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The light-dark task in zebrafish confuses two distinct factors: Interaction between background shade and illumination level preference

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ABSTRACT

The light-dark preference task has been commonly used in rodents to screen for anxiogenic and anxiolytic drugs. However, recent adaptations of the light-dark preference test for zebrafish have produced inconsistent results. Several studies have reported that zebrafish exhibit a preference for light, while others have found a preference for black. We suggest the inconsistencies may be the result of confusing certain parameters of the test leading to improper interpretation. For example, researchers often use "light" interchangeably with "white" and "dark" with "black" when these are two distinct factors: level of illumination vs. background shade. In the current study, we use specifically designed preference tanks to investigate the influence of background shade (i.e. white vs. black) and level of illumination (i.e. light vs. dark) on preference and anxiety-like behaviour. Furthermore, we pharmacologically validate our results by quantifying the effects of ethanol, a drug with known anxiety-altering properties, on anxiety-like behaviours. Here we report that zebrafish's preference varies depending upon background shade and level of illumination. We also found that ethanol administration altered behavioural responses in an illumination- and background shade-dependent manner. Our findings reinforce the need to correctly differentiate between these factors when interpreting results obtained with this behavioural paradigm. Lastly, our results show that simple modifications to the experimental tank in which anxiety-related responses are measured can significantly alter behaviour of zebrafish, supporting the need for standardized testing procedures and/or for detailed description of experimental procedures and the apparatus.

1. Introduction

The use of animal models in behavioural pharmacology has become important for understanding the mechanisms underlying a number of different human disorders. With the increasing prevalence of anxiety-related disorders, there is an ever-growing need for high throughput drug-screens to identify efficacious anxiolytics that can be used as treatment options (Gelfuso et al., 2014; Schwartz et al., 2005). A commonly used behavioural task to quantify anxiety-related behaviours in rodents is the light-dark preference task (Bourin and Hascoett, 2003). Studies that employ the light-dark task have found that rodents exhibit a strong preference for the dark compared to the light compartment, likely due to rodents' nocturnal nature (Bourin and Hascoett, 2003; Hascoet et al., 2001). Higher levels of anxiety-like behaviours are associated with more time spent in the dark compartment, a natural anti-predatory response that is found both in and out of the lab (Sousa et al., 2006). Using this simple and robust behavioural paradigm, researchers could quickly and effectively screen anxiolytic and anxiogenic drugs that alter this anxiety-like behavioural response (Anisman and Matheson, 2005; Belzung and Griebel, 2001).

Although the light-dark preference task was originally developed for rodents, this behavioural paradigm has recently been adapted for zebrafish (*Danio rerio*) due to their increasing popularity in behavioural pharmacology (Blaser and Penalosa, 2011; Gerlai et al., 2000; Lieschkem and Currie, 2007; Maximino et al., 2007; Serra et al., 1999). Zebrafish are effective for high throughout drug screens due to their small size, high fecundity, and the non-invasive drug administration techniques (i.e. immersion method) developed for this species (Champagne et al., 2010; Clark et al., 2011; Gerlai et al., 2000; Howe et al., 2013; Klee et al., 2012; Vacaru et al., 2014). However, unlike rodents, the zebrafish is diurnal, a species-specific idiosyncrasy that may complicate interpretation of results obtained using the light-dark

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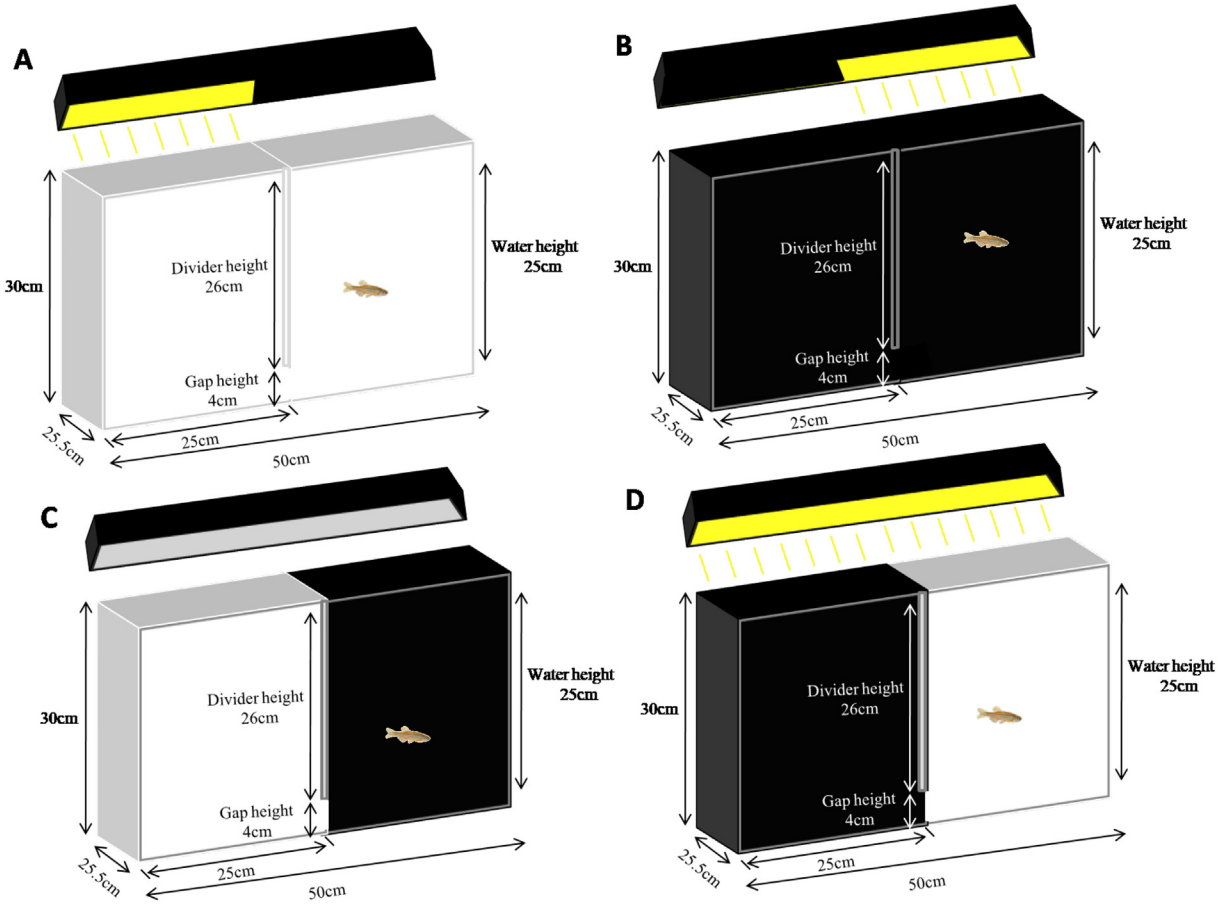

Fig. 1. Behavioural testing tanks illustrating our 2 × 2 (illumination level x background shade) experimental design (tank dimensions are also indicated). (A) All-white tank and (B) all-black tank with high vs. low illumination sides. (C) Low illumination and (D) high illumination tanks with black vs. white sides. All tanks had clear glass lids. Note that the side of high vs low illumination or black vs. white was varied randomly across experimental subjects.

preference task (Champagne et al., 2010; Gerlai et al., 2000). Recent studies that have employed the light-dark preference task in zebrafish have found conflicting results: some researchers report a preference for black (Blaser and Penalosa, 2011; Facciol et al., 2017; Maximino et al., 2007; Maximino et al., 2010; Serra et al., 1999) while others report a preference for light (Blaser and Penalosa, 2011; Champagne et al., 2010; Gerlai et al., 2000).

In the current study, we investigate several factors that may underlie these behavioural inconsistencies. One potential issue is the lack of distinction between level of illumination (i.e. light vs. dark) and background shade (i.e. white vs. black). It has become increasingly common for researchers to use these two terms interchangeably even within the same study, using white interchangeably with light and black with dark (Blaser and Penalosa, 2011; Maximino et al., 2007; Serra et al., 1999). Another limitation in the current literature is the lack of consistency between testing apparati, making it difficult to compare results between studies. Some methods employ overhead lighting in a dark room (Blaser and Penalosa, 2011; Facciol et al., 2017; Gerlai et al., 2000) some use only ambient light (Blaser and Penalosa, 2011). Some studies use no floor substrate (Champagne et al., 2010; Facciol et al., 2017; Gerlai et al., 1999; Maximino et al., 2007; Serra et al., 1999), while others use grey gravel (Blaser and Penalosa, 2011; Blaser and Rosemberg, 2012). Lastly, some studies use a divider to separate the testing chambers (Blaser and Penalosa, 2011; Facciol et al., 2017; Maximino et al., 2007) while others do not (Champagne et al., 2010; Gerlai et al., 2000; Serra et al., 1999). Specific to the light-dark preference task (i.e. illumination preference), researchers commonly cover one entire side of the testing apparatus with cardboard or paper to create the "dark" compartment (Blaser and Penalosa, 2011; Champagne et al., 2010; Gerlai et al., 2000), a manipulation that may further complicate interpretation of results because it introduces the potential confound of a choice between a cave-like vs. open environment rather than illumination level.

In a previous study, we showed that zebrafish exhibited a significant preference for black over white background shade, which was distinctly different from preference for illumination level (i.e. light vs. dark). However, we were unable to show that zebrafish exhibit a preference for light vs. dark, possibly due to the introduction of a divider to separate the compartments (Facciol et al., 2017). In the current study, we employed modified behavioural testing tanks to further investigate preference for background shade and/or illumination level, as well as the possible interaction between these two factors. In addition, in the current study we also investigate whether preference for background shade and/or illumination level is altered by the administration of ethanol, a commonly used pharmacological compound with anxiogenic and anxiolytic properties depending on dose, duration, and context of exposure (Tran and Gerlai, 2013).

