Vision of conspecifics decreases the effectiveness of ethanol on zebrafish behaviour

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Aquatic organisms in pharmacology and toxicology research are often exposed 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 and anxiety-like behaviour, we observed significantly greater effects of ethanol in isolated fish, compared to fish with a view of conspecifics during dosing. These results were not explained by altered locomotion during exposure, which might otherwise increase drug uptake. This highlights the need to consider the social environment during exposure when conducting and interpreting behavioural research involving drug or toxicant exposure.

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37   **ABSTRACT**
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39   Aquatic organisms in pharmacology and toxicology research are often exposed to compounds in

40   isolation prior to physiological or behavioural testing. Recent evidence suggests that the presence

41   of conspecifics during a stressful event can modulate behavioural outcomes (called 'social

42   buffering') when testing occurs within the same context. It is unknown, however, whether the

43   social environment during exposure interacts with the efficacy of anxiety-altering substances

44   when subsequently tested in the absence of conspecifics. In this study, zebrafish were

45   individually exposed to habitat water or ethanol (1.0% vol/vol) while untreated conspecifics

46   were visually present or absent during dosing. Using the novel object approach test, a validated

47   test of boldness and anxiety-like behaviour, we observed significantly greater effects of ethanol

48   in isolated fish, compared to fish with a view of conspecifics during dosing. These results were

49   not explained by altered locomotion during exposure which might otherwise increase drug

50   uptake. This highlights the need to consider the social environment during exposure when

51   conducting and interpreting behavioural research involving drug or toxicant exposure.

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58 **INTRODUCTION**

59   Living in a social environment offers many evolutionary advantages. Belonging to a

60 group can facilitate reproduction, enable earlier detection and evasion of predators, and improve

61 resource efficiency (Rubinstein, 1978). Social cues are commonly the mechanism that conveys

62 these messages between conspecifics and can guide responses in uncertain situations (Suboski et

63 al., 1990). Another benefit of conspecifics is a decrease in stress level that minimizes the impact

64 of stressful situations (Kikusui, Winslow & Mori, 2006). This phenomenon, known as 'social

65 buffering', has been experimentally demonstrated in many species including cats (Masserman,

66 1943), goats (Liddell, 1949), rats (Davitz & Donald, 1955; Latané, 1969), humans (Hostinar,

67 Johnson & Gunnar, 2015), and, recently, zebrafish (Oliveira & Faustino, 2017; Faustino, Tacão-

68 Monteiro & Oliveira, 2017).

69   The zebrafish has become a popular model organism for use in a variety of scientific

70 disciplines including pharmacology and toxicology. Behavioural neuroscience tests can be used

71 to analyze a wide variety of cognitive processes in zebrafish including episodic-like memory

72 (Hamilton et al., 2016), object recognition memory (May et al., 2016), classically conditioned

73 memory (Sison & Gerlai, 2010), fear (Speedie & Gerlai, 2008), boldness (Dean et al., 2020), and

74 anxiety-like behaviour (Maximino, de Brito & da Silva Batista, 2010). To test anxiety-like

75 behaviour there are a variety of paradigms available, with the most common being the light/dark

76 preference and novel tank diving tests (for a review see (Maximino, de Brito & da Silva Batista,

77 2010). Due to the reliability of these tests and the practical simplicity in which

78 psychopharmacological substances can be administered to zebrafish (Gerald, Lee & Blaser,

79 2006), adaptive behavioural responses can be easily manipulated with anxiolytic (anxiety-

80 reducing) and anxiogenic (anxiety-enhancing) compounds (Collier & Echevarria, 2013).

81   Ethanol is a classic anxiolytic compound that has been shown to reduce zebrafish

82   anxiety-like behaviour in a variety of paradigms. For instance, 0.25%, 0.5% and 1.0% ethanol

83   increase the amount of time zebrafish spend exploring the light zone of the light/dark test

84   (Gebauer et al., 2011; Fontana et al., 2020) and 0.3% and 0.5% ethanol increase time spent in the

85   top zone of a novel tank dive test (Egan et al., 2009; Müller et al., 2020), both indications of the

86   anxiolytic nature of ethanol. In the novel object approach test, used to test boldness and anxiety-

87   like behaviour, 1.0% ethanol reduces the amount of time zebrafish spend in the outer

88   'thigmotaxis zone' adjacent to the wall of the arena (Johnson & Hamilton, 2017) further

89   exemplifying the anxiolytic nature of ethanol. Using the same test, 1.5% ethanol also increases

90   the time zebrafish spend in the inner zone exploring a novel object (Hamilton et al., 2017b), thus

91   increasing boldness. Taken together, these findings illustrate zebrafish display less anxiety and

92   more boldness following an acute administration of ethanol. Recent evidence, however, suggests

93   that the social environment in which anxiety-altering compounds are administered and/or tested

94   in may influence the behavioural effects of these substances in zebrafish (Faustiona, Tacão-

95   Monteiro & Oliveira, 2017), which may complicate conclusions.

96   Visual and olfactory conspecific cues can decrease the response of zebrafish to an

97   anxiogenic compound when exposure and testing occurs within the same environment (Faustino,

