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1 Anxiolytic effects of Fluoxetine and 2 Nicotine exposure on exploratory behavior 3 in Zebrafish

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9 ABSTRACT

10 Zebrafish (*Danio rerio*) have emerged as a popular model for studying pharmacological effects on behavior
11 and anxiety. While there have been numerous studies documenting the anxiolytic and anxiogenic effects
12 of common drugs in zebrafish, many do not report or test for behavioral differences between the sexes.
13 Previous studies of zebrafish have indicated that males and females differ in their behavioral responses
14 to anxiety. In this study, we test for sex-dependent effects of fluoxetine and nicotine. We exposed fish to
15 system water (control), 10 mg/L fluoxetine, or 1 mg/L nicotine for three minutes prior to being subjected
16 to four minutes in an open-field drop test. Video recordings were tracked using ProAnalyst. Fish from
17 both drug treatments reduced swimming speed, increased vertical position, and increased use of the
18 top half of the open field when compared with the control, though fluoxetine had a larger effect on depth
19 related behaviors while nicotine mostly affected swimming speed. A significant sex effect was observed
20 where females swam at a slower and more constant speed than males in all treatments. No interactions
21 between sex and the drugs were observed across the entire study.

22 Keywords: behavior, anxiety, fluoxetine, nicotine

23 INTRODUCTION

24 The zebrafish (*Danio rerio*) is a popular research model for studying pharmacology (summarized in
25 Barros et al., 2008; Langheinrich, 2003) and behavior (Gerlai, 2015), particularly with regard to stress
26 and anxiety. The zebrafish provides a vertebrate model that breeds rapidly, is easy to maintain in large
27 numbers, and can be administered drugs through immersion. Zebrafish also share many of the same
28 neurotransmitters (Shin and Fishman, 2002) and stress pathways as humans, utilizing cortisol rather than
29 corticosteroids as used by rats and mice (Barcellos et al., 2007). These features have facilitated zebrafish
30 studies on addiction (Mathur and Guo, 2010), learning (Sison and Gerlai, 2010), social behavior (Buske
31 and Gerlai, 2014; Gerlai, 2014) and anxiety behaviors (Mathur and Guo, 2010; Maximino et al., 2010).

32 Anxiety-related behaviors are known to vary by sex in zebrafish and other model organisms, and these
33 differences may be explained by gonadal hormones (Zimmerberg and Farley, 1993; Palanza, 2001). Male
34 and female rats differ in their time spent in the center of an open field and a plus maze, though the nature
35 of these differences are also dependent on the strain observed (Mehta et al., 2013). In zebrafish, females
36 tend to be less anxious, or more bold, than males when measuring location preferences in the presence of
37 a human observer (Benner et al., 2010; Oswald et al., 2013a).

38 Drugs are used to manipulate anxiety and related disorders in humans and are also utilized as tool for
39 understanding behavior. Fluoxetine, for example, is a drug used to treat depression and anxiety. It works
40 by blocking the reuptake of serotonin in the brain (Beasley et al., 1992). Serotonin and its transporters
41 have been associated with anxiety (Graeff et al., 1997; Lesch et al., 1996). Nicotine is naturally found in
42 tobacco products and binds to nicotinic cholinergic receptors (nAChRs) to release dopamine (Benowitz
43 et al., 2009). The result is an anxiolytic response (Picciotto et al., 2002).

44 Observations of male and female differences in anxiety-related behavior have led us to ask whether
45 the effects of anxiolytic substances also differ by sex. There is evidence that the effectiveness of anxiolytic

46 drugs may vary with sex in mammals. Differential responses have been observed in humans utilizing
47 Sertraline, a selective serotonin reuptake inhibitor (SSRI) where females showed an enhanced response
48 compared to males. (Kornstein et al., 2000). Sex-specific differences were observed in the effectiveness
49 of the SSRI Fluoxetine in humans (Martényi et al., 2001), and studies utilizing rats (Mitic et al., 2013;
50 Leuner et al., 2004; Lifschytz et al., 2006) and mice (Monleón et al., 2002; Hodes et al., 2010) have shown
51 a discrepancy between the sexes in both the physiological and behavioral responses to this drug where
52 efficacy tends to be greater in females than in males. Evidence in rats also suggest that nicotine's effects
53 on stress and anxiety may also differ between the sexes with males exhibiting a greater anxiolytic effect
54 (Faraday et al., 1999). This is important from a pharmacological standpoint in that effective doses may
55 differ between males and females. On a broader level, studies utilizing a single sex, or ignoring the effect
56 of sex altogether ought not to be used to draw broad conclusions about the effects of that drug.

