spent significantly more time in the transition and inner zones compared to fish in the *Isolated-CTL* condition ($p < 0.0001$; Fig. 3B; $p < 0.0001$; Fig. 3C). Fish in the *Isolated-Ethanol* condition spent significantly less time in the thigmotaxis zone compared to fish in the *Isolated-CTL* condition ($p < 0.0001$; Fig. 3A). Distance moved was not found to differ between *Isolated-CTL* and *Isolated-Ethanol* groups ($p = 0.4964$; Fig. 3D). Differences in immobility between these groups, however, were found to be highly significant. Compared to *Isolated-CTL*s, fish in the *Isolated-Ethanol* condition spent significantly more time immobile ($p = 0.0002$; Fig. 3E).

Ethanol was not found to have a significant effect on any of the behavioural measures when fish could view conspecifics during exposure. Specifically, *In-view-CTL* and *In-view-*

Ethanol groups did not differ in the time spent in the thigmotaxis ($p = 0.1929$; Fig. 3A), transition ($p = 0.0946$; Fig. 3B), or inner ($p > 0.9999$; Fig. 3C) zones, the distance fish moved ($p > 0.9999$; Fig. 3D), or time spent immobile ($p = 0.5543$; Fig. 3E).

Distance moved and side-preference during exposure

The distance fish moved while in the dosing container did not significantly differ between *Isolated-Dosing* and *In-view-Dosing* groups ($t_{28} = 1.255$, $p = 0.2198$; Fig. 4A). A highly significant preference for the conspecific side of the dosing container was found in fish from the *In-view-Dosing* group ($t_{28} = 10.21$, $p < 0.0001$; Fig. 4B).

DISCUSSION

To examine whether the social condition during dosing impacts behavioural effects of anxiety-altering substances when later tested in isolation, we exposed zebrafish to ethanol (1.0%) either while fish were isolated or able to observe conspecifics. Following dosing, anxiety-like behaviours were tested in the novel object approach test. The behavioural effects of ethanol were found to be highly dependent on the social condition in which it was administered. Ethanol only affected anxiety-like behaviour and boldness in isolated fish and did not have an effect in fish that were able to view conspecifics during dosing.

Ethanol exposure significantly increased the time isolated fish spent in the zones closest to the novel object (transition and inner; Fig. 3B-C), consistent with previous research demonstrating ethanol increased boldness (Hamilton et al., 2017; Johnson & Hamilton, 2017). Ethanol also decreased time spent in the thigmotaxis zone; an indication that anxiety-like behaviour was

decreased. The same pattern also emerged in ethanol's influence on locomotion. Ethanol did not impact the distance *Isolated* or *In-view* fish moved (Fig. 3D), or the time *In-view* fish spent immobile; it only increased immobility in *Isolated* fish (Fig. 3E). This suggests that social isolation either increases sensitivity to ethanol's anxiolytic and depressant effects, or the presence of conspecifics suppresses these effects.

In an attempt to understand how the social context contributes to differences observed in behavioural outcomes, we analyzed the behaviours of a second group of fish while in the dosing container during the 30-minute dosing period. Because mobility may affect the rate of intake when fish are dosed via immersion, with greater physiological demands resulting in more ventilation and therefore the drug moving in through the gills at a higher rate (Blaser & Vira, 2014), we wanted to determine whether the heightened effect of ethanol observed in *Isolated* fish could be explained by greater movement during dosing. To examine the effect of social condition, we chose to analyze the behaviours of fish exposed to habitat water while isolated or within view of conspecifics. Interestingly, no differences were observed in the distance fish moved (Fig. 4A), indicating differences in locomotion during dosing could not explain behaviours observed in the novel object approach test. Not surprisingly, zebrafish spent significantly more time on the side of the dosing container closest to conspecifics when in view (Fig. 4B), demonstrating their preference to remain near other zebrafish.