98   Tacão-Monteiro & Oliveira, 2017). Specifically, conspecific water and alarm substance along

99   with a visual of untreated conspecifics induced significantly less freezing and erratic movements

100   than when the adjacent tank was empty and no conspecific water was added (Faustino, Tacão-

101   Monteiro & Oliveira, 2017). When the effectiveness of each type of cue was tested, visual cues

102   were superior to olfactory cues in reducing aversive behaviours and promoting 'social buffering'

103   (Faustino, Tacão-Monteiro & Oliveira, 2017). However, it is unknown whether the presence of

104   conspecifics can alter behavioural response when the fish are subsequently removed from the

105   compound and tested in a separate arena. In other words, does the effect of social buffering

106   persist beyond the exposure to the cues?

107   In the majority of acute pharmacological experiments that test individual fish behaviour,

108   substances are administered while fish are physically isolated, and the exposed fish is then

109   transferred to a behavioural arena for testing (Stewart et al., 2012). Moreover, almost all of the

110   studies published that have examined acute ethanol exposure on behaviour of individual fish

111   have not specified whether conspecifics were within view during dosing (Blaser and Peñalosa,

112   2011, Echevarria et al., 2008; Egan et al., 2009, Fontana et al., 2020, Gerlai et al., 2008).

113   Moreover, only a few other studies have stated that fish were isolated during ethanol dosing

114   (Hamilton et al., 2017b, Johnson and Hamilton 2010). To the best of our knowledge, no study

115   has examined whether the view of conspecifics during dosing may influence the anxiety level of

116   a fish subsequently tested in an isolated testing arena. It is also unknown whether social

117   buffering may also act to alter the effects of anxiety *reducing*, 'anxiolytic' substances. To test

118   these questions, we exposed individual zebrafish to either habitat water or ethanol (1.0% vol/vol)

119   while untreated conspecifics were visually present or absent for the entire exposure period.

120   Following exposure, the fish were transferred to the novel object approach test for quantification

121   of anxiety-like behaviour and boldness (Dean et al., 2020; Krook et al., 2019; Leighton et al.,

122   2018). Finally, we tested whether fish move at different rates and remain closer to conspecifics

123   during the dosing period itself, in order to determine whether the social condition (*Isolated* vs.

124   *In-view*) influences behaviour during drug exposure.

125

126

127   **METHODS**

128 **Subjects and housing**

129

130 Short-fin wild-type zebrafish (n = 90) were acquired from Aquatic Imports (Calgary, AB) at a

131 minimum age of 9-months. Fish were experimentally naïve and comprised of mixed males and

132 females (~50/50 ratio). Following a month-long quarantine period, the fish were held in either 3

133 or 10L polypropylene tanks within a three-shelf bench top system (Aquatic Habitats, Aquatic

134 Ecosystems, Inc. Apopka, FL, USA) which was controlled for filtration and aeration. No fish

135 was ever housed in isolation and tank capacities never exceeded five fish per liter. Temperature

136 and pH remained between 26 - 30°C and 6.0 – 8.0, respectively. Lights were kept on a 12-hour

137 light/dark cycle with lights on at 8AM and off at 8PM. Fish were fed dry brine shrimp (Omega

138 One Freeze Dried Mysis Shrimp nutri-treat, OmegaSea Ltd., Germany) once per day, and after

139 experimentation on test days. All experiments were approved by the MacEwan University

140 Animal Research Ethics Board (AREB) under protocol number 05-12-13 in compliance with the

141 Canadian Council for Animal Care (CCAC) guidelines for the care and use of experimental

142 animals.

143

144 **Experimental design**

145 *Experiment 1:* This experiment used a 2 x 2 factorial design. The between-subject experimental

146 variables included visual access to conspecifics (Fig. 1A,B, *Isolated or In-view*) and the type of

147 substance the fish were exposed to (habitat water (*CTL*) or *Ethanol*) while in the dosing

148 containers. Prior to experimentation, fish were randomly assigned to one of four groups:

149 *Isolated-CTL*, *Isolated-Ethanol*, *In-view-CTL*, and *In-view-Ethanol.* Following exposure,

150 behaviour was tested in the novel object approach test (Fig. 1C,D) to examine whether the social

151 environment during exposure influences the efficacy of ethanol.

152 *Experiment 2:* A follow-up experiment was performed in which we tested the movement of

153 individual zebrafish within the dosing containers in the *Isolated or In-view* conditions while

154 exposed to control water (Fig. 2A,B).