57 While zebrafish are becoming a model for pharmacological research, literature describing sex-
58 dependent effects of anxiolytic drugs in this system are lacking. In this experiment, we test the hypothesis
59 that zebrafish exhibit sex-dependent responses to fluoxetine and nicotine. These substances were chosen
60 because they have known anxiolytic effects across a wide variety of model systems including humans
61 (Gilbert, 1979; Griffin and Mellon, 1999), rats (Cohen et al., 2009; Zhang et al., 2000) and zebrafish (Ben-
62 can and Levin, 2008; Bencan et al., 2009; Cachat et al., 2010; Levin et al., 2007), and while sex-specific
63 effects have been observed in mammals, studies in zebrafish utilizing these substances largely ignore the
64 effects of sex.

65 METHODS

66 Subjects

67 Experimental fish were bred from adult Scientific Hatcheries strain (Huntingdon, CA) that has been
68 maintained in our facility. Water in our Aquaneering Inc. (San Diego, CA) system was constantly
69 circulating and kept at a temperature of 28.5 °C on a 14 hour light:10 hour dark cycle. The fish were fed a
70 diet of brine shrimp twice and flake food (Tetramin) once for a total of three daily feedings. At the time of
71 data collection, the fish were four months old and housed in three-liter tanks in groups of five to achieve
72 maximal growth rates. Though zebrafish stocked at this density are known to develop social hierarchies
73 that can influence stress and behavior (Pavlidis et al., 2013), we randomly assigned individuals to a drug
74 treatment group such that these effects should be equally distributed across treatments. All aspects of
75 this study were approved by the University of Idaho's Animal Care and Use Committee under protocol
76 2014-14.

77 Dosing

78 Fluoxetine (generic (Teva Pharmaceuticals) from Wal Mart) and nicotine (Sigma Aldrich) treatments were
79 administered at concentrations of 10 mg/L for the fluoxetine and 1 mg/L for the nicotine. These doses
80 vary from standard doses in the zebrafish literature. Fluoxetine is often given at concentrations up to 100
81 $\mu\text{g/L}$, but administered chronically over a two-week period (Egan et al., 2009). We used a higher dose
82 than the chronic concentrations reported in the literature, however it is important to note that this choice
83 could yield non-target effects due to higher concentrations. Nicotine is often administered as a ditartrate
84 salt at concentrations up to 100 mg/L (Levin et al., 2007). We used pure nicotine and were unsure at the
85 time of the experiment how the two forms compared with each other. We chose our dose based on the
86 LD50 concentration (4 mg/L) to avoid lethal effects on our subjects. Each drug was dissolved in system
87 water to make a working solution each morning of administration. A third treatment of only system
88 water served as a control. Fish were netted from their home tank and immediately placed into a beaker
89 containing 100mL of one of the three treatments. After three minutes of exposure to the drug dose, the
90 fish were transferred to an open field test tank filled with untreated system water for behavioral recording.
91 Dosing and behavioral observations were made on one fish at a time and the treatment type and order
92 were randomized across individuals.

93 Behavior Assay & Video tracking

94 The fish were placed in a rectangular tank with interior dimensions measuring 25cm wide, 12 cm high
95 (from water level to bottom), and 6 cm thick (front to back). The volume of water in the tank was
96 approximately 2 L. Each fish was filmed for four minutes (240 seconds) at 25 frames/second beginning
97 from the time that the subject entered the water. The camera and operator were hidden behind a blind

98 during the recorded observation time. The tank was backlit with an opaque diffuser for the purposes of
99 creating a silhouetted object for motion tracking. After the four-minute period, the fish was netted out of
100 the test tank, placed into its own individual 1.5 L housing and returned to the main system. Observations
101 were recorded over three days between the hours of 10:00 am and 2:00 pm. After all subjects had been
102 recorded, weight and standard length measurements were obtained by first anesthetizing the individual in
103 MS-222 solution and blotting excess water with a paper towel. At this time, we also recorded the sex of
104 the individual using visual cues: larger, rounded abdomen and dull fins for females, smaller and leaner
105 abdomen and bright yellow fins for males.

106 Videos were digitally tracked using ProAnalyst[®] (Xcitex, Cambridge, MA). Tracking began with the
107 frame in which the fish hit the surface of the water, and proceeded to the end of the video. The tracking
108 data were imported into R for cleaning and processing. Each track was truncated to exclude the first
109 five seconds during which the fish would sink, but remain otherwise motionless, as it recovered from the
110 initial shock of being released from the net. Tracks were then standardized to 4 minutes, or 6000 frames.
111 We computed velocity from the x-y data points. Since the tracking software did not always track the exact
112 same position on the fish, velocity was estimated using the change in coordinates between two frames
113 before and two frames after the focal frame. This algorithm sufficiently smoothed the speed data while
114 retaining detail at small time intervals.