2. Methods

2.1. Animals and housing

132 adult wildtype zebrafish (*Danio rerio*) of mixed sexes were used in this experiment. Zebrafish were purchased from Petsmart (Oakville, ON, Canada), and housed in 100 L tanks in groups of 50–60 per tank. The rationale for using such a genetically undefined population of fish is that these fish likely do not exhibit strain-specific idiosyncrasies. The rationale for using pet-store purchased fish acclimatized to our facility,

instead of breeding these fish and using their offspring, is that numerous zebrafish investigators do not have the capacity to maintain large stocks of zebrafish and often use pet-store bought animals. Fish were fed daily with a mixture of dry flake food (2:1 Tetramin:Spirulina) twice a day. Water quality was maintained at optimal levels and checked daily (salinity: 150-500uS; pH: 6.5–7.5; temperature: 27–29 °C). A 14:10 light dark cycle was maintained with lights on at 7:00 h. Behavioural testing occurred between 9:00 to 18:00 h.

2.2. Tank design

Four 37 L behavioural testing tanks as described in Facciol et al. (2017) were used with minor modifications (Fig. 1). Behavioural testing was conducted in a completely dark room with one overhead light per tank (15 W bulb). Tanks 1 and 2 were used to investigate illumination preference while controlling for background shade. Tank 1 was all-white (with the front side left uncovered for video recording) with a white divider down the center separating the light and dark chambers. Fish were able to cross between chambers by either swimming below (4 cm gap) or through 4 holes in the divider (each hole was 4 × 4 cm, two positioned closer to the surface and two closer to the bottom, spaced approximately 5 cm apart). Tank 2 was all black (with the front side left uncovered for video recording) with a black divider designed similar to the all-white divider. A dark chamber for both Tanks 1 and 2 was created by covering half of the overhead tank light with 4 layers of printer paper instead of the tank itself, avoiding the confound of creating a cave-like environment. The paper allowed for enough light to enter for video-tracking purposes yet still created a strong contrast between the dark and the light side. Light intensity inside and outside each tank was measured using a REED ST-1301 Precision Lux Meter (REED Instruments; Toronto, Canada). Light intensity outside the all-white tank ranged from 1017 (light side) to 265 Lux (dark side), and light intensity inside the all-white tank ranged from 1650 (light side) to 266 Lux (dark side). Light intensity outside the all black tank ranged from 355 (light side) to 21 Lux (dark side), and light intensity inside the all black tank ranged from 750 (light side) to 52 Lux (dark side).

Tanks 3 and 4 were used to investigate white-black preference while controlling for illumination level by using two half white half black tanks. Tank 3 had low illumination throughout, achieved by covering the entire overhead tank light with 4 layers of printer paper, allowing only enough light for video-tracking to work. Tank 4 had high illumination as the overhead tank light was left uncovered. Tanks 3 and 4 also had dividers down the center identical in physical measures to those described for Tanks 1 and 2 to maintain methodological consistency. However, the divider for Tanks 3 and 4 had a black side and a white side to match the background shade of their respective compartments. Light intensity outside the low illumination tank ranged from 39 (white side) to 14Lux (black side), and light intensity inside the low illumination tank ranged from 65 (white side) to 45 Lux (black side). Light intensity outside the high illumination tank ranged from 867 (white side) to 335 Lux (black side), and light intensity inside the high illumination tank ranged from 1201 (white side) to 550 Lux (black side).

2.3. Behavioural testing procedure

Zebrafish were netted from their 100 L home tanks and placed into a 1.5 L pre-exposure tank containing either 0 or 1% vol/vol ethanol solution for 30 min. After this 30-min exposure period, zebrafish were placed into a 500 mL transportation tank briefly (containing the same concentration of ethanol) before being placed into the 37 L testing tanks with the same concentration of ethanol (0 or 1%) for 30 min, a total of 60 min alcohol exposure length. Sample sizes were as follows: All-white tank control (n = 20), all-white tank ethanol (n = 19), all-black tank control (n = 18), all-black tank ethanol (n = 12), low illumination control (n = 15), low illumination ethanol (n = 17), high illumination control (n = 15), high illumination ethanol (n = 16). Note the low sample size for the all-black ethanol group was due to the high levels of freezing and dark preference following ethanol administration, making video-tracking difficult. The alcohol dose and exposure period were chosen based upon prior studies that investigated the temporal trajectory of changes of the behaviour and of neurochemicals induced by alcohol exposure correlating with the concentration of alcohol in the brain (Tran and Gerlai, 2013; Tran et al., 2015). The compartment in which the fish was placed (i.e. white or black compartment; light or dark compartment) was randomized to control for potential side bias. For tanks 1 and 2 (constant background shade), the “light” side was randomized in between trials by rotating the overhead light. For tanks 3 and 4 (constant illumination), side bias (i.e. whether the black compartment was on the right or left side of the tank) was controlled for by randomizing black on left vs. black on right tanks between Tanks 3 and 4 (i.e. tanks were randomized between low illumination with black on left, low illumination with black on right, high illumination with black on left, and high illumination with black on right; Fig. 1C/D). Each 30-min behavioural testing session was recorded from the front view using a JVC video recorder.

2.4. Quantification of behaviour

Behavioural responses were quantified using Ethovision XT 8.5, an automated video tracking software. For Tanks 1 and 2, we analyzed the time spent in the dark compartment and for Tanks 3 and 4, we analyzed time spend in the black compartment (Champagne et al., 2010). In addition to preference for a given compartment, we also quantified a number of behavioural parameters that have been associated with anxiety-like responses in zebrafish, including freezing (a measure of immobility), distance to bottom (a measure of bottom dwelling), absolute turn angle (a measure that correlates with erratic movement), and velocity (a measure of locomotion) (Ahmed and Richardson, 2013; Blaser and Rosemberg, 2012; Wong et al., 2010). These 4 behaviours were analyzed across the entire 30-min trial and averaged during the first and last 3 min of the session. During the first 3 min, fish are expected to exhibit maximal fear/anxiety responses due to handling and novelty induced stress (Facciol et al., 2017; Tran and Gerlai, 2013). While during the last 3 min of the session, fish should show the least amount of fear/anxiety as a result of habituation to the environment and the time that elapsed between the handling episode and the behavioural recording. Lastly, the above behaviours were also compared between compartments (i.e. in the light vs. dark compartments and in the white vs. black compartments).

2.5. Statistical analysis

The time-course of behavioural responses was analyzed using a two-way repeated measures ANOVA with ethanol (two levels: 0 and 1% ethanol) and illumination (two levels: light vs. dark – For Experiment 1) or background shade (two levels: white vs. black – For Experiment 2) as between-subject factors, and “time” (30 levels: 30 1-min intervals) as the repeated measures factor. In the case of a significant main effect or interaction, and since Tukey Post-hoc analyses are not appropriate for repeated measures ANOVA, behavioural responses were averaged in the first and last 3 min of recording to compare treatment groups across these periods. Tukey Post-hoc analyses were subsequently conducted to compare all four groups (2 alcohol treatment groups x 2 periods) to each other with significance reported at $p \leq 0.05$ (Group 1 = first 3 control, Group 2 = first 3 ethanol, Group 3 = last 3 control, Group 4 = last 3 ethanol). The rationale for focussing on the first 3 and last 3 min of the session is given in detail by Facciol et al., 2017 and Tran and Gerlai, 2013. Briefly, these periods are expected to represent maximal fear and maximal habituation-related responses. To compare behavioural changes between the different compartments (i.e. between the light vs. dark and white vs. black), behavioural responses were averaged over the entire duration that zebrafish were in those

Table 1
Results summary of Experiment 1: effects of background shade on light vs. dark preference.

Behaviour	Tank	First 3 vs. last 3 min	Compartment
Duration in dark	1 (All-white)	No significant differences	N/A
(sec)	2 (All-black)	Ethanol increased time in dark in first 3 but not last 3	N/A
Distance to bottom	1 (All-white)	Ethanol increased bottom dwelling in first 3 but not last 3	No significant differences
(cm)	2 (All-black)	Ethanol increased bottom dwelling in first 3 but not last 3	No significant differences
Freezing	1 (All-white)	No significant differences	Ethanol increased freezing in the dark compartment
(sec)	2 (All-black)	Ethanol increased freezing in first 3 but not last 3	Ethanol increased freezing in the dark compartment
Absolute turn angle	1 (All-white)	Ethanol increased turn angle in the first 3 but not last 3	Ethanol increased turn angle in the dark compartment
(deg)	2 (All-black)	Ethanol reduced turn angle in the first 3 but not last 3	Controls decreased turn angle in the dark compartment
Velocity	1 (All-white)	No significant differences	No significant differences
(cm/s)	2 (All-black)	No significant differences	Controls decreased velocity in the dark compartment

respective compartments. Tukey Post-hoc tests were conducted to compare the 4 groups to each other (i.e. Group 1 = dark or black compartment control, Group 2 = dark or black compartment ethanol, Group 3 = light or white compartment control, Group 4 = light or white compartment ethanol). To examine preference for dark (Group 1 and 2) and black (Group 3 and 4), the total duration of time spent in each compartment during the first and last 3 min were compared to random chance. A one-sample *t*-test (Test value = 90) was used to compare each of the 4 groups to random chance using a Bonferroni-Holm correction to reduce type 1 error.