155

156 **Experiment 1: Experimental conditions**

157 *Ethanol exposure:* On the day of experimentation, fish were carried in their habitat tanks into the

158 experimental room prior to feeding and were given at least 10 minutes to acclimatize to this new

159 environment. A white corrugated plastic barrier was set up surrounding habitat tanks to limit

160 external stimuli. Following the habituation period, fish were individually netted from their

161 habitat tanks and placed into one of two experimental dosing containers (600 mL). Each dosing

162 container contained 500 mL of solution and was also surrounded by white corrugated plastic

163 barriers (Fig. 1A). Two dosing containers were used rather than one to increase testing efficiency

164 by allowing two fish to be dosed with a staggered schedule. Once in the dosing container, a

165 square piece of the same plastic was placed on top to prevent evaporation of the solution and to

166 ensure fish remained inside (Cachat et al., 2010; Holcombe et al., 2013). Fish exposed to control

167 water (*Isolated-CTL* (n = 15) or *In-view-CTL* (n = 15)), were placed into dosing containers that

168 only contained habitat water (500 mL). Fish in the ethanol groups (*Isolated-Ethanol* (n = 15) or

169 *In-view-Ethanol* (n = 15)) were placed into dosing containers with 1.0% ethanol. Solutions for

170 each compound were made fresh each day by mixing 5.26 mL of non-denatured, 95% ethanol

171 into 495 mL of habitat water in the respective dosing containers. The selected concentration and

172 duration of ethanol exposure was based on previous experiments in zebrafish (Johnson &

173 Hamilton, 2017).

174   _View of conspecifics:_ Fish were assigned to one of the two _Isolated_ conditions (_Isolated-CTL_ (n

175   = 15), or _Isolated-Ethanol_ (n = 15)) with no view of conspecifics while in the dosing container,

176   or fish were assigned to the _In-view_ conditions (_In-view-CTL_ (n = 15) or _In-view-Ethanol_ (n

177   =15)), with a view of a tank containing 12 untreated conspecifics during dosing. The dosing

178   containers used in the _In-view_ conditions were positioned in front of each other to ensure fish in

179   both dosing containers had equal view of their conspecifics. The same group of conspecifics

180   were used for each _In-view_ condition. A white corrugated plastic barrier covered the remaining

181   two sides of the conspecific tank (Fig. 1B) and water temperatures were maintained between 26

182   and 30°C by seedling heat mats (Hydrofarm Horticultural Products, Petaluma CA). Fish in the

183   _Isolated_ condition were surrounded fully by a white plastic barrier which was also placed on the

184   heat mat. Fish in all conditions remained in the dosing containers for 30 minutes, after which the

185   solution (including the fish) was carefully poured into a net, with a second dosing container

186   collecting the solution. Once in the net, the fish was placed into the adjacent behavioural arena

187   for testing. No data was acquired during dosing in experiment 1.

188

189   **Experiment 1: Behavioural testing**

190   Fish were individually tested in the novel object approach test following the 30-minute exposure

191   period. The behavioural arena used in this experiment was circular and made from white opaque

192   plastic (Ø = 34 cm; depth = 15 cm; Fig.1C). The arena was placed on top of a heat mat to

193   maintain habitat water temperatures and was surrounded by a three-sided white corrugated

194   plastic enclosure to limit external stimuli during testing. Habitat water was added to the arena up

195   to a maximum height of 5 cm and was replaced with fresh habitat water every four hours. An

196   equal amount of heated habitat water was also exchanged whenever temperatures fell below

197   26°C. The object used in this study was a 2 cm x 4.25 cm Lego figurine which was multi-

198   coloured to rule out possible colour preferences (Fig. 1D; Dean et al., 2020; Hamilton et al.,

199   2017b; Johnson & Hamilton, 2017) and was adhered using velcro to the bottom of the arena's

200   center. Prior to testing, three virtual zones representing the thigmotaxis (the outermost zone, 23-

201   34 cm in diameter), inner (center zone of 12 cm in diameter) and transition sones (in between the

202   other two zones, 12-23 cm in diameter) were defined using EthoVision XT motion tracking

203   software (Fig. 1E; version 11.0, Noldus, VA, USA). All experimental procedures occurred

204   between 9AM and 6PM prior to feeding. The time, in seconds, fish spent in each zone

205   (thigmotaxis, transition, inner) was recorded and used as a proxy for anxiety-like behaviour (i.e.

206   increased time in the thigmotaxis zone related to increased anxiety) and boldness behaviour (i.e.

207   increased time in the inner zone is related to increased boldness) (Ou et al., 2015). Locomotion

208   was also assessed by tracking the distance moved(cm) and immobility(s). Fish were tested

209   individually for a period of 10 minutes following dosing and recording began as soon as the fish

210   was placed into the transition zone facing the object.

211

212   **Experiment 2: Experimental conditions and behavioural testing**

213   After the first round of experimentation we sought to determine if the social context during

214   exposure affected the distance fish moved while in the dosing container; possibly this could

215   account for any differences in behavioural outcomes observed? In a second experiment, we

216   tested the activity of a new group of fish in the dosing containers while in either the *Isolated-*

217   *CTL* (n=15) or *In-view-CTL* (n=15) condition. We also quantified whether fish preferred the half

218   of the dosing container close to conspecifics (conspecific side). Following a 10-minute

219   habituation period, one fish was individually netted from their habitat tank and placed into a 600

220   mL dosing container with habitat water (500 mL), identical to procedures in our first experiment.