115 **Analysis**

116 **Freezing**

117 Freezing time was defined as the time a subject spent motionless on the bottom of the tank. We defined
118 motionless as maintaining a velocity of less than .01 cm/frame for more than 20 consecutive frames. Any
119 short bursts of motion flanked by considerable freezing times were verified in the video to be true motion.
120 If a time period of activity was less than 40 frames, it was re-categorized as part of the freezing time
121 as this motion is likely an artifact of the automated tracking. The freezing time was then calculated by
122 counting the total number of frames marked as frozen. We also characterized freezing behavior as a binary
123 ‘yes’ or ‘no’ response as the propensity to show any freezing behavior can be considered an independent
124 response from duration of freezing.

125 **Speed**

126 We computed the average speed for each individual using only the active (non-frozen) data points from
127 the swim tracks. Freezing behaviors can cause a high degree of correlation with average swimming
128 behaviors such as speed and depth use. Since we analyzed freezing behavior separately, we chose to
129 analyze the effects of anxiolytic drugs on velocity during active swimming only. We predicted that anxious
130 individuals would swim slower on average than less anxious individuals (Gerlai et al., 2009). In addition,
131 we computed the variance in velocity for the active data points. The variance represents the consistency in
132 swim speed within an individual. Less anxious individuals should display more consistency in velocity
133 than more anxious individuals due to erratic behavior (Gerlai et al., 2009).

134 **Depth**

135 Depth was measured by the y-coordinate position in the swim track. We aligned the y origin with the
136 water’s surface, and measured depth as increasing negatively toward the bottom of the tank. As with
137 velocity, depth variables were calculated using only the active points in the tracks. We analyzed both the
138 mean and variance (consistency) of depth. We predicted that anxious individuals should spend more time
139 near the bottom of the tank and should have a lower variance in depth (Levin et al., 2007; Oswald et al.,
140 2013b). Conversely, we predicted that less anxious individuals will position themselves higher in the
141 water on average and spend more time exploring the entire tank, resulting in a larger variance in depth
142 usage. We also quantified at the number of times an individual entered the top half of the tank from the
143 bottom half. Such behavior may be indicative of anxiety, as anxious individuals tend to enter the top half
144 less often than less anxious individuals (Egan et al., 2009). We also expected that anxious individuals
145 would spend a smaller proportion of active swimming time in the top half, and that they would exhibit a
146 longer latency to enter the top from the beginning of the trial (Egan et al., 2009). The threshold between
147 the top and bottom halves was defined at -6 cm.

148 **Horizontal Place Preference**

149 The width of the tank was divided into three equal sections and the proportion of time in the middle section
150 calculated to differentiate preference to be located in the center versus the edge of the test environment.

151 While we had clear expectations for location preference with respect to depth, it was unclear at the
152 time of analysis whether the middle or the edges represent a “safe” zone with respect to horizontal
153 preference. Experiments with rodents have found that stressed individuals prefer the edges of their arenas
154 (thigmotaxis), but that this behavior is analogous to stressed fish preferring the bottom (Levin et al., 2007).

155 **Statistical Analysis**

156 We began with a MANOVA on all continuous variables where all individuals could be included. We
157 applied transformations where they were required to conform to the assumptions of normality in the
158 residuals (see Results for transformations). The initial model included the effects of weight as a covariate,
159 sex, drug treatment, and the sex by drug interaction. No significant effect of weight was observed,
160 and there was no improvement to the model by keeping the term, so we excluded weight from all
161 subsequent analyses. We performed individual ANOVAs on each of the continuous variables. Since
162 freezing occurrence is a binary response, it was analyzed using a logistic GLM to estimate and compare
163 the probability that an individual will freeze based on a given treatment group. In order to accurately assess
164 freezing time, only individuals that froze were used (N=52). All tests were performed with a significance
165 threshold of $\alpha = 0.05$. When a significant effect of drug treatment was detected, we performed pairwise
166 T-tests among the three treatments with a Tukey correction.

167 **RESULTS**

168 We recorded observations from 90 individuals divided equally and randomly among the 3 treatments
169 (n=30 per treatment). Due to complications with the filming, observations on three of the individuals had
170 to be removed leaving us with final sample size of 87 individuals broken down by treatment and sex as
171 follows: 29 in the control treatment (17 females and 12 males), 30 in the fluoxetine treatment (16 females
172 and 14 males), and 28 in the nicotine treatment (14 females and 14 males).