3. Results

3.1. Experiment 1: effect of background shade on light vs. dark preference

Table 1 summarizes the results of Experiment 1. Fig. 2 panels A and B show the time zebrafish spent in the dark compartment of the all-white or of the all-black tank over the entire 30-min testing trial. Repeated measures ANOVA revealed a significant main effect of time ($F(29,1885) = 2.745$, $p < 0.001$) and ethanol ($F(1,65) = 7.574$, $p = 0.008$) but no significant main effect of background shade ($p = 0.201$). There was a significant two-way interaction between time and ethanol ($F(29,1885) = 2.050$, $p = 0.001$) and a three-way interaction between time, background shade and ethanol ($F(29,1885) = 1.759$, $p = 0.008$) but no significant interaction between time and background shade ($p = 0.598$) or background shade and ethanol ($p = 0.150$). Time-course analysis revealed ethanol exposed zebrafish in the all-black tank (Fig. 2D) spent significantly more time in the dark compartment compared to controls in the first 3 min ($p < 0.001$), a behavioural response that was also significant when compared to random chance (control group significantly below chance $p = 0.024$; ethanol group significantly above chance $p = 0.004$). Additionally, in the all-black tank, the difference between first and last 3 min ethanol exposed zebrafish spent in the dark compartment was found only to approach but not to reach significance ($p = 0.068$).

Fig. 3 panels A and B show the distance to bottom during the entire 30-min trial. Repeated measures ANOVA found a significant time-dependent change in zebrafish bottom dwelling behaviour ($F(29,1885) = 13.273$, $p < 0.001$), as well as a main effect of ethanol ($F(1,64) = 6.291$, $p = 0.015$) and background shade ($F(1,64) = 5.547$, $p = 0.022$). There was also significant interaction between time and ethanol ($F(29, 1885) = 5.525$, $p < 0.001$) but no significant

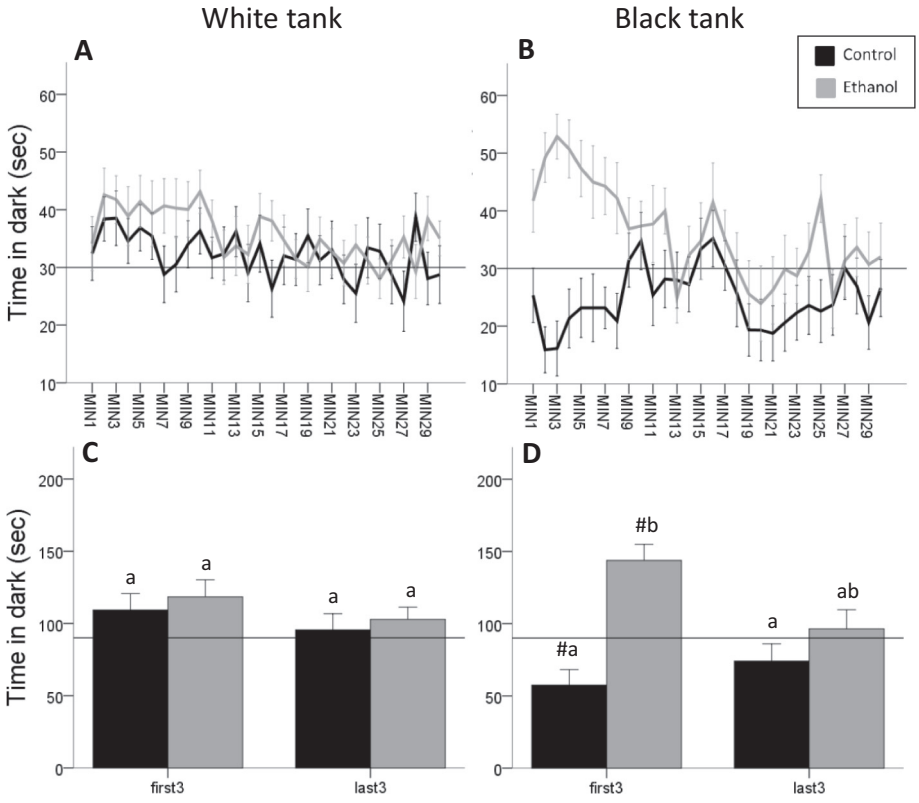

Fig. 2. Mean ± S.E.M. time zebrafish spent on the dark side of the illumination preference in the all-white tank. (A) during the entire 30-min testing session and (C) during the first and last 3 min. Mean ± S.E.M. time zebrafish spent on the dark side of the illumination preference in the all-black tank (B) during the entire 30-min testing session and (D) during the first and last 3 min. Letters that differ from each other indicate significance at $p \leq 0.05$. # indicates significant ($p < 0.05$) departure from chance level performance (indicated by solid line).

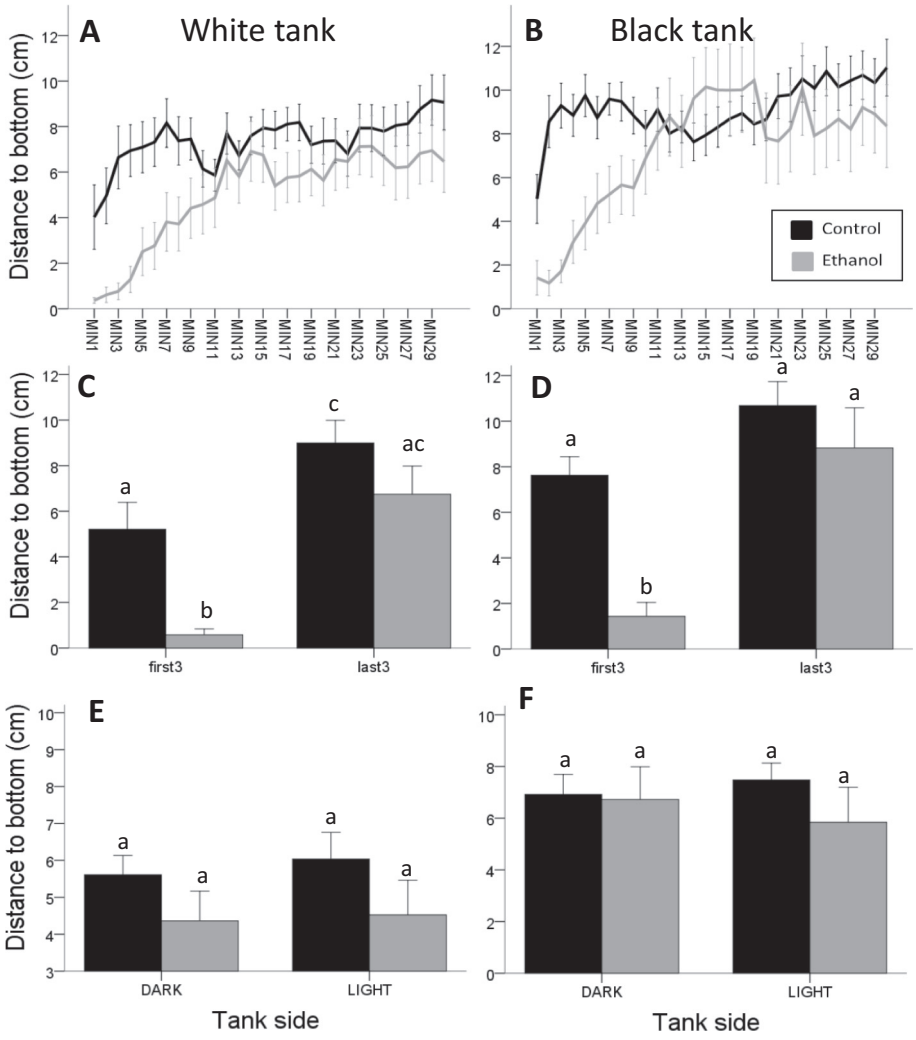

Fig. 3. Mean + S.E.M. distance to bottom in the all-white tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the light vs. dark compartment. Mean + S.E.M. distance to bottom in the all-black tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the light vs. dark compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

interactions between time, background shade and ethanol ($p = 0.343$) or background shade and ethanol ($p = 0.778$) or time and tank ($p = 0.871$). Tukey Post-hoc analysis revealed that in the all-white tank (Fig. 3C), both control ($p = 0.041$) and ethanol ($p < 0.001$) treated zebrafish stayed closer to the bottom in the first 3 min compared to the last 3 min. However, in the first 3 min ethanol exposed zebrafish were significantly closer to the bottom compared to controls ($p = 0.009$) (Fig. 3C). In the all-black tank (Fig. 3D), only ethanol exposed zebrafish showed greater bottom dwelling in the first 3 compared to the last 3 min ($p < 0.001$). Similar to the all-white tank, ethanol exposed zebrafish had higher levels of bottom dwelling in the first 3 min compared to controls ($p = 0.001$) (Fig. 3D). There were no significant differences detected in bottom dwelling when comparing the light vs. dark compartments (Fig. 3E/F).

Fig. 4, panels A and B show the average duration of freezing during the entire 30-min trial. Repeated measures ANOVA revealed significant main effects of time ($F(29,1885) = 5.493$, $p < 0.001$) and ethanol ($F(1,65) = 4.392$, $p = 0.040$), a significant interaction between time and ethanol ($F(29,1885) = 2.624$, $p < 0.001$), and a three-way interaction between time, background shade and ethanol ($F(29,1885) = 1.644$, $p = 0.017$). There was no significant main effect of background shade ($p = 0.426$) and no significant interaction between background shade and ethanol ($p = 0.525$) or time and background shade ($p = 0.841$). In the all-white tank (Fig. 4C), there was no significant difference between the first and last 3 min for either ethanol or untreated control zebrafish. However, in the all-black tank (Fig. 4D), ethanol exposed zebrafish had significantly higher levels of freezing in the first 3 compared to the last 3 min ($p = 0.001$), and ethanol exposed zebrafish had higher levels of freezing in the first 3 min compared to controls ($p < 0.001$). In both the all-white ($p = 0.053$) and all-black tanks (0.011) (Fig. 4E/F), ethanol exposed zebrafish had higher levels of freezing in the dark compartment compared to the light compartment, although the former did not reach significance.