221   A rectangular piece of white corrugated plastic was placed beneath the dosing container to assist

222   with motion tracking. As in the novel object approach test, a three-sided enclosure was set up

223   during behavioural tracking and seedling heat mats maintained water temperatures. To ensure

224   these fish received the same treatment as fish in the *Isolated-CTL* and *In-view-CTL* conditions in

225   the first experiment, a white piece of corrugated plastic was also set up across the front of the

226   three-sided enclosure (Fig. 2A). For fish in both the *Isolated-Dosing* and *In-view-Dosing*

227   conditions, EthoVision was set up to record the distance (cm) each fish moved throughout the

228   30-minute exposure period while in the dosing container. For fish in the *In-view-Dosing*

229   condition, a habitat tank containing the same conspecifics (n = 12) that were used in the other *In-*

230   *view* conditions was positioned to the right of the beaker. Using EthoVision, the beaker was then

231   vertically split into two equal-sized virtual sections to compare the amount of time, in seconds,

232   fish explored the side of the beaker closest to conspecifics (conspecific side) and the side farthest

233   from conspecifics (empty side; Fig.2B). To rule out external variables potentially contributing to

234   a side preference, the habitat tank was placed to the left of the beaker for the final three of fifteen

235   trials per condition. No differences were observed in the time spent exploring either side of the

236   beaker regardless of whether the habitat tank was on the right or left side of the beaker (Mann-

237   Whitney; conspecific side, U = 10, p = 0.2549; opposite side, U = 10, p = 0.2945) so these were

238   combined for analysis.

239

240   **Statistical analysis**

241   R (version 4.0.2) was used to analyze the data. The majority of the utilized functions are a part of

242   the *stats* library. The exceptions are the *brunnermunzel.test* and *leveneTest* functions, which are a

243 part of the *brunnermunzel* and *car* libraries, respectively (Fox, 2016; Fox and Weisberg, 2019;

244 Munzel and Brunner, 2000). Prior to the model fitting process, an exploratory data analysis was

245 performed on all variables. In particular, we used the *cor.test* function to determine if there was

246 evidence of statistically significant correlations among the measurements in the thigmotaxis,

247 transitions, and inner zones and among the measurements in the distance moved and immobility

248 variables. To analyze the effect of social condition and/or ethanol on anxiety levels, two-way

249 ANOVAs were fitted to each variable using the *aov* function. The normality and constant

250 variance assumptions were checked using Shapiro-Wilks' and Levene's tests, respectively, via

251 the *shapiro.test* and *leveneTest* functions. If either assumption was violated, a Gamma

252 Generalized Linear Model (GLM) with log link was fitted to the data using the *glm* function. If

253 there was evidence that the Gamma GLM did not sufficiently fit the data, then Wilcoxon rank-

254 sum (WRS) tests were fitted to compare both main effects and the treatment combinations of

255 interest via the *wilcox.test* and *pairwise.wilcox.test* functions. In cases where the Gamma GLM

256 fit sufficiently, the Brunner-Munzel test (Munzel and Brunner, 2000) was used to analyze

257 differences between the treatment combinations of interest via the *brunnermunzel.test* function.

258 Significance across all tests was determined using a 5% significance level. In what follows, we

259 use t(df) to designate a Student's t test statistic with df degrees of freedom, F(df1,df2) to

260 represent a F-test statistic with df1 and df2 degrees of freedom, W to represent the test statistic

261 from a Wilcoxon Rank Sum test, and W_{BM} to represent the test statistic from the Brunner-

262 Munzel test.

263

264 **RESULTS**

265 **Analysis of Correlation**

266   Time spent in the thigmotaxis zone was negatively correlated with time spent in the transition

267   zone (r = -0.816, t(58) = -10.74, p < 0.001) and time spent in the transition zone (r = -0.754, t(58)

268   = -8.74, p < 0.001). On the contrary, time spent in the inner zone was positively correlated, but

269   the result was not statistically significant (r = 0.235, t(58) = 1.84, p = 0.071). Distanced moved

270   was significantly negatively correlated with time spent immobile (r = -0.643, t(58) = -6.400, p <

271   0.001). While the results show that there are significant correlations between the times spent in

272   each zone and between distance moved and immobility we follow Hamilton et al., (2017) and

273   Thomson et al., (2020) and study individual behavioural measures rather than consider a

274   principal component analysis as it has been shown that these behavioural measures have a large

275   variance across reports from different research groups.

276

277   **Effect of social context**

278   Social context was found to have a significant effect on behaviour when comparing the time fish

279   in the *In-view* and *Isolate* conditions spent in the thigmotaxis, transition, and inner zones.

280   Specifically, fish spent more time in the thigmotaxis zone in the *In-view* condition than they did

281   in the *Isolate* condition ($t(56) = 4.26$, $p < 0.0001$; Fig. 3A), and less time in the transition zone

282   ($t(56) = 3.98$, $p < 0.001$; Fig. 3B) and the inner zone ($t(56) = 3.40$, $p = 0.001$; Fig. 3C), on

283   average. No significant differences were found between the mean distance fish in the *In-view* and

284   *Isolate* conditions moved ($F(1,56) = 2.44$, $p = 0.12$; Fig. 3D) nor between the median time fish in

285   these groups spent immobile ($W = 470$, $p = 0.77$; Fig. 3E).