An explanation for the anxiolytic effect of ethanol in *Isolated* but not *In-view* groups may be related to 'social buffering.' Previous research in zebrafish has shown that the presence of conspecifics helps to suppress anxiety evoked by a fearful stimulus (Faustino, Tacão-Monteiro & Oliveira, 2017). Faustino, Tacão-Monteiro and Oliveira (2017) first demonstrated this in zebrafish by exposing fish to a conspecific alarm substance with or without the presence of con-

specific cues. They found that the anxiogenic effects were dampened by the presence of olfactory and/or visual cues. In other words, fish that could observe or smell their conspecifics showed less anxiety in response to the alarm substance (Faustino, Tacão-Monteiro & Oliveira, 2017). The mechanisms of social buffering have not been well explored in zebrafish; however, it is possible that zebrafish use the behaviours of their conspecifics as a source of information to guide their own responses in unfamiliar or fearful environments. This would explain why there was no effect of ethanol in the *in-view* condition in our experiment. Fish in the dosing container were observing their conspecifics behaving normally and the effects of ethanol were minimized. However, social buffering has only been shown to decrease stress responses and in our study anxiolysis was reduced. The effect of ethanol may have been 'buffered' by the presence of conspecifics but the mechanism would be due to behavioural mimicry. Future studies could examine how manipulating the emotional state of conspecifics that are within view during dosing affects the behavioural outcomes of fish observing them. It would also be valuable to explore the neurochemical basis of the decreased response to ethanol with analysis of brain chemistry after dosing in either of these social conditions.

CONCLUSIONS

The presence of conspecifics lessens the effects of ethanol suggesting social buffering can also blunt the effect of anxiolytics in zebrafish. These findings have important implications in the fields of pharmacology, toxicology and behavioural neuroscience as isolated drug administration seems to be more effective in eliciting a behavioural response. Additionally, it is not uncommon for behavioural findings involving fish to be inconsistent, yet researchers rarely specify whether

or not conspecifics are within view during dosing (Table. 1). Therefore, social buffering may offer a potential explanation for these discrepancies and necessitates more detailed explanations of methods used within these experiments. Overall, this study provides the first evidence that the social condition during dosing effects the efficacy of anxiolytic substances when subsequently tested in isolation and highlights the need to consider the social environment during exposure when conducting or interpreting behavioural research in the future.

Figure legends:

FIGURE 1. Experimental dosing set-up. (A) *Isolated* and (B) *In-view* dosing. An Individual fish was netted from the holding tank and placed into one of the two dosing containers. *In-view* fish had visual access to 12 conspecifics held in the conspecific tank but were not able to see the other fish being dosed. Fish remained in the dosing containers for 30-minutes prior to behavioural testing. C) The circular arena used was 34 cm in diameter and 16 cm in height. D) The novel object used was a multi-coloured LEGO figurine. E) The thigmotaxis, transition and inner zones were calibrated to 34, 23 and 12 cm in diameter respectively.

FIGURE 2. Experimental set up for tracking behaviour during testing. (A) *Isolated* dosing, or (B) *In-view* dosing. The circle in the bottom left of figure (B) represents the virtual zones created in Ethovision to test whether fish spend more time on the side of the beaker closest to conspecifics when in view.

FIGURE 3. Effects of social context and ethanol on zone preference. The time, in seconds, fish spent in the thigmotaxis (A), transition (B) and inner zones (C). (D) and (E) represent the effect of social context and substance on the distance fish moved (D) and the time fish spent immobile (E). Individual data points represent mean values (n = 15 per group). Error bars represent the SE and * identifies significant differences between group means using 95% C.I. $^{**}P < 0.01$;$^{***}P < 0.001$; $^{****}P < 0.0001$

FIGURE 4. Distance and side preferences during the dosing procedure. (A) The distance, *Isolated* and *In-view-CTL* fish moved and the (B) amount of time, in seconds, *In-view-CTL*s

spent on either side of the dosing container during dosing. The social condition did not have a significant effect on the distance fish moved, however when in-view, fish had a significant preference for the side of the dosing container closest to conspecifics. Data was analyzed using independent *t*-tests. Individual data points (n = 15 per group) represent mean values ± SEM. Error bars represent SE and * identifies significant differences between group means using 95% C.I. ****$P < 0.0001$.