Fig. 5, panels A and B show mean absolute turn angle during the entire 30-min trial. Repeated measures AVONA found a significant main effect of background shade ($F(1,65) = 368.710$, $p < 0.001$) and time ($F(29, 1885) = 2.245$, $p < 0.001$) but no significant main effect of ethanol ($p = 0.963$). There were significant interactions between time and background shade ($F(29,1885) = 4.860$, $p < 0.001$), time and ethanol ($F(29,1885) = 1.494$, $p = 0.044$) and a three-way interaction between time, background shade and ethanol ($F(29, 1885) = 3.456$, $p < 0.001$), however, no significant interaction between background shade and ethanol ($p = 0.282$). Over the entire 30-min trial, control ($p < 0.001$) and ethanol treated zebrafish ($p < 0.001$) in the all-black tank exhibited higher average absolute turn angle when compared to control and ethanol zebrafish in the all-white tank (Fig. 5A/B). In the all-white tank (Fig. 5C), Tukey Post-hoc analysis revealed that ethanol exposed zebrafish had significantly

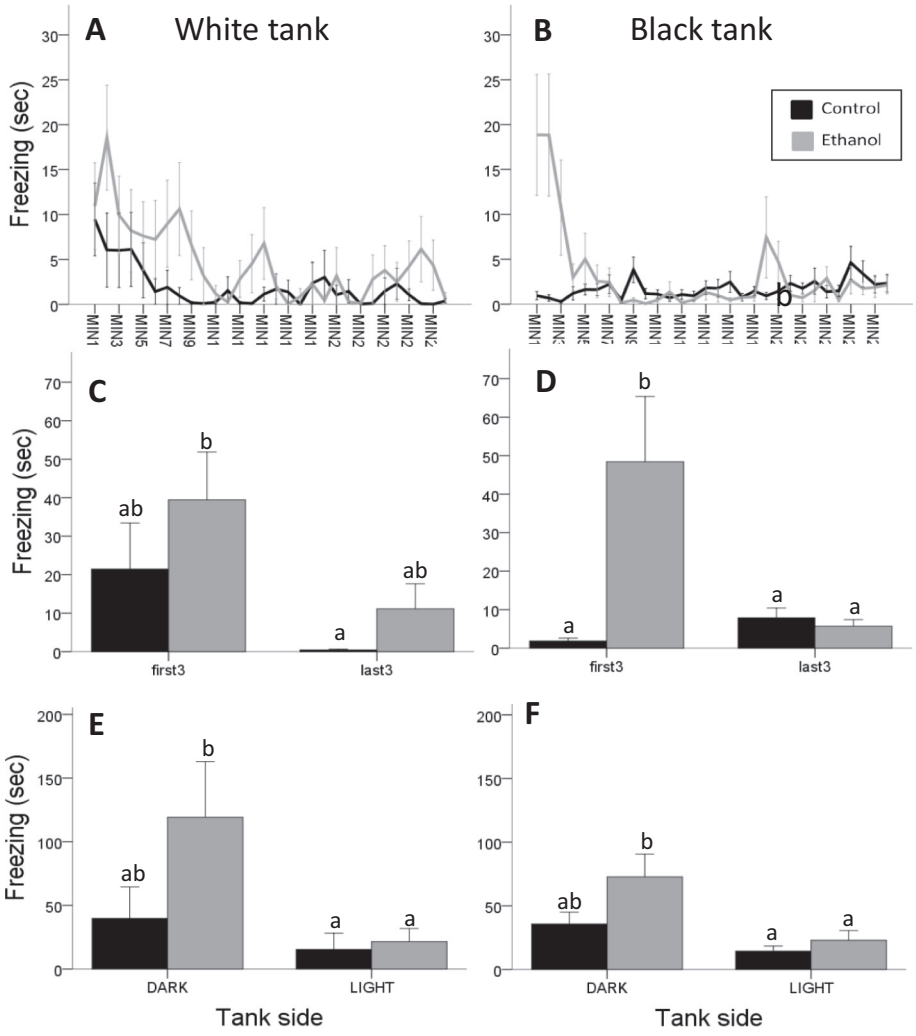

Fig. 4. Mean + S.E.M. freezing in the all-white tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the light vs. dark compartment. Mean + S.E.M. freezing in the all-black tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the light vs. dark compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

higher levels of erratic movement in the first 3 compared to the last 3 min ($p = 0.033$). However, in the all-black tank (Fig. 5D), ethanol exposed zebrafish had significantly lower levels of erratic movement in the first 3 compared to the last 3 min ($p = 0.041$). Also, in the all-black tank, control zebrafish had significantly higher levels of erratic movement compared to ethanol only in the first 3 min ($p < 0.001$). In the all-white tank (Fig. 5E), ethanol exposed zebrafish had significantly higher levels of erratic movement in the dark compartment compared to the light compartment ($p = 0.008$), while in the all-black tank (Fig. 5F), control zebrafish had significantly higher levels of erratic movement in the light compared to the dark compartment ($p < 0.001$).

Fig. 6 panels A and B show the velocity of zebrafish during the entire 30-min testing trial. Repeated measures ANOVA revealed a significant effect of time ($F(29,1885) = 4.708$, $p < 0.001$) and background shade ($F(1,65) = 11.632$, $p = 0.001$) but no main effect of ethanol ($p = 0.608$). There were also no significant 2-way or 3-way interactions. Post-hoc analyses revealed no significant differences between the first and last 3 min of testing for either tank for both control and ethanol treated zebrafish (Fig. 6C/D). In the all-black tank (Fig. 6F), control zebrafish had a higher average velocity in the light compartment compared to the dark compartment ($p = 0.005$), however, there was no significant difference in the all-white tank (Fig. 6E).

3.2. Experiment 2: effect of illumination level on white vs. black preference

Table 2 summarizes the results of Experiment 2. Fig. 7 panels A and B show the amount of time zebrafish spent in the black compartment of the white-black preference tank under low and high illumination over the entire 30-min testing trial. Repeated measures ANOVA revealed a significant main effect of ethanol ($F(1,59) = 18.118$, $p < 0.001$) but no main effect of time ($p = 0.215$) or tank ($p = 0.106$). There was a significant interaction between time and illumination ($F(29,1711) = 1.543$, $p = 0.033$) but no significant interaction between time and ethanol ($p = 0.605$) or illumination and ethanol ($p = 0.477$), however the interaction between time, illumination and ethanol approached significance ($p = 0.069$). Under low illumination (Fig. 7C), ethanol exposed zebrafish spent more time in the black compartment compared to controls in the last 3 min of the trial ($p = 0.048$). Under high illumination, only ethanol exposed zebrafish exhibited a significant preference for the black compartment compared to random chance ($p = 0.016$). However, there were no significant differences between groups in the high illumination condition (Fig. 7D).

Fig. 8 panels A and B show the mean distance to bottom of zebrafish under low and high illumination in the white-black preference task over the entire 30-min trial. Repeated measures ANOVA revealed a significant main effect of illumination ($F(1,59) = 5.946$, $p = 0.018$), time ($F(29,1711) = 9.962$, $p < 0.001$) and of ethanol ($F(1,59) = 13.632$,

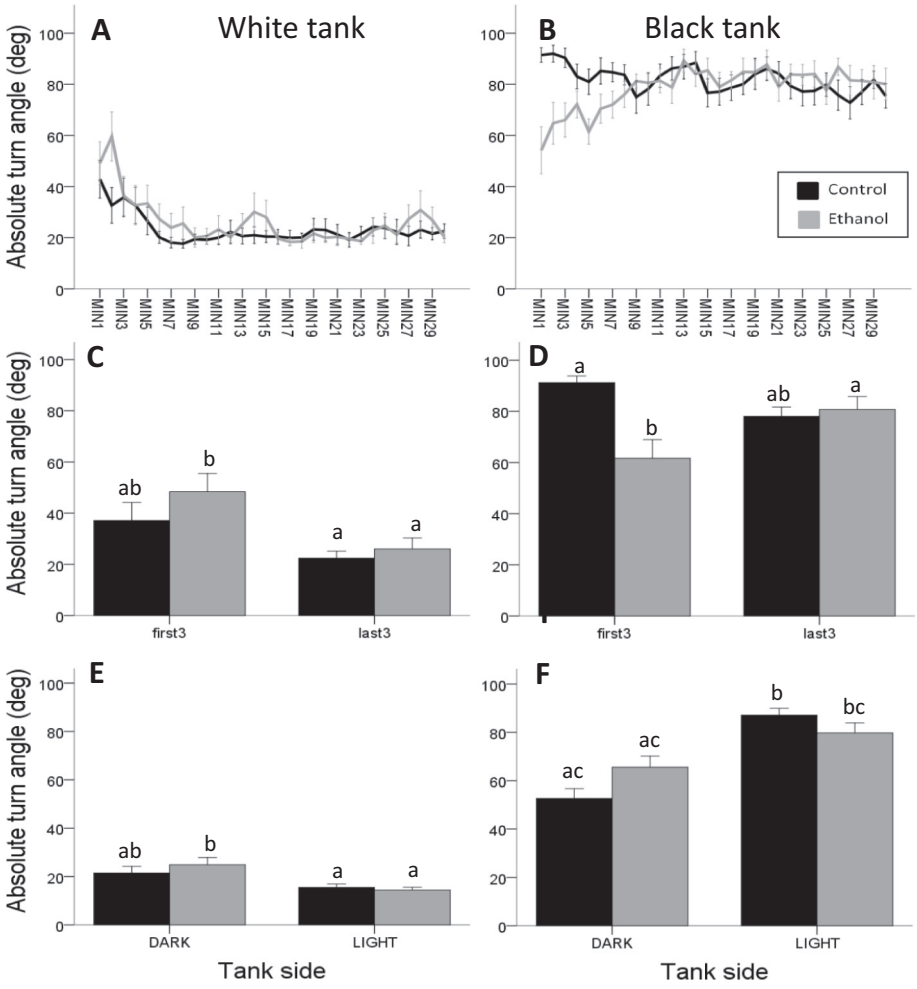

Fig. 5. Mean + S.E.M. absolute turn angle in the all-white tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the light vs. dark compartment. Mean + S.E.M. absolute turn angle in the all-black tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the light vs. dark compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