286

287   **Investigating social context interactions**

288   Among the control (CTL) groups, the Brunner-Munzel test provided sufficient evidence

289   to conclude that fish in the *Isolate-CTL* group spent more time in the thigmotaxis zone than fish

290   in the *In-View-CTL group* (W_{BM} = -4.34, p < 0.001, Fig. 3A). Whereas, fish in the *In-View-CTL*

291   group spent more time in both the inner and transition zones than fish in the *Isolate-CTL* group

292   (W_{BM} = 3.99, p < 0.001, Fig. 3C and W_{BM} = 4.92, p < 0.0001, Fig. 3B respectively).

293   Among the ethanol groups, the Brunner-Munzel test provided sufficient evidence to

294   conclude that fish in the *In-view-Ethanol* group spent more time in the thigmotaxis zone than fish

295   in the *Isolate-Ethanol group* (W_{BM} = 2.07, p = 0.05, Fig. 3A). Whereas, fish in the *Isolate-*

296   *Ethanol* group spent more time in the inner zone compared to fish in the *In-View-Ethanol* group

297   (W_{BM} = -2.34, p = 0.03, Fig. 3C). No differences were found when comparing the time fish in the

298   *Isolate-Ethanol* and *In-View-Ethanol* groups spent in the transition zone (W_{BM} = -0.93, p = 0.361,

299   Fig. 3B).

300

301   **Effect of type of solution**

302   The type of solution was found to have a significant effect on fish behaviour when comparing the

303   time fish spent in their habitat water (CTL) to the time fish spent in ethanol. In particular, we

304   found that fish dosed in the ethanol solution spent less time in the in the thigmotaxis zone (t(56)

305   = 8.78, p < 0.0001; Fig. 3A), and more time in the transition zone (t(56) = 7.20, p < 0.0001; Fig.

306   3B) and the inner zone (t(56) = 9.31, p < 0.0001; Fig. 3C), on average, compared to fish dosed in

307   control water. In addition, a significant difference was also detected between the median time

308   spent immobile when comparing fish in their habitat water to those in ethanol (W = 169.5, p <

309   0.0001). No significant difference was found when comparing the mean distance moved for

310   these two groups (F (1,56) = 3.75, p = 0.058; Fig. 3D).

311

312   **Investigating solution interactions**

313   Investigating the treatment combinations revealed that time spent in the thigmotaxis zone

314   was statistically greater for fish in the *In-view-CTL* group compared to fish in the I*n-view-*

315   *Ethanol* group (W_{BM} = -3.24, p = 0.003, Fig. 3A). Whereas, time spent in the transition zone was

316   statistically greater for fish in the *In-view-Ethanol* group compared to fish in the I*n-view-CTL*

317   group (W_{BM} = 3.52, p = 0.002, Fig. 3B). A significant difference was not found when comparing

318   the time fish in these two groups spent in the inner zone (W_{BM} = 1.82, p = 0.079, Fig. 3C). In

319   addition, the Wilcoxon rank-sum test concluded that the median time spent immobile for fish in

320   the *In-view-Ethanol* group was not significantly different compared to fish in *In-view-CTL* group

321   (W = 69, p = 0.074, Fig. 3E).

322   Post hoc comparison of the isolated treatment groups using the Brunner-Munzel test

323   indicated that fish in the *Isolate-CTL* group spent more time in the thigmotaxis zone than fish in

324   the *Isolate-Ethanol* group (W_{BM} = -32.19, p < 0.0001, Fig. 3A). Whereas, fish in the *Isolate-*

325   *Ethanol* group spent more time in both the transition zone (W_{BM} = 24.80, p < 0.0001, Fig. 3B)

326   and the inner zone (W_{BM} = 13.16 , p < 0.0001, Fig. 3C) compared to fish in *Isolate-CTL* group.

327   The Wilcoxon rank-sum test indicated that the median time spent immobile by fish in *Isolate-*

328   *CTL* group was significantly greater than median time spent in the in *Isolate-Ethanol* group (W =

329   16.5, p < 0.001, Fig. 3E).

330

331   **Distance moved and side-preference during exposure**

332   The distance fish moved while in the dosing container did not significantly differ

333   between *Isolated-Dosing* and *In-view-Dosing* groups ($t_{28} = 1.255$, $p = 0.2198$; Fig. 4A). A highly

334   significant preference for the conspecific side of the dosing container was found in fish from the

335   *In-view-Dosing* group ($t_{28} = 10.21$, $p < 0.0001$; Fig. 4B).