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Author contributions

RD conducted all experiments. Data analysis, the design of the study, and writing of the manuscript was done by RD, NHR, and TJH. TJH contributed all experimental compounds. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

References

Bernardo HT, Agostini JF, Zuehl Dal Toe HC, Vieira KM, Baldin SL, Schuck PF, Uribe-Cruz C, Rico EP. 2019. Cholinergic system and exploratory behavior are changed after weekly binge ethanol exposure in zebrafish. *Pharmacology, Biochemistry and Behavior,* 186: 172790. DOI: https://doi.org/10.1016/j.pbb.2019.172790

Blaser RE, Pénalisa YM. 2011. Stimuli affecting zebrafish (Danio rerio) behavior in the light/dark preference test. *Physiology & Behavior,* 104: 831-838. DOI: 10.1016/j.physbeh.2011.07.029

Cachat J, Canavello P, Elegante M, Bartels B, Hart P, Bergner C, Egan R et al. 2010. Modeling withdrawal syndrome in zebrafish. *Behavior Brain Research,* 208: 371-376. DOI: https://doi.org/10.1016/j.bbr.2009.12.004.

Collier AD, Echevarria DJ. 2013. The utility of the zebrafish model in conditioned place preference to assess the rewarding effects of drugs. *Behaviour Pharmacology,* 24: 375-383. DOI: https://doi.org/10.1097/FBP.0b013e328363d14a.

Da Silva Chaves SN, Costa BPD, Gomes GCV, Lima-Maximino M. Rico EP, Maximino C. 2020. NOS-2 participates in the behavioral effects of ethanol withdrawal in zebrafish. *Neuroscience Letter,* 728: 134952. DOI: https://doi.org/10.1016/j.neulet.2020.134952

Davitz JR, Donald JM. 1955. Socially facilitated reduction of a fear response in rats. *Journal of Comparative Psychology,* 48: 149-151. DOI: https://doi.org/10.1037/h0046411.

Dean R, Duperrault E, Newton D, Krook J, Ingraham E, Gallup J, Franczak BC, Hamilton TJ. 2020. Opposing effects of acute and repeated nicotine exposure on boldness in zebrafish. *Scientific Reports,* 10: 8570. DOI: https://doi.org/10.1038/s41598-020-65382-6

Dlugos CA, Rabin RA. 2003. Ethanol effects on three strains of zebrafish: model system for genetic investigations. *Pharmacology Biochemistry and Behavior,* 74: 471-480. DOI: 10.1016/S0091-3057(02)01026-2

Echevarria DJ, Hammock CM, Pratt DW, Hosemann, JD. 2008. A novel Behavioural test battery to assess global drug effects using the zebrafish. *International Journal of Comparative Psychology,* 21: 19-35.

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV. 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research,* 205: 38-44. Doi: 10.1016/j.bbr.2009.06.022

Faustino AI, Tacão-Monteiro A, Oliveira RF. 2017. Mechanisms of social buffering of fear in zebrafish. *Scientific Reports,* 7: 44329. DOI: https://doi.org/10.1038/srep44329.

Fontana BD. Duarte T, Muller TE, Canzian J, Ziana PR, Mezzomo NJ, Parker MO, Rosemberg DB. 2020. Concomitant taurine exposure counteracts ethanol-induced changes in locomotor and anxiety-like responses in zebrafish. *Psychopharmacology,* 237: 735-743. DOI: https://doi.org/10.1007/s00213-019-05410-0

Gebauer DL, Pagnussat N, Piato AL, Schaefer IC, Bonn CD, Lara DR. 2011. Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. *Pharmacology Biochemistry and Behavior,* 99: 480- 487. DOI: 10.1016/j.pbb.2011.04.021

Gerald R, Lee V, Blaser R. 2006. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (Danio rerio). *Pharmacology Biochemistry and Behavior,* 85: 752-761. DOI: https://doi.org/10.1016/j.pbb.2006.11.010.