173 **Multivariate**

174 The full model Type-II MANOVA included the effects of weight, sex, drug treatment, and the sex
175 by drug interaction on average depth, variance of depth, average speed, variance of speed, percent
176 of time spent in the top half, number of crosses into the top half, latency to enter the top half, and
177 proportion of time spent in the middle third horizontally (ie, away from the edges). There was a non-
178 significant effect of sex ($\Lambda = 0.17896$, $F_{8,73} = 1.9889$, $p = 0.05974$) and a significant effect of drug
179 treatment ($\Lambda = 0.56646$, $F_{16,148} = 3.6551$, $p = 0.00001305$) on behavior, but no significant interaction.
180 There was no significant effect of weight as a co-variate, and including weight in the model showed no
181 improvement over removing it ($\Lambda = 0.95793$, $F_{5,76} = 0.66755$, $p = 0.6492$). With the reduced model,
182 we observed a significant effect of sex ($\Lambda = 0.22404$, $F_{8,74=2,6707}$, $p = 0.01237$) and drug treatment
183 ($\Lambda = 0.56659$, $F_{16,150} = 3.7057$, $p = 0.00001014$). Therefore, for all subsequent analyses we considered
184 only the effects of sex, drug treatment, and the interaction term.

185 **Individual components of behavior**

186 We observed no significant interactions between sex and drug treatment in any of the individual behavior
187 components (see Table 1), consistent with the results of the MANOVA above. All components indicated a
188 significant effect of drug treatment ($p < 0.05$) except for freezing occurrence and freezing duration. The
189 subsequent descriptions describe the results of the post-hoc pairwise comparisons of the drug treatments
190 using the least-squared means and Tukey adjusted p -values based on 3 tests. We also observed a significant
191 effect of sex with regard to average swimming speed ($F_{1,81} = 10.7178$, $p = 0.001562$) and consistency
192 (variance) of swimming speed ($F_{1,81} = 13.9196$, $p = 0.0003528$). Males were on average faster than
193 females, but also exhibited less consistency in their swimming speeds. These were the only instances in
194 which the sexes differed in their behavior.

195 **Freezing behavior**

196 Freezing behavior is a commonly observed anxiety related behavior in zebrafish (Egan et al., 2009). Of the
197 87 individuals observed, 52 exhibited freezing behavior. Though males tend to be more likely to freeze than
198 females on average, this difference was not statistically significant ($\chi^2 = 3.7866$, $p = 0.05167$). We also
199 failed to observe a significant effect of drug treatment on freezing occurrence ($\chi^2 = 3.7964$, $p = 0.14983$)
200 as well as a sex by drug interaction ($\chi^2 = 0.3949$, $p = 0.82083$). For freezing duration, or latency to
201 explore, we only included the 52 individuals that exhibited freezing behavior (control: F=11, M=10;

	Sex	Drug	Interaction	Fluoxetine	Nicotine
Freezing Time	0.17	0.26	0.76	0.99	0.33
Average Speed	0.00	0.01	0.63	0.13	0.00
Variance Speed	0.00	0.02	0.47	0.57	0.02
Average Depth	0.98	0.00	0.94	0.00	0.04
Variance Depth	0.62	0.01	0.91	0.01	0.19
Proportion in Top	0.86	0.00	0.72	0.00	0.22
Crosses to Top	0.57	0.00	0.89	0.00	0.45
Latency to Top	0.64	0.00	0.89	0.03	0.00
Proportion in Center	0.19	0.00	0.36	0.00	0.99

Table 1. Table of P-values summarizing results. **Bold** items are considered to show significant differences among treatment groups ($\alpha = 0.05$). P-values for the Fluoxetine and Nicotine columns represent pairwise comparisons with the control and are adjusted using the Tukey method for 3 comparisons.

202 fluoxetine: $F=7$, $M=8$; nicotine: $F=6$, $M=10$). This improved the assumptions of normality required for the
 203 ANOVA. Results of the type II ANOVA suggest that neither sex nor drug treatment have any significant
 204 effect on freezing duration (Sex: $F_{1,46} = 1.9604$, $p = 0.1682$; Drug: $F_{2,46} = 1.3707$, $p = 0.2641$). Figure
 205 1 shows the results of freezing behaviors.

206 **Speed**

207 When analyzing only the active swimming data from the trials, fish from both drug treatments appear to
 208 reduce their average swimming speed compared with the control, however this pattern is only significant in
 209 the nicotine treatment ($t = 3.373$, $p = 0.0032$, see figure 2). Drugged fish also swam at a more consistent
 210 speed than the undrugged control fish ($F_{2,81} = 4.0654$, $p = 0.0207731$), but again this trend was only
 211 significant in the nicotine treatment ($t = 2.818$, $p = 0.0166$).

212 **Depth**

213 Both the subjects dosed with nicotine and fluoxetine positioned themselves higher in the water column than
 214 the control fish (nicotine: $t = -2.462$, $p = 0.0417$; fluoxetine: $t = -4.711$, $p < .0001$). Fish dosed with
 215 fluoxetine explored more of the water column than control subjects ($t = -3.172$, $p = 0.0060$). Subjects
 216 dosed with nicotine also exhibited more variation in depth use on average than the control subjects, but
 217 this difference was not significant (see figure 3).