336

337   **DISCUSSION**

338   To examine whether the sight of conspecifics during dosing impacts effectiveness of anxiety-like

339   behaviour measurements and anxiety-altering substances, we exposed zebrafish to habitat water

340   or ethanol (1.0%) while fish were isolated or able to observe conspecifics. Following dosing,

341   behaviour was tested in the novel object approach test. Fish that were able to view conspecifics

342   during dosing with control water had significantly less anxiety-like behaviour and increased

343   boldness compared to fish dosed in isolation. The behavioural effects of ethanol also varied

344   depending on the social condition in which it was administered. Ethanol had a significantly

345   greater effect on anxiety-like behaviour and boldness in isolated fish compared to fish that were

346   able to view conspecifics during dosing.

347   Both the sight of conspecifics and ethanol exposure significantly increased the time fish

348   spent in the zones closest to the novel object (transition and inner; Fig. 3B-C), consistent with

349   previous research demonstrating that ethanol increases boldness (Hamilton et al., 2017b; Johnson

350   & Hamilton, 2017). Ethanol and sight of conspecifics also both decreased time spent in the

351   thigmotaxis zone; an indication that anxiety-like behaviour was decreased. The most pronounced

352   change in behaviour occurred with the combination of ethanol exposure and isolation during

353   dosing compared to ethanol exposure and view of conspecifics; anxiety-like behaviour was

354   significantly reduced (Fig. 3A, P<0.0001) and boldness was increased (Fig. 3C, P<0.0001). In

355   fish with a view of conspecifics, ethanol still did decrease anxiety-like behaviour, however to

356   much less of an extent than in isolated fish, and had little effect on approach to the novel object.

357   A similar pattern emerged in ethanol's influence on locomotion. Ethanol did not impact the

358   distance *Isolated* or *In-view* fish moved (Fig. 3D), or the time *In-view* fish spent immobile; it

359   only increased immobility in *Isolated* fish (Fig. 3E). This suggests that social isolation either

360   increases sensitivity to ethanol's anxiolytic and depressant effects, or the presence of

361   conspecifics suppresses these effects.

362   In an attempt to understand how the social context contributes to differences observed in

363   behavioural outcomes, we analyzed the behaviour of a second group of fish while in the dosing

364   container during the 30-minute dosing period. Because mobility may affect the rate of intake

365   when fish are dosed via immersion, with greater physiological demands resulting in more

366   ventilation and therefore increased uptake of the drug through the gills (Blaser & Vira, 2014), we

367   sought to determine whether the heightened effect of ethanol observed in *Isolated* fish could be

368   explained by greater movement during dosing. We analyzed the behaviours of fish exposed to

369   habitat water while isolated or within view of conspecifics while they were in the dosing

370   container. Interestingly, no differences were observed in the distance fish moved (Fig. 4A),

371   indicating differences in locomotion during dosing could not explain the behaviours we

372   observed. Not surprisingly, zebrafish spent significantly more time on the side of the dosing

373   container closest to conspecifics when in view (Fig. 4B), demonstrating their preference to

374   remain near other zebrafish.

375   An explanation for the increased anxiolytic effect of ethanol in *Isolated* relative to *In-*

376   *view* groups may be related to 'social buffering.' Previous research in zebrafish has shown that

377   the presence of conspecifics helps to suppress anxiety evoked by a fearful stimulus (Faustino,

378   Tacão-Monteiro & Oliveira, 2017). Faustino, Tacão-Monteiro and Oliveira (2017) first

379   demonstrated this in zebrafish by exposing fish to a conspecific alarm substance with or without

380   the presence of conspecific cues. They found that the anxiogenic effects were dampened by the

381   presence of olfactory and/or visual cues. In other words, fish that could observe or smell their

382   conspecifics showed less anxiety in response to the alarm substance (Faustino, Tacão-Monteiro

383   & Oliveira, 2017). The mechanisms of social buffering have not been well explored in zebrafish;

384   however, it is possible that zebrafish use the behaviours of their conspecifics as a source of

385   information to guide their own responses in unfamiliar or fearful environments. This would

386   explain why there was less of an effect of ethanol in the *In-view* condition in our experiment (ie.

387   higher time in the thigmotaxis zone and less time near the object) compared to the *Isolated*

388   condition. Presumably, fish in the dosing container were observing their conspecifics behaving

389   normally and this may have minimized the effect of ethanol. However, social buffering has only

390   been shown to decrease stress responses and in our study anxiolysis was reduced. The effect of

391   ethanol may have been 'buffered' by the presence of conspecifics. The sight of conspecifics did

392   significantly lower time spent in the thigmotaxis zone independent of the presence of ethanol,

393   which suggests that the sight of conspecifics may lower anxiety overall. However, if this were

394   the only factor at play then the effect of ethanol should be greater in the *In-view* condition

395   compared to the *Isolated* condition, because of the combined anxiolytic effect of conspecifics

396   and the anxiolytic effect of ethanol, but this is the opposite of what we observed. Somehow the

397   visual cues from conspecifics decreased the effectiveness of ethanol, and the candidate

398   mechanism is social mimicry, which has been demonstrated in zebrafish (Dresoti et al., 2015).