Gerlai R, Lahav M, Guo L, Rosenthal A. 2001. Drinks like a fish: Zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. *Pharmacology Biochemistry and Behavior,* 67: 773-783. DOI: 10.1016/S0091-3057(00)00422-6

Gerlai R, Ahmad F, Prajapati S. 2008. Differences in acute alcohol-induced behavioral responses among zebrafish populations. *Alcoholism, Clinical and Experimental Research,* 32: 1763-177s. DOI: 10.1111/j.1530-0277.2008.00761.x

Hamilton TJ, Morrill A, Lucas K, Gallup J, Harris M, Healey M, Pitman T, Schalomon M, Digweed S, Tresguerres M. 2017. Establishing zebrafish as a model to study the anxiolytic effects of scopolamine. *Scientific Reports,* 7: 1-9. DOI: https://doi.org/10.1038/s41598-017-15374-w.

Holcombe A, Howorko A, Powell RA, Schalomon M, Hamilton TJ. 2013. Reversed scototaxis during withdrawal after daily-moderate, but not weekly-binge, administration of ethanol in zebrafish. *PloS one,* 8. DOI: https://doi.org/10.1371/journal.pone.0063319.

Hostinar CE, Johnson AE, Gunnar MR. 2015. Early social deprivation and the social buffering of cortisol stress responses in late childhood: An experimental study. *Developmental Psychology*, 51: 1597-1608. DOI: https://doi.org/10.1037/dev0000029.

Johnson A, Hamilton TJ. 2017. Modafinil decreases anxiety-like behaviour in zebrafish. *PeerJ, 5*. DOI: https://doi.org/10.7717/peerj.2994.

Kikusui T, Winslow JT, Mori Y. 2006. Social buffering: relief from stress and anxiety. *Philosophical Transactions: Biological Sciences,* 1476: 2215-2228. DOI: http://doi.org/10.1098/rstb.2006.1941.

Krook JT, Duperreault E, Newton D, Ross MS, Hamilton TJ. 2019. Repeated ethanol exposure increases anxiety-like behaviour in zebrafish during withdrawal. *PeerJ,* 7. DOI: https://doi.org/10.7717/peerj.6551.

Kurta A, Palestis BG. 2010. Effects of ethanol on the shoaling behavior of zebrafish. *Dose-Response,* 8. DOI: 10.2203/dose-response.10-008.Palestis

Blaser RE, Vira DG. 2014. Experiments on learning in zebrafish (Danio rerio): a promising model of neurocognitive function. *Neuroscience & Biobehavioural Reviews,* 42: 224-231. DOI: https://doi.org/10.1016/j.neubiorev.2014.03.003.

Latané B. Gregariousness and fear in laboratory rats. 1969. *Journal of Experimental Social Psychology,* 5: 61-69. DOI: https://doi.org/10.1016/0022-1031(69)90006-7.

Leighton PLA, Nadolski NJ, Morrill A, Hamilton TJ, Allison WT. 2018. An ancient conserved role for prion protein in learning and memory. *Biology Open,* 7. DOI: https://doi.org/10.1242/bio.025734.

Liddell H. 1949. Some specific factors that modify tolerance for environmental stress. *Research publications-Association for Research in Nervous and Mental Disease,* 29: 155-171.

Masserman JH. 1943. Behavior and neurosis: An experimental psychoanalytic approach to psychobiologic principles. Chicago, IL: University of Chicago Press.