218 We also divided the tank into two discrete and equal vertical zones and compared the proportion of
 219 time spent in the upper half (figure 4). Subjects dosed with fluoxetine tended to spend more than twice as
 220 much time in the upper half as control subjects and this difference is significant ($t = -3.883$, $p = 0.0006$).
 221 Subjects in both the nicotine and fluoxetine treatments exhibited a reduced latency time to first enter
 222 the top half than control subjects (nicotine: $t = 3.333$, $p = 0.0037$; fluoxetine: $t = 2.652$, $p = 0.0258$).
 223 When comparing the total number of visits to the top half, only the fluoxetine group showed a significant
 224 increase over the control ($t = -3.801$, $p = 0.0008$).

225 **Horizontal Place Preference**

226 All subjects spent most of their time near the edges avoiding the center (figure 4), consistent with the
 227 concept of thigmotaxis. However, subjects dosed with fluoxetine spent less time in the center and more
 228 time near the edges than subjects in the control and nicotine treatments ($t = 3.257$, $p = 0.0046$) which
 229 is inconsistent with a reduction in thigmotaxis resulting from a reduction in stress. At this time we are
 230 unsure how these results relate to anxiolytic properties of the drug.

231 **DISCUSSION**

232 **Differences in fluoxetine and nicotine behavioral responses**

233 Small prey fish such as zebrafish tend to behave in such a way as to reduce risk of predation. When placed
 234 in a novel open field, such behavioral strategies include diving to the bottom and remaining motionless
 235 (Egan et al., 2009), and avoiding potentially risky locations such as the surface of the water (Wilson and
 236 Godin, 2009; Oswald et al., 2013b). Exposure to anxiolytic drugs alters these behaviors in ways that

237 may indicate an association between anxiety related behaviors and risk management. We observed a
238 decrease in bottom dwelling and an increase in time spent in the top half of the tank in fish exposed to
239 fluoxetine (figures 3 and 4). This is consistent with patterns observed by Egan et al. (2009) who also
240 report an increased use of the top of the water column by zebrafish exposed to fluoxetine. However, the
241 study by Egan et al. (2009) also reports a reduction in freezing bouts and freezing time, a pattern we
242 failed to observe. One explanation for this discrepancy could be differing effects of chronic and acute
243 dosing. Fluoxetine is metabolized into norfluoxetine, its active metabolite, in the liver by cytochrome
244 P450 enzymes (Rasmussen et al., 1995). It then travels through the bloodstream to the brain where it
245 blocks the reuptake of serotonin (Beasley et al., 1992). Metabolism of the drug could delay its effect
246 until after the animal had already recovered from freezing behavior. While most fluoxetine studies utilize
247 chronic exposure, we have shown that similar behavioral changes can occur with just a single acute
248 dose. Acute exposure to fluoxetine has also been shown to reduce cortisol levels of zebrafish exposed
249 to a stressful environment (de Abreu et al., 2014). We speculate that the behaviors we observed may be
250 due to a reduction in physiological stress response resulting from exposure to the drugs, though more
251 experiments are needed to confirm this.

252 We observed changes in swimming speed, average depth, and latency to enter the top in fish exposed
253 to nicotine. Fish exposed to nicotine were quicker to enter the top and swam higher in the water column on
254 average compared to control fish. This is consistent with a reduction in anxiety related behaviors as seen
255 in the fluoxetine treatment group. Exposure to nicotine and fluoxetine appeared to decrease swimming
256 speed while increasing the consistency at which the fish swam. The increased consistency (reduction
257 of individual variance) might be explained by a reduction in anxiety, where individuals that are calm
258 should move at a fairly normal and constant pace, while anxious individuals may constantly alter their
259 swimming speeds in an erratic fashion Gerlai et al. (2009). Egan et al. (2009) reported an increase in
260 average swim speed with exposure to fluoxetine, which contrasts with our observations of slower average
261 swim speeds with exposure to either fluoxetine or nicotine. Sackerman et al. (2010) suggests that nicotine
262 may have sedating effects which could account for the slower swim speeds. However, we also observe
263 slower average swim speeds in the fluoxetine treatment, and though the difference is not statistically
264 different from the control, it is also not different from the nicotine effect. We observed a similar pattern in
265 the nicotine treatment with respect to the time spent at the top and the variation in depth use, where the
266 nicotine treatment was statistically indistinguishable from both the control and the fluoxetine treatments.
267 In these two instances, it is likely that the nicotine is having an anxiolytic effect, but that we used too low
268 of a dose to observe an effect that is different from the control. Sackerman et al. (2010) also failed to
269 observe an effect of nicotine on swim depth using a low dose of 25 mg/L, but noted that higher doses
270 such as 50 mg/L and 100 mg/L do produce a significant effect (Levin et al., 2007). Our dose of 1 mg/L
271 is noticeably lower than other studies of nicotine in adult Zebrafish, accounting for the our use of pure
272 nicotine liquid while the other studies used a nicotine tartrate salt (Levin et al., 2007; Sackerman et al.,
273 2010). It should be noted that the relationship between the tartrate salt and pure form is about 0.325, such
274 that a concentration of 100mg/l of the tartrate equates to a concentration of 32.5mg/l of pure nicotine
275 (Matta et al., 2007).