$p < 0.001$). There was a significant interaction between illumination and ethanol ($F(1,59) = 9.684$, $p = 0.003$) but no significant interactions between time and illumination ($p = 0.473$), time and ethanol ($p = 0.795$), time, illumination and ethanol ($p = 0.416$). Under low illumination (Fig. 8C), ethanol exposed zebrafish stayed closer to the bottom on the tank in the first 3 compared to the last 3 min ($p = 0.004$). Under high illumination (Fig. 8D), both control ($p = 0.005$) and ethanol ($p = 0.002$) treated fish stayed closer to the bottom in the first compared to the last 3 min of the session. Additionally, ethanol exposed zebrafish remained closer to the bottom of the tank compared to controls in both the first ($p = 0.024$) and last 3 min ($p = 0.038$). Lastly, under high illumination (Fig. 8F), ethanol exposed zebrafish stayed closer to the bottom compared to controls in both the black ($p = 0.001$) and white ($p < 0.001$) compartments.

Fig. 9 panels A and B show the duration of freezing of zebrafish under low and high illumination over the entire 30-min in the white-black preference task. Repeated measures ANOVA revealed a significant main effect of time ($F(29,1711) = 4.752$, $p < 0.001$) and ethanol ($F(1,59) = 8.296$, $p = 0.006$), however no main effect of illumination ($p = 0.835$). There was significant interactions between time and illumination ($F(29,1711) = 3.365$, $p < 0.001$) but no significant interaction between time and ethanol ($p = 0.922$), illumination and ethanol ($p = 0.362$) or time, illumination and ethanol ($p = 0.215$). Post-hoc analysis revealed that ethanol exposed zebrafish had significantly higher levels of freezing in the first 3 compared to the last 3 min under high illumination (Fig. 9D) ($p = 0.034$), but no significant differences under low illumination (Fig. 9C). In the low illumination tank, ethanol exposed zebrafish had higher levels of freezing compared to controls in the black side of the tank ($p = 0.012$) (Fig. 9E).

Fig. 10 panels A and B show absolute turn angle during the entire 30-min white-black preference test in the high and the low illumination tanks. Repeated measures ANOVA showed a significant main effect of time ($F(29,1711) = 4.782$, $p < 0.001$) and illumination ($F(1,59) = 176.156$, $p < 0.001$), but no main effect of ethanol ($p = 0.150$). There was a significant interaction between time and illumination ($F(29,1711) = 6.365$, $p < 0.001$) and time and ethanol ($F(29,1711) = 1.639$, $p = 0.018$) but no significant interactions between time, illumination and ethanol ($p = 0.720$). The interaction between illumination and ethanol approached significance ($p = 0.054$). Post-hoc analysis found that ethanol exposed zebrafish have significantly higher absolute turn angle in the first 3 compared to the last 3 min ($p = 0.006$) under low illumination (Fig. 10C), but no significant differences under high illumination (Fig. 10C). Under low illumination tank (Fig. 10E), ethanol exposed zebrafish had significantly lower levels of absolute turn angle compared to controls, however, this effect was only significant when fish were in the white compartment of the tank ($p = 0.040$).

Fig. 11 panels A and B show the mean velocity of zebrafish during the entire 30-min testing session in the high and low illumination tanks during the white-black preference test. Repeated measures ANOVA revealed a significant effect of time ($F(29,1711) = 10.551$, $p < 0.001$) but no main effect of ethanol ($p = 0.699$) or illumination ($p = 0.121$).

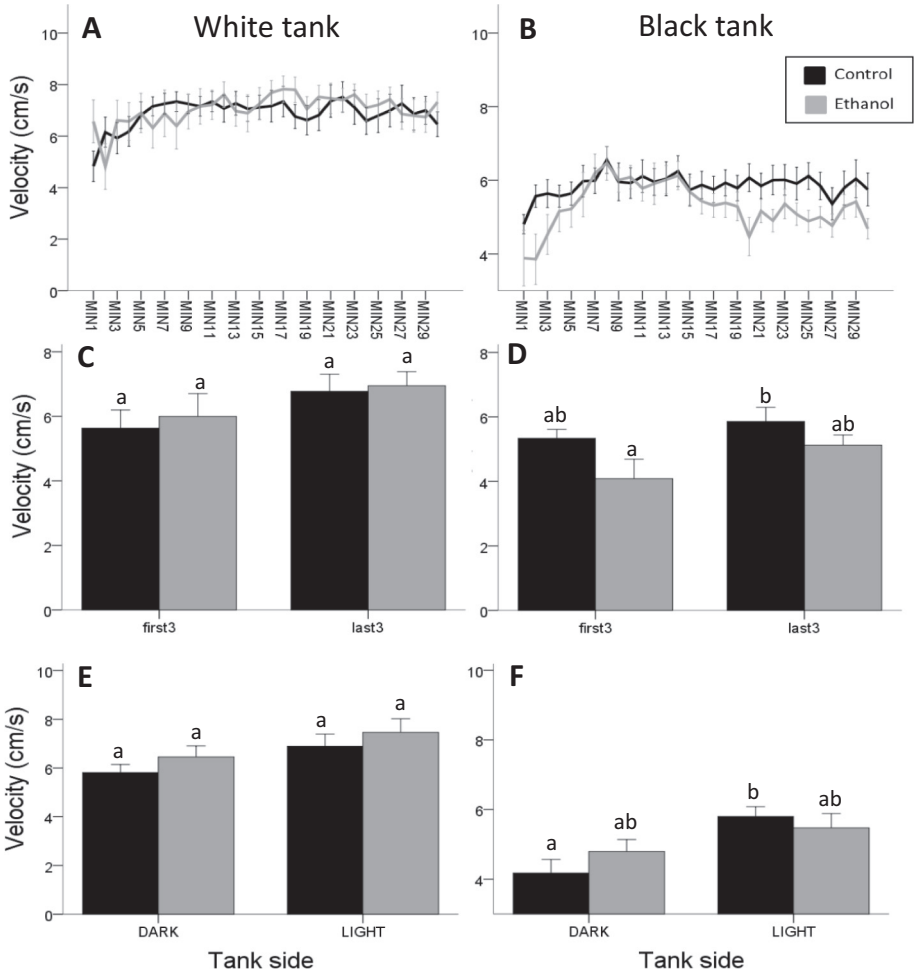

Fig. 6. Mean + S.E.M. velocity in the all-white tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the light vs. dark compartment. Mean + S.E.M. velocity in the all-black tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the light vs. dark compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

Table 2
Results summary of Experiment 2: effect of illumination level on white vs. black preference.

Experiment 2: Effect of illumination level on white vs. black preference			
Behaviour	Tank	First 3 vs. last 3 min	Compartment
Duration in black (sec)	3 (Low)	Ethanol increases time in black in the last 3	N/A
	4 (High)	No significant differences	N/A
Distance to bottom (cm)	3 (Low)	Ethanol increases bottom dwelling in first 3 but not last 3	No significant differences
	4 (High)	Ethanol increases bottom dwelling in the first and last 3	Ethanol increased bottom dwelling in both compartments
Freezing (sec)	3 (Low)	No significant difference between groups	Ethanol increased freezing in the black compartment
	4 (High)	Ethanol increased freezing in the first 3 but not last 3	No significant differences
Absolute turn angle (deg)	3 (Low)	No significant differences	Ethanol increased turn angle in black compartment
	4 (High)	Ethanol increased turn angle in the first 3 but not last 3	No significant difference between groups
Velocity (cm/s)	3 (Low)	No significant difference between groups	Ethanol decreased velocity in the white compartment
	4 (High)	No significant differences	No significant differences

There was a significant interaction between time, illumination and ethanol ($F(29,1711) = 2.330$, $p < 0.001$), but no significant interactions between time and illumination ($p = 0.059$), time and ethanol ($p = 0.467$), or illumination and ethanol ($p = 0.180$). There were no significant differences between control and ethanol treated zebrafish in the first 3 compared to the last 3 min under either high or low illumination (Fig. 11C/D). Under low illumination (Fig. 11E), control zebrafish had a greater velocity on the white side of the tank compared to the black side ($p = 0.009$).However, ethanol exposed zebrafish had significantly lower velocity compared to controls when inside the white compartment ($p = 0.011$), but not the black compartment ($p > 0.05$).