Maaswinkel H, Le X, He L, Zhu L, Weng, W. 2013. Dissociating the effects of habituation, black walls, buspirone and ethanol on anxiety-like behavioral responses in shoaling zebrafish. A 3D approach to social behavior. *Pharmacology Biochemistry and Behavior,* 108: 16-18. DOI: 10.1016/j.pbb.2013.04.009

Maximino C, de Brito TM, da Silva Batista AW, Herculano AM, Morato S, Gouveia Jr A. 2010. Measuring anxiety in zebrafish: a critical review. *Behavior Brain Research,* 214: 157-171. DOI: https://doi.org/10.1016/j.bbr.2010.05.031.

May Z, Morrill A, Holcombe A, Johnston T, Gallup J, Fouad K, Schalomon M, Hamilton TJ. 2016. Object recognition memory in zebrafish. *Behavior Brain Research,* 296: 199-210. DOI: https://doi.org/10.1016/j.bbr.2015.09.016.

Miller N, Greene K, Dydinski A, Gerlai R. 2013. Effects of nicotine and alcohol on zebrafish (Danio rerio) shoaling. *Behavioural Brain Research,* 240: 192-196. DOI: 10.1016/j.bbr.2012.11.033

Muller TE, Nunes SZ, Silveira A, Loro VL, Rosenberg DB. 2017. Repeated ethanol exposure alters social behavior and oxidative stress parameters of zebrafish. *Progress in Neuropsychopharmacology & Biological Psychiatry,* 79: 105-111. DOI: http://dx.doi.org/10.1016/j.pnpbp.2017.05.026

Muller, TE, Ziana PR, Fontana BD, Duarte T, Stefanello FV, Canzian J, Santos ARS, Rosemberg, DB. 2020. Role of the serotonergic system in ethanol-induced aggression and anxiety: A pharmacological approach using the zebrafish model. *European Neuropsychopharmacology,* 32: 66-76. DOI: https://doi.org/10.1016/j.euroneuro.2019.12.120

Oliveira RF, Faustino AI. 2017. Social information use in threat perception: Social buffering, contagion and facilitation of alarm responses. *Communicative & Integrative Biology,* 10: 44329. DOI: https://doi.org/10.1080/19420889.2017.1325049.

Parker MO, Millington ME, Combe FJ, Brennan CH. 2012. Housing conditions differentially affect physiological and behavioural stress responses of zebrafish, as well as the response to anxiolytics. *PLoS ONE*, 7: 1-9. DOI: 10.1371/journal.pone.0034992

Paiva MI, Sartori BM, Castro TFD, Lunkes LC, Virote BDCR, Murgas LD, De Souza RP, Brunialti-Godard AL. 2020. Behavioral plasticity and gene regulation in the brain during an intermittent ethanol exposure in adult zebrafish population. *Pharmacology, Biochemistry and Behavior,* 192: 172909. DOI: https://doi.org/10.1016/j.pbb.2020.172909

Rubinstein DI. 1978. On predation, competition, and the advantages of group living. *Social Behavior,* 205-231. DOI: https://doi.org/10.1007/978-1-4684-2901-5_9.

Sison M, Gerlai R. 2010. Associative learning in zebrafish (Danio rerio) in the plus maze. *Behavior Brain Research,* 207: 99-104. DOI: https://doi.org/10.1016/j.bbr.2009.09.043.

Speedie N, Gerlai R. 2008. Alarm substance induced behavioral responses in zebrafish (Danio rerio). *Behavior Brain Research,*188: 168-177. DOI: https://doi.org/10.1016/j.bbr.2007.10.031.

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, Kalueff AV. 2012. Modeling anxiety using adult zebrafish: a conceptual review. *Neuropharmacology,* 62: 135-143. DOI: https://doi.org/10.1016/j.neuropharm.2011.07.037.

Suboski MD, Bain S, Carty AE, McQuoid LM, Seelen MI, Seifert M. 1990. Alarm reaction in acquisition and social transmission of simulated predator recognition by zebra danio fish (Brachydanio rerio). *Journal of Comparative Psychology,* 104: 101-112. DOI: 10.1037/0735-7036.104.1.101