276 Both nicotine and fluoxetine affected behavior in ways indicative of a reduction of anxiety. However,
277 the two drugs also appear to affect different components of behavior. Nicotine had its highest effect on
278 swimming speed, while fluoxetine mostly affected behaviors related to vertical positioning. This suggests
279 that anxiety is not a simple condition, but rather a complex idea encompassing a number of components
280 that are sometimes correlated, but not always connected. These behavioral components may be separated
281 by different physiological pathways which could explain why different classes of drugs affect specific
282 behaviors.

283 **The effect of sex on behavior and drug efficacy**

284 Sex differences in anxiety behaviors have been described in a number of species including rats (Mehta
285 et al., 2013), stickleback (King et al., 2013), and guppies (Harris et al., 2010). While most of these studies
286 find that males are typically more bold (less anxious) than females, our lab has previously observed the
287 opposite trend in the Scientific Hatcheries strain of zebrafish with regard to association with humans,
288 vertical position, and feeding latency in individual home-tank observations (Oswald et al., 2013a,b; Benner
289 et al., 2010). These differences are the basis for our inquiry as to whether substances known to alter these
290 behaviors might work at different efficacy in males and females. In the present study, we only observe

291 significant behavioral differences between the sexes with respect to swimming speed. While males swim
292 slightly faster than females, it's the females that swim at a more constant rate. In addition, males seem to
293 show a higher probability to exhibit freezing behavior across all three treatments, and even though this
294 trend isn't statistically significant, it still leads us to suggest that males **could be** behaving with higher
295 anxiety levels than females.

296 With the active swimming behaviors, we fail to observe differences between the sexes, and across all
297 of the behaviors, the data do not suggest any indications of sex-specific effects of either drug. There is
298 plenty of literature in mammalian models that contradict these findings (Mitic et al., 2013; Leuner et al.,
299 2004; Lifschytz et al., 2006; Monleón et al., 2002; Hodes et al., 2010). One possible explanation for our
300 lack of sex-specific effects stems from our general lack of sex differences in the behaviors analyzed, and
301 perhaps a baseline difference in behavior is necessary to elicit a sex-specific effect. The results of Mitic
302 et al. (2013); Leuner et al. (2004) and Lifschytz et al. (2006) in rats all observe sex-specific responses to
303 fluoxetine only when the sexes differed in behaviors without the drug. We do not have adequate data to
304 confirm this explanation and more experimentation along with physiological data would be necessary.

305 Another possible explanation for our lack of sex-specific drug effects could be our choice of dose.
306 Our choice of 1mg/L of nicotine is quite low compared with other studies in zebrafish (Levin et al., 2007;
307 Sackerman et al., 2010), and while our dosage of fluoxetine was much higher than is typically reported
308 (Egan et al., 2009; Wong et al., 2013), it is typically administered chronically. We would also like to note
309 that the sex-specific results of Faraday et al. (1999) utilizing nicotine in rats was only observed in one of
310 the two strains used. Zebrafish are highly genetically diverse (Parichy, 2015) and strain differences in
311 behavior (Benner et al., 2010; Egan et al., 2009) and drug efficacy (Sackerman et al., 2010) have been
312 reported. Therefore the possibility exists for sex-dependent drug effects to be observed in another strain.

313 Finally, we cannot dismiss the possibility that zebrafish simply don't exhibit sex-specific effects with
314 fluoxetine or nicotine. While there is no literature in this species to compare our results with, a recently
315 published study utilizing medaka (*Oryzias latipes*), another small teleost fish from southeast Asia, fails
316 to find sex-specific effects of chronic fluoxetine on many of the same behaviors described in the present
317 study (Ansai et al., 2016). More research is necessary to confirm any of the explanations given for our
318 lack of observed sex-drug interactions. The absence of studies considering sex-specific effects of drugs is
319 problematic if zebrafish are to remain a relevant model of pharmacology research. The topic has become
320 a concern in all animal models that NIH is going to start requiring all animal research to include sex as
321 part of the study unless deemed unnecessary (Clayton and Collins, 2014). If it turns out that strain is a
322 major factor influencing our results, then the abundance of genetically diverse populations could make
323 zebrafish an exciting tool to aid in the growing field of pharmacogenetics and personalized medicine in
324 which genetic background, among other traits, will be important for determining what drugs will be most
325 effective for treating disorders.