4. Discussion

Numerous factors may influence choice zebrafish make in the light-dark task. For example, the number of fish tested together has been shown to have an effect (Mansur et al., 2014). In the current study we decided to test a single subject at a time in the light-dark task. The primary goal of this task is to measure fear and/or anxiety responses. Given that the zebrafish is a shoaling species, and for this species being

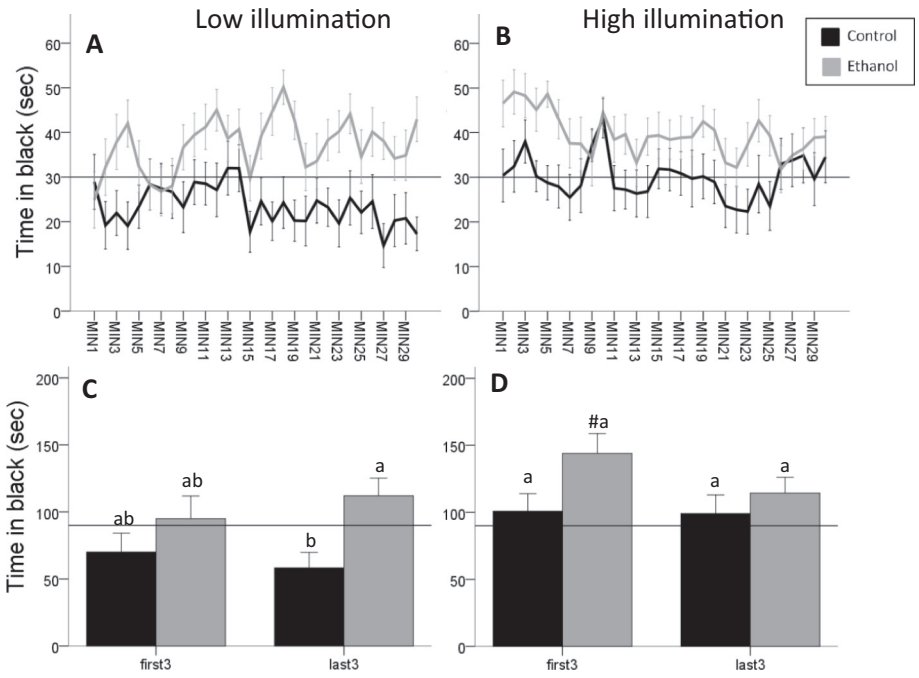

Fig. 7. Mean ± S.E.M. time zebrafish spent on the black side of the illumination preference in the low illumination tank (A) during the entire 30-min testing session and (C) during the first and last 3 min. Mean ± S.E.M. time zebrafish spent on the black side of the illumination preference in the high illumination tank (B) during the entire 30-min testing session and (D) during the first and last 3 min. Letters that differ from each other indicate significance at $p \leq 0.05$.# indicates significant ($p < 0.05$) departure from chance level performance (indicated by solid line).

in a group reduces fear and anxiety, we argued that testing multiple subjects at a time would defeat the purpose of the light-dark paradigm. Although the effects of a variety of psychoactive compounds, e.g. anxiolytic and antidepressant drugs, have been investigated in zebrafish using the light-dark task (e.g. Magno et al., 2015), the results obtained with the paradigm were often controversial as some found "light" (e.g. Blaser and Penalosa, 2011; Champagne et al., 2010; Gerlai et al., 2000) while others found "dark" preference depending on specific

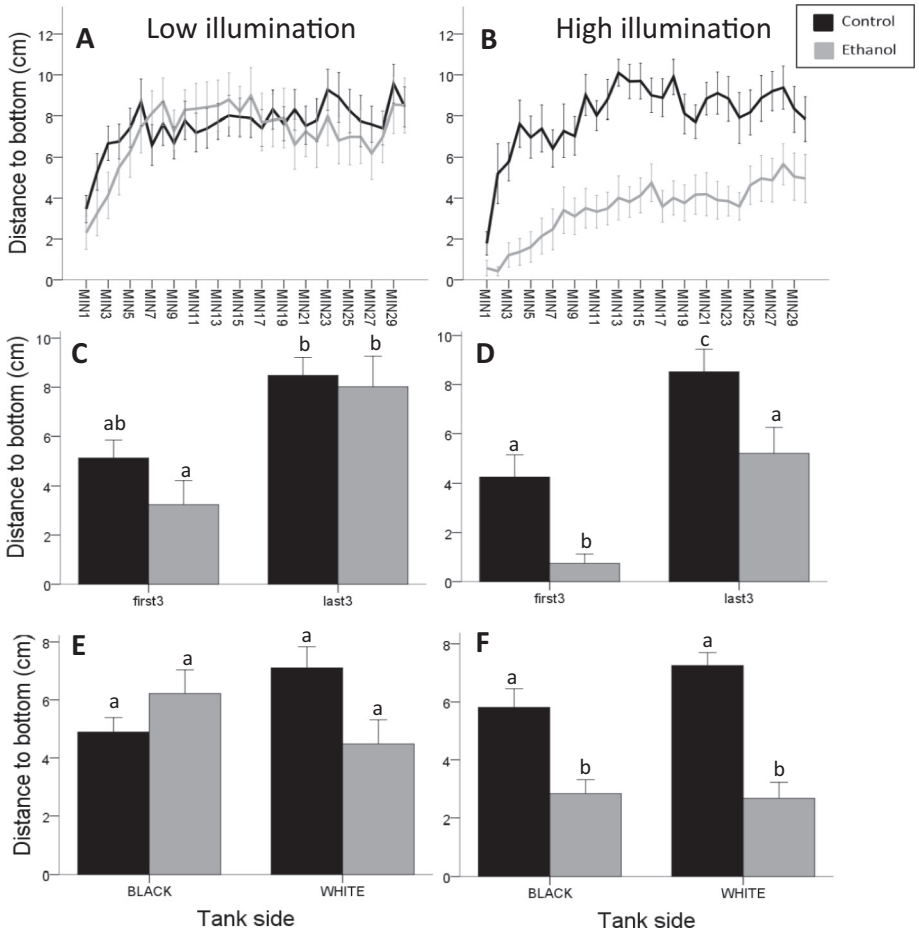

Fig. 8. Mean + S.E.M. distance to bottom in the low illumination tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the white vs. black compartment. Mean + S.E.M. distance to bottom in the high illumination tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the white vs. black compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

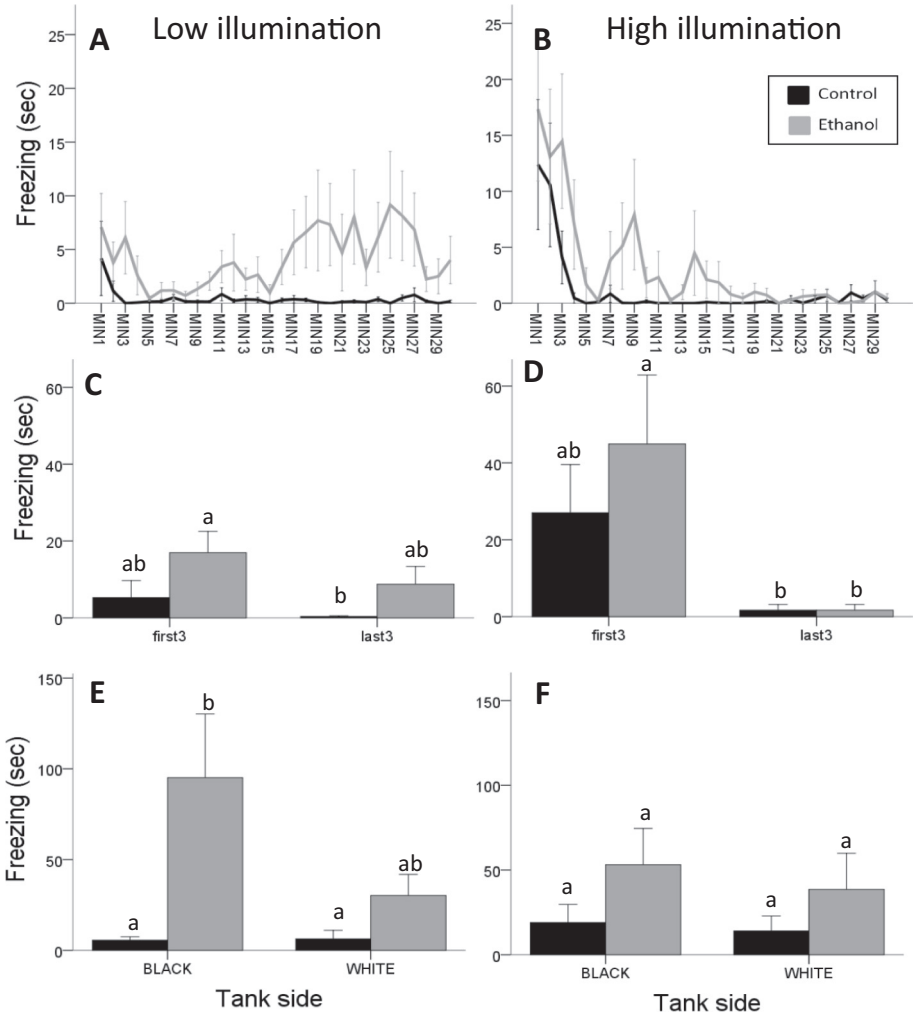

Fig. 9. Mean + S.E.M. freezing in the low illumination tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the white vs. black compartment. Mean + S.E.M. freezing in the high illumination tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the white vs. black compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

experimental parameters (Blaser and Penalosa, 2011; Facciol et al., 2017; Maximino et al., 2007; Maximino et al., 2010; Serra et al., 1999). In the current study, we attempted to disentangle the effects of two potentially distinct factors: the effect of background shade vs. the effect of the level of illumination using a 2 × 2 experimental design. We also studied how performance of zebrafish in the light-dark task changed in response to acute ethanol administration. We found that the effect of background shade and of the level of illumination influenced the choice zebrafish made as well as other behaviours zebrafish exhibited in a non-additive manner, i.e., we found a significant interaction between these factors. We also found that these effects were modified by acute alcohol administration.