399   To further investigate this future, studies could manipulate the emotional state of conspecifics

400   with stress or pharmacology then examine how these *In-view* fish affect the behavioural

401 outcomes of the individual fish observing them. In this study, we did not examine sex differences

402 and we did not manipulate the number of fish used as conspecifics nor the distance of the

403 conspecific tank from the focal fish. These factors may also impact the strength of the social

404 buffering response. It would also be valuable to explore the neurochemical basis of social

405 buffering with analysis of brain chemistry after dosing in these social conditions.

406

407 **CONCLUSIONS**

408 Taken together, the sight of conspecifics decreases anxiety, and more so, buffers the anxiolytic

409 effect of ethanol. These findings have important implications in the fields of pharmacology,

410 toxicology and behavioural neuroscience as isolated drug administration seems to be more

411 effective in eliciting a behavioural response. Additionally, zebrafish behavioural research

412 findings can be inconsistent across laboratories, and this may be due a lack of detailed

413 methodological reporting, including whether conspecifics are within visual range during drug

414 exposure. Social buffering may offer a potential explanation for at least some of these

415 discrepancies and necessitates detailed descriptions of dosing conditions in future experiments.

416 Overall, this study provides the first evidence that the social condition during dosing effects the

417 efficacy of anxiolytic substances when subsequently tested in isolation. This highlights the need

418 to consider the social environment during exposure when conducting or interpreting behavioural

419 research.

420

421   Figure legends:

422   **FIGURE 1.** Experimental dosing set-up. (A) *Isolated* and (B) *In-view* dosing. During dosing an

423   individual fish was netted from the holding tank and placed into one of the two dosing

424   containers. *In-view* fish had visual access to 12 conspecifics held in the conspecific tank but were

425   not able to see the other fish being dosed. Focal fish remained in the dosing containers for 30-

426   minutes prior to behavioural testing. C) The circular arena used was 34 cm in diameter and 16

427   cm in height. D) The novel object used was a multi-coloured LEGO® figurine. E) The

428   thigmotaxis, transition and inner zones were calibrated to 34, 23 and 12 cm in diameter

429   respectively.

430

431   **FIGURE 2.** Experimental set-up for motion-tracking during exposure. (A) *Isolated* dosing, or

432   (B) *In-view* dosing. The circle in the bottom left of figure (B) represents the virtual zones created

433   in EthoVision to test whether fish spend more time on the side of the beaker closest to

434   conspecifics when in view.

435

436   **FIGURE 3.** Effects of social context and ethanol on zone preference. The time, in seconds, fish

437   spent in the thigmotaxis (A), transition (B) and inner zones (C). (D) and (E) represent the effect

438   of social context and substance on the distance fish moved (D) and the time fish spent immobile

439   (E). Graphs show individual data points (n = 15 per group) with horizontal lines that represent

440   mean values ± SEM. The number of * symbols identifies significant differences between groups

441   at varying levels of significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

442

443   **FIGURE 4.** Distance and side preferences during the dosing procedure. (A) The distance,

444   *Isolated-CTL* and *In-view-CTL* fish moved and the (B) amount of time, in seconds, *In-view-*

445   *CTL*s spent on either side of the dosing container during dosing. The social condition did not

446   have a significant effect on the distance fish moved, however when in-view, fish had a

447   significant preference for the side of the dosing container closest to conspecifics. Data was

448   analyzed using independent *t*-tests. Graphs show individual data points (n = 15 per group)

449   with horizontal lines that represent mean values ± SEM. The number of * symbols identifies

450   significant differences between groups at varying levels of significance: ****$P < 0.0001$.

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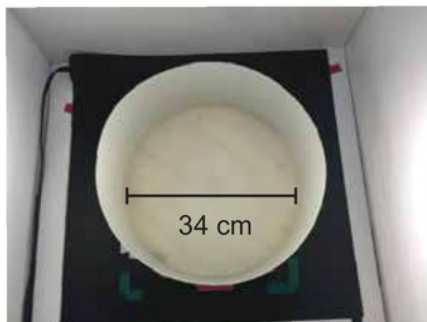
Figure 1

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.

A

B

C

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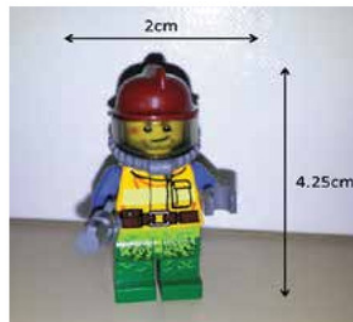
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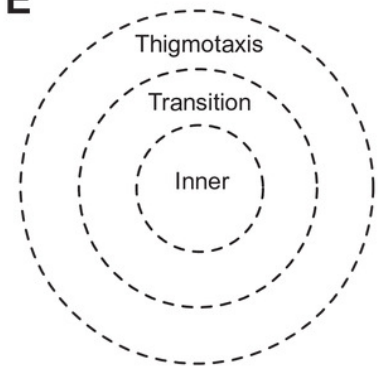
Figure 2

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.