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462 FIGURES

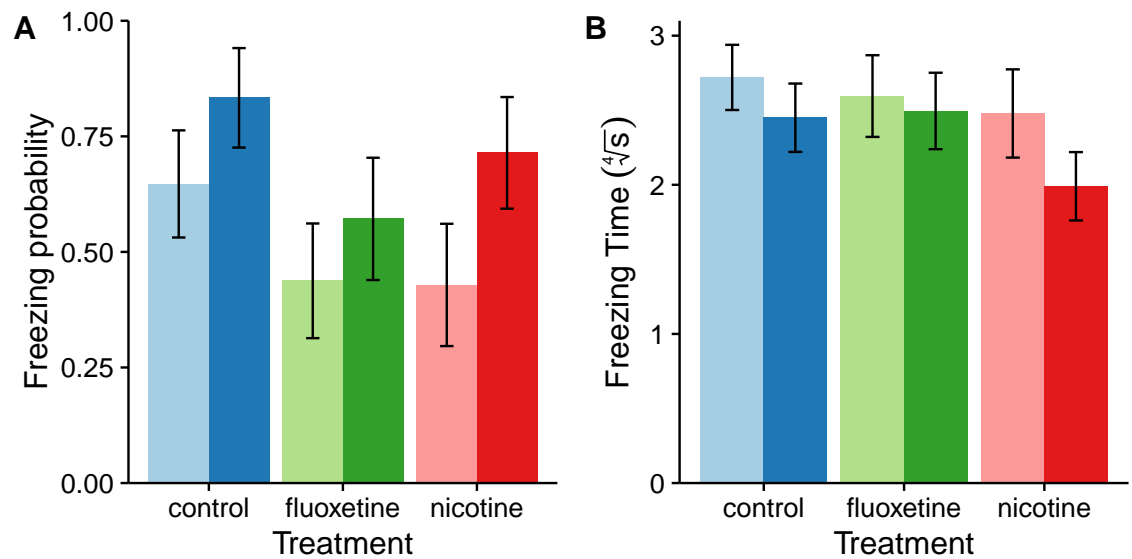


Figure 1. Freezing behaviors (motionless at the bottom of the tank) appear not to be affected by exposure to fluoxetine or nicotine. These graphs show the probability of freezing \pm SE. (A) and the mean time spent frozen \pm SE (B) for both sexes in each drug treatment group. Females are represented as light bars and males as dark bars. The freezing probability was calculated from a logistic GLM and transformed back into probabilities for this figure using the 'lsmeans' package in R. Freezing time was transformed using a fourth root in order to meet the assumptions of normality in the ANOVA.