In the all-black tank, control zebrafish initially preferred the well illuminated compartment compared to the dark compartment, a finding not replicated in the all-white tank. This high illumination preference in the black tank was reversed by acute ethanol treatment (Fig. 2B). These findings suggest that fear and/or anxiety manifests in control fish as dark (low illumination level) avoidance as found previously by Blaser and Penalosa (2011), Champagne et al. (2010) and Gerlai et al. (2000). This dark avoidance, i.e. light preference, was found to diminish with time, a result likely due to habituation of fear/anxiety. Interestingly, when the tank's background was white, we could not detect a significant ethanol induced change in response. In fact, we also could not detect a preference by control fish for high illumination. Conceivably, the all-white background was too aversive for the experimental fish, leading to no preference for any side, a hypothesis supported by previous studies showing a tank with a white background to be more aversive, eliciting elevated anxiety-like behaviours compared to tanks with transparent or black backgrounds (Blaser and Rosemberg, 2012; Blaser et al., 2010).

In addition to tank side preference, we also recorded other behaviours that helped us further interpret our findings. As mentioned above, in the tank with the black background we found ethanol treated zebrafish to spend significantly more time in the dark compartment compared to control zebrafish during the first 3 min of the preference test, a response which habituated over time (Fig. 2D). We also found the ethanol exposed zebrafish to show reduced distance to bottom (Fig. 3D) and higher levels of freezing (Fig. 4D) compared to controls, responses which also habituated by the end of the testing session. Ethanol exposed zebrafish also had significantly higher levels of freezing in the dark compared to the light compartment (Fig. 4F). Notably, control zebrafish exhibited greater turn angle (Fig. 5F) and velocity (Fig. 6F) in the light compared to the dark compartment. Our results with ethanol are also in line with previous studies that have demonstrated anxiogenic effects of ethanol at high concentrations (i.e. 1% vol/vol) (Tran and Gerlai, 2013). Although greater bottom dwelling and freezing among ethanol treated zebrafish could be interpreted as a sedative effect, this is unlikely the case because there was no significant reduction in average velocity between ethanol exposed and control zebrafish in the all-white tank (Fig. 6A), and also elevated freezing and decreased distance to

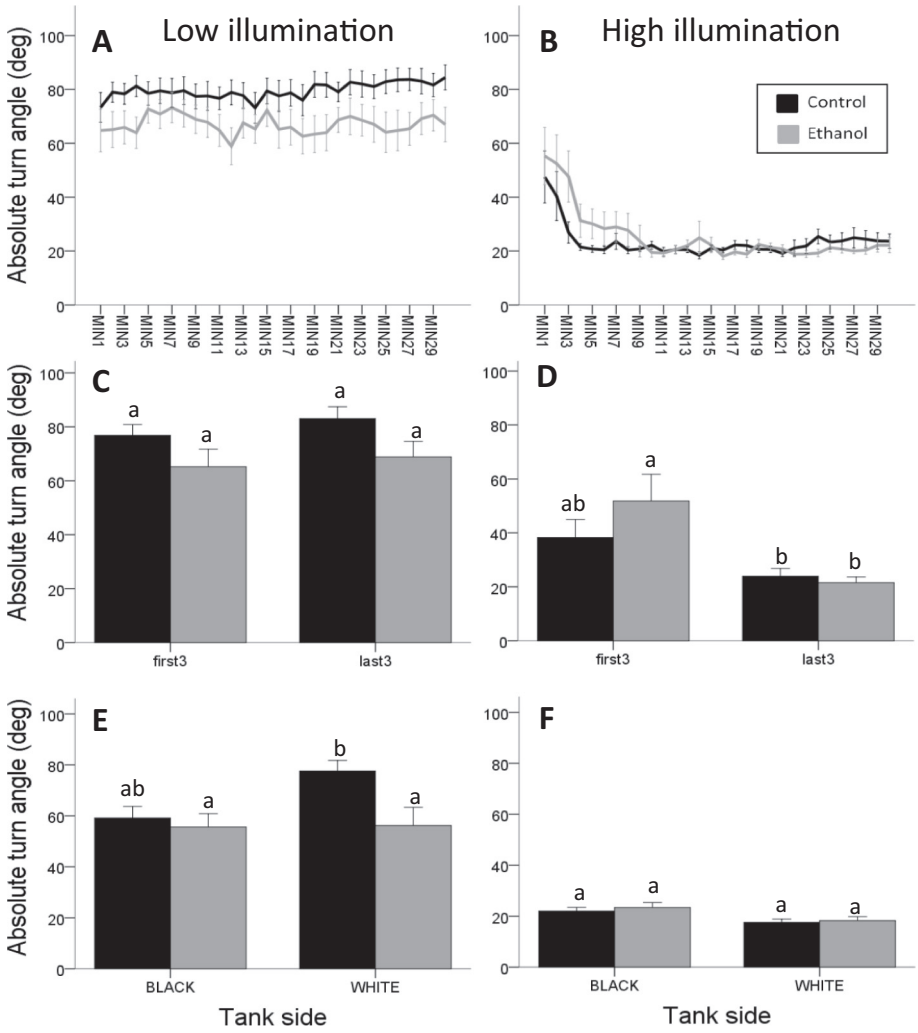

Fig. 10. Mean + S.E.M. absolute turn angle in the low illumination tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the white vs. black compartment. Mean + S.E.M. absolute turn angle in the high illumination tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the white vs. black compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

bottom habituated with time. Thus, taken together, our results suggest that ethanol, as administered here, was anxiogenic and enhanced scototaxis by increasing preference for the dark compartment in the all black tank, a conclusion that has been supported by a number of studies (Blaser and Penalosa, 2011; Facciol et al., 2017; Maximino et al., 2010).

An important question, however, remains unresolved. If elevated anxiety induced by handling and being exposed to a novel test tank, as we argued, led to dark avoidance in control fish, why did anxiety further elevated by ethanol lead to the opposite choice, i.e., dark preference in the ethanol treated fish (Fig. 2B)? The two, but somewhat related, working hypotheses we propose to answer this question are as follows. One, light vs dark preference is not absolute but depends upon the level of anxiety. When lower level of anxiety is induced, zebrafish may prefer well illuminated places, but when higher level of anxiety is induced, low illumination level is preferred. Related to this hypothesis is the second possibility that under low anxiety active avoidance response (escape) is preferred, for which zebrafish need to use their vision (hence the high illumination preference). Under high anxiety level, on the other hand, passive avoidance (hiding) may be preferred, hence the low illumination preference, working hypotheses whose validity will be ascertained empirically in the future.

In Experiment 2, we investigated preference for white vs. black (background shade) in a well illuminated and in a dimly illuminated experimental tank. Unlike previously reported in Facciol et al. (2017), in the current study we did not find a preference for black vs. white under either low or high illumination conditions among control zebrafish. However, we report a significant effect of ethanol on white-black preference and anxiety-related behaviours under high illumination. Under low illumination, ethanol exposed zebrafish appeared to show an initial preference for the black compartment, demonstrated by significantly more time spent in the black compartment, a response which habituated over time (Fig. 7C). This initial preference for black was also accompanied by higher levels of anxiety-related behaviours, such as freezing (Fig. 9A). However, this is not believed to be a sedative effect as we found similar levels of turn angle (Fig. 10A) and velocity (Fig. 11A) between ethanol and control zebrafish in the low illumination condition. Ethanol exposed zebrafish also had higher levels of freezing in the black compared to the white compartment (Fig. 9E), again suggesting that preference for black is driven by anxiety similar to what has been found in previous studies (Blaser and Penalosa, 2011; Facciol et al., 2017; Maximino et al., 2010).

Under high illumination, only ethanol exposed zebrafish exhibited a significant preference for the black compartment compared to random chance (Fig. 7B). Ethanol treated zebrafish also exhibited a significant preference for the black compartment in the first 3 min of the recording session, but not in the last 3 min (Fig. 7D). This initial preference for black is also accompanied by reduced distance to bottom (Fig. 8D). Although this anxiety-like behavioural response habituated over time,

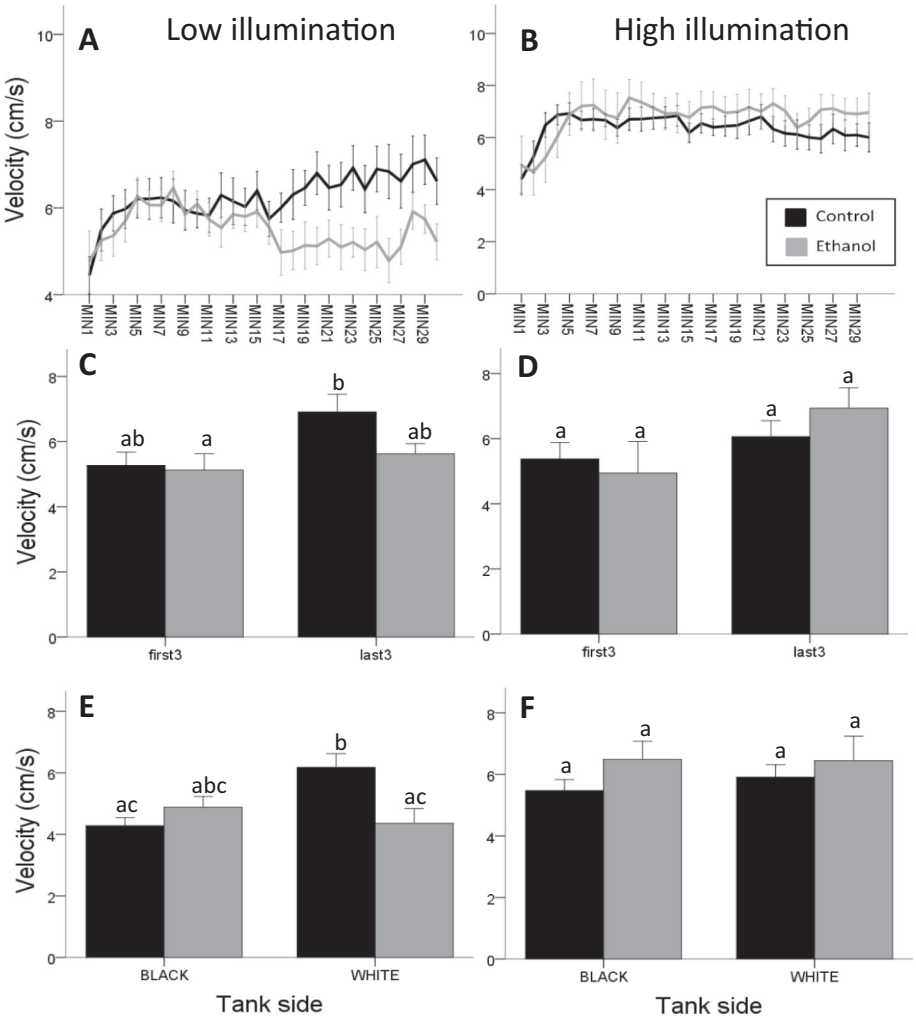

Fig. 11. Mean + S.E.M. velocity in the low illumination tank (A) during the entire 30-min testing session, (C) during the first and last 3 min, and (E) in the white vs. black compartment. Mean + S.E.M. velocity in the high illumination tank (B) during the entire 30-min testing session, (D) during the first and last 3 min, and (F) in the white vs. black compartment. Letters that differ from each other indicate significance at $p \leq 0.05$.