A

B
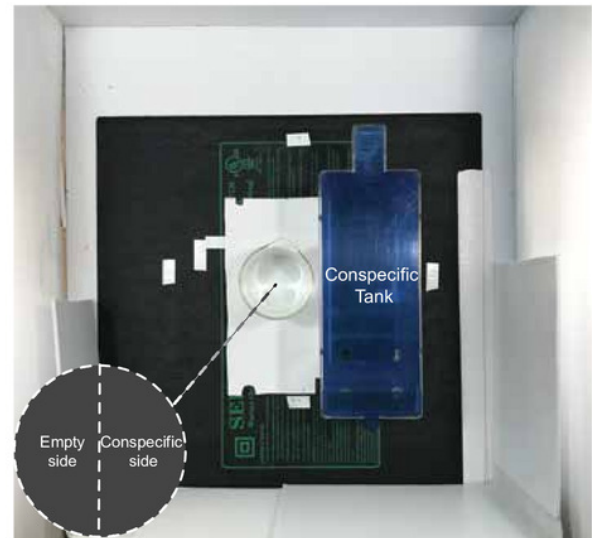
Figure 3

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. **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$
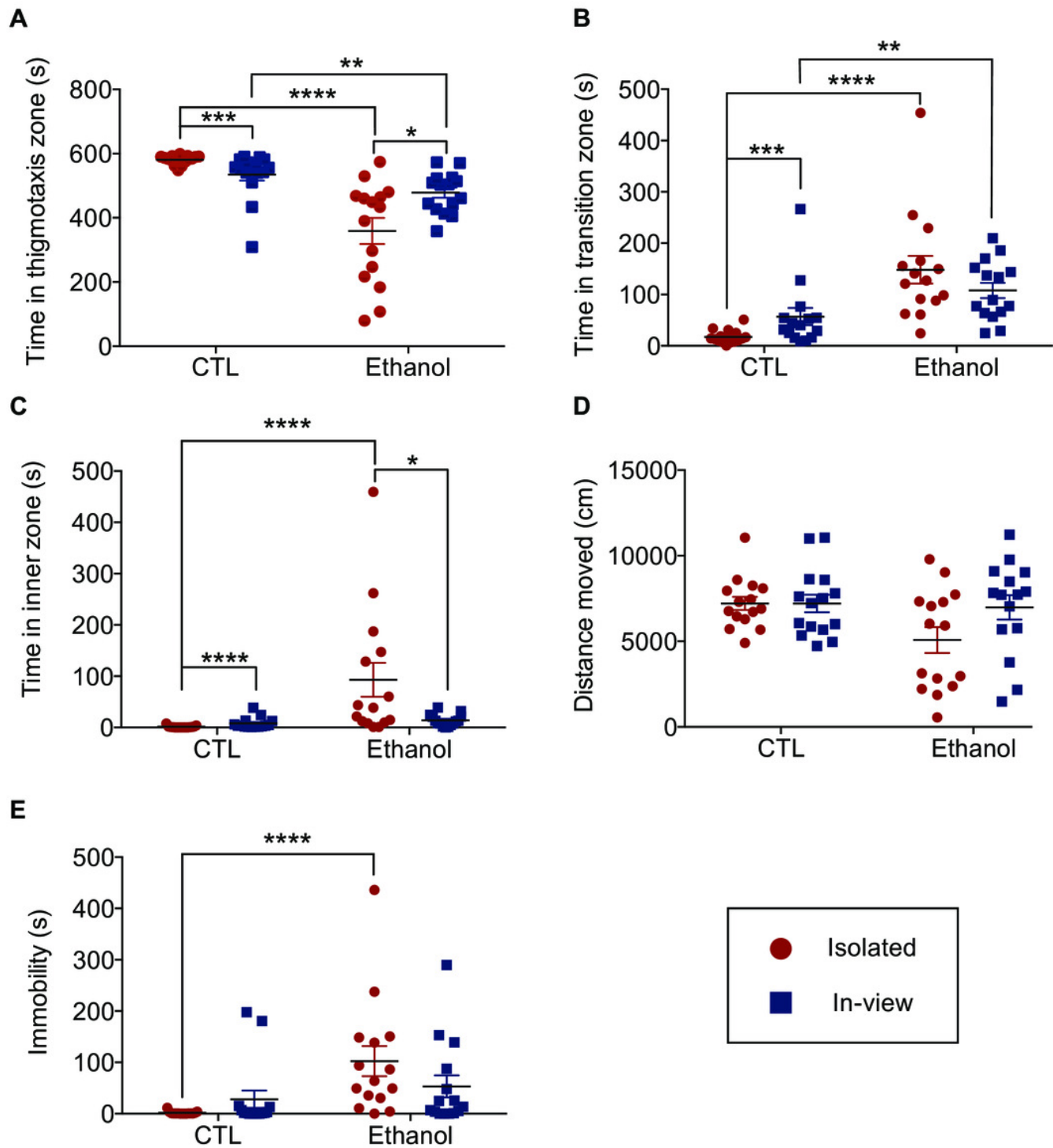
Figure 4

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. ****$P < 0.0001$.