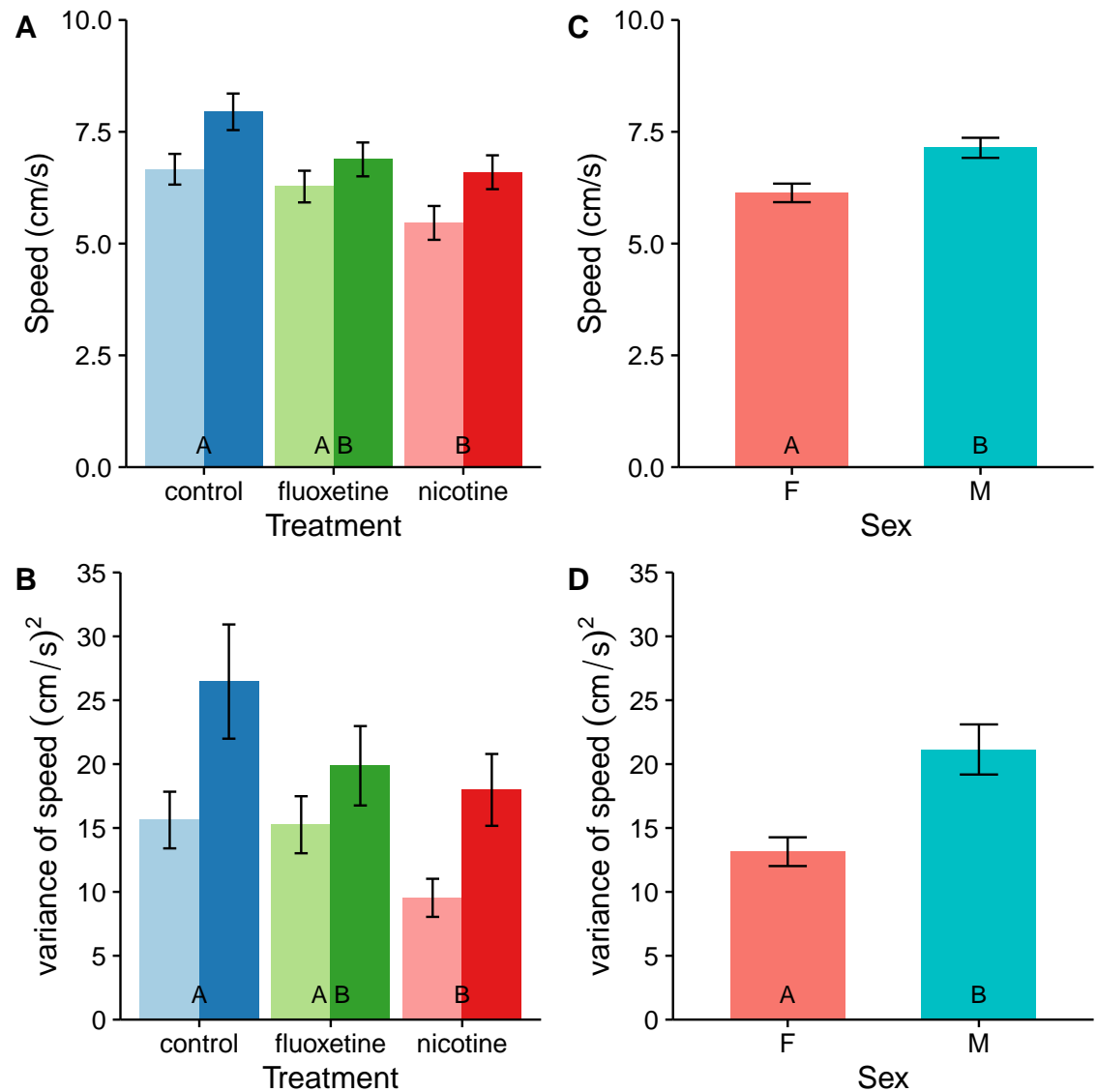


Figure 2. Average swimming speed (top) and consistency (individual variance) of swimming speed (bottom) are affected by fluoxetine and nicotine (A & B) as well as by sex (C & D). The fluoxetine treatment is not statistically different from the control, but is also not different from the nicotine treatment. Means \pm SE are reported. Results of the Tukey pairwise comparisons of drug treatment groups are delineated with letter groupings where similar letters represent a non-significant difference between treatments ($p > 0.05$). In panels A & B, females are represented with light bars and males with dark bars.

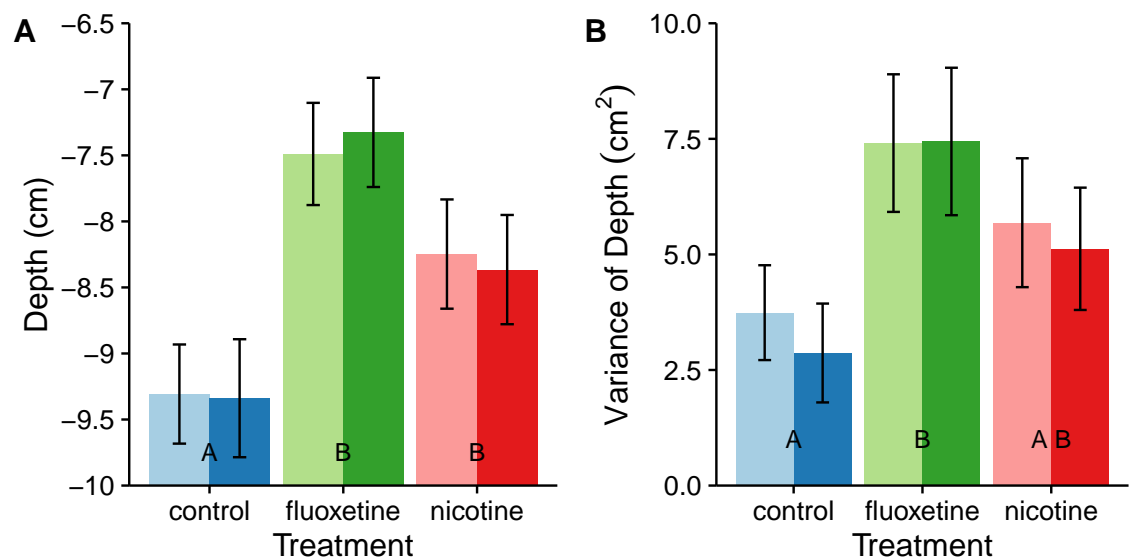


Figure 3. Average swimming depth (A) and average consistency (individual variance) of vertical usage (B) are affected by fluoxetine and nicotine. The nicotine treatment was not significantly different than the control with depth variance, but was also not different from the fluoxetine treatment. Means \pm SE are reported and the results of the Tukey pairwise comparisons of drug treatment groups are delineated with letter groupings where similar letters represent a non-significant difference between treatments ($p > 0.05$). Sex is distinguished by females with light bars and males with dark bars.