ethanol exposed zebrafish remained closer to the bottom compared to controls (Fig. 8B), suggesting ethanol's anxiogenic effect attenuated habituation to the aversive environment. Higher levels of freezing (Fig. 9D) and erratic movement (Fig. 10D) were also observed to habituate over time in ethanol exposed zebrafish. Together, these behavioural results suggest that under high illumination, ethanol treated zebrafish exhibit an initial preference for black over white, a response likely resulting from ethanol-induced anxiety.

Our results show that factors background shade and level of illumination significantly alter how zebrafish respond in the "light-dark" preference task. Furthermore, using ethanol as an anxiogenic agent, along with behavioural measures of anxiety in zebrafish, we could pharmacologically validate the light-dark paradigm and attribute preference behaviour as being driven by anxiety. In Experiment 1, the combination of a forced exposure to a white background and high illumination appeared to produce a highly aversive environment, so much so that it overcame the expected influence of ethanol. It is common practice, especially in zebrafish research, to perform behavioural testing using a white background, as it is easier for video-tracking software to track zebrafish. Our findings suggest that although this makes behavioural tracking easier, the combination of a white background and high illumination may also lead to elevated anxiety altering expected baseline behaviours to which responses of zebrafish treatment groups are compared.

In Experiment 2, ethanol was found to produce an initial anxiety-like response only in the high illumination condition, a response that habituated over time. In the low illumination condition, only bottom dwelling of ethanol exposed zebrafish showed a habituation response (Fig. 8C). While in the high illumination tank, all behaviours except velocity (Fig. 11D) showed a significant habituation response over time. Similar to Experiment 1, the high illumination condition may be aversive. However, in the absence of a forced exposure to a white background, we were able to see more robust effects of ethanol. The aversive high illumination condition may compound with the anxiety-producing effects of ethanol, however, only for a short time, as evidenced by habituation of the anxiety-like behavioural responses.

Interestingly, we did not observe significant black or white preference in this experiment, contradicting previous studies that found a significant preference for black to white (Blaser and Penalosa, 2011; Facciol et al., 2017). The reason for this discrepancy may lie in the testing tank design. In the above cited previous studies, there was no divider separating the black and white compartments. In our current experiment, both the high and low illumination tanks had a divider with the black and white sides facing the respectively coloured compartments.

In summary, although the terms "white" is often used interchangeably with "light" and "black" with "dark" our current results demonstrate that background shade (black vs. white) and level of illumination (dark vs. light) represent separate factors that influence the effects of ethanol in a non-additive, interacting, manner. It is thus

important to separate these factors when one uses the so-called "light-dark" test. We also note that the terms "light" vs. "dark" or "white" vs "black" should not be used in absolute sense, i.e., zebrafish likely exhibit their preference dependent upon illumination or background shade levels. What the most and least preferred illumination levels or background shades may be for zebrafish will have to be established using experimental procedures in which these levels are systematically and continuously varied. Last, we emphasize that in addition to the complications arising from not distinguishing illumination level vs background shade as separate factors, seemingly unimportant variation in methodology, e.g. the use of tank dividers in our case, may also lead to inconsistent results, a notion that must be considered in the context of standardization of methods and the often-discussed reproducibility crisis in science (Gerlai, 2018).

Acknowledgements

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References

Ahmed, F., Richardson, M.K., 2013. Exploratory behaviour in the open field test adapted for larval zebrafish: impact of environmental complexity. Behav. Process. 92, 88–98.
Anisman, H., Matheson, K., 2005. Stress, depression, and anhedonia: caveats concerning animal models. Neurosci. Biobehav. Rev. 29, 525–546.
Belzung, C., Griebel, G., 2001. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav. Brain Res. 125, 141–149.
Blaser, R.E., Penalosa, Y.M., 2011. Stimuli affecting zebrafish (*Danio rerio*) behaviour in the light/dark preference test. Physiol. Behav. 104, 831–837.
Blaser, R.E., Rosemberg, D.B., 2012. Measures of anxiety in zebrafish (*Danio rerio*): dissociation of black/white preference and novel tank test. PLoS One 7, e36931.
Blaser, R.E., Chadwich, L., McGinnis, G.C., 2010. Behavioral measures of anxiety in zebrafish (*Danio rerio*). Behav. Brain Res. 208, 56–62.
Bourin, M., Hascoett, M., 2003. The mouse light/dark box test. Eur. J. Pharmacol. 463, 55–65.
Champagne, D., Hoefnagels, C., de Kloet, R., Richardson, M., 2010. Translating rodent behavioural repertoire to zebrafish (*Danio rerio*): relevance for stress research. Behav. Brain Res. 214, 332–342.
Clark, K.J., Boczek, N.J., Ekker, S.C., 2011. Stressing zebrafish for behavioural genetics. Rev. Neurosci. 22, 49–62.
Facciol, A., Tran, S., Gerlai, R., 2017. Re-examining the factors affecting choice in the light-dark preference test in zebrafish. Behav. Brain Res. 327, 21–28.
Gelfuso, E.A., Rosa, D.S., Fachin, A.L., Mortari, M.R., Cunha, A.O., Beleboni, R.O., 2014. Anxiety: a systematic review of neurobiology, traditional pharmaceuticals and novel alternatives from medicinal plants. CNS Neurol. Disord. Drug Targets 13, 150–165.
Gerlai, R., 2018. Reproducibility and replicability in zebrafish behavioral neuroscience research. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2018.02.005. pii: S0091-3057(17)30608-1 (In Press).
Gerlai, R., Lahac, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebrafish (*Danio rerio*) as a behaviour genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67, 773–782.
Hascoet, M., Bourin, M., Dhonnchadha, B., 2001. The mouse light-dark paradigm: a review. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 25, 141–166.
Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., et al., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503.
Klee, E.W., Schneider, H., Clark, K.J., Cousin, M.A., Ebbert, J.O., Hooten, W.M., et al., 2012. Zebrafish: a model for the study of addiction genetics. Hum. Genet. 131, 977–1008.
Lieschkem, G., Currie, P., 2007. Animal models of human disease: zebrafish swim into view. Nat. Rev. Genet. 8, 353–367.
Magno, L.D., Fontes, A., Gonçalves, B.M., Gouveia Jr., A., 2015. Pharmacological study of the light/dark preference test in zebrafish (Danio rerio): waterborne administration. Pharmacol. Biochem. Behav. 135, 169–176.
Mansur, Bde M., Dos Santos, B.R., Dias, C.A., Pinheiro Mde, S., Gouveia Jr., A., 2014. Effects of the number of subjects on the dark/light preference of Zebrafish (Danio rerio). Zebrafish 11, 560–566.
Maximino, C., Marques, T., Dias, F., Cortes, F.V., Taccolini, I.B., Pereira, P.M., et al., 2007. A comparative analysis of the preference for dark environments in five telosts. Int. J. Comp. Psychol. 20, 351–367.
Maximino, C., de Brito, T.M., Dias, C.A.G.M., Gouveia, A., Morato, S., 2010. Scototaxis as anxiety-like behaviour in fish. Nat. Protoc. 5, 209–216.
Schwartz, T.L., Nihalani, N., Simionescu, M., Hopkins, G., 2005. History repeats itself: pharmacodynamic trends in the treatment of anxiety disorders. Curr. Pharm. Des. 11, 255–263.
Serra, E.L., Medalha, C.C., Mattioli, R., 1999. Natural preference of zebrafish (*Danio rerio*) for a dark environment. Braz. J. Med. Biol. Res. 32, 1551–1553.
Sousa, N., Almeida, O., Wotjak, C., 2006. A hitchhiker's guide to behavioural analysis in laboratory rodents. Genes Brain Behav. 5, 5–24.
Tran, S., Gerlai, R., 2013. Time-course behavioural changes induced by ethanol in zebrafish (*Danio rerio*). Behav. Brain Res. 252, 204–213.
Tran, S., Chatterjee, D., Gerlai, R., 2015. An integrative analysis of ethanol tolerance and withdrawal in zebrafish (Danio rerio). Behav. Brain Res. 276, 161–170.
Vacaru, A.M., Unlu, G., Spitzner, M., Mione, M., Knapik, E.W., Sadler, K.C., 2014. In vivo cell biology in zebrafish – providing insights into vertebrate development and disease. J. Cell Sci. 127, 485–495.
Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., et al., 2010. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behav. Brain Res. 208, 450–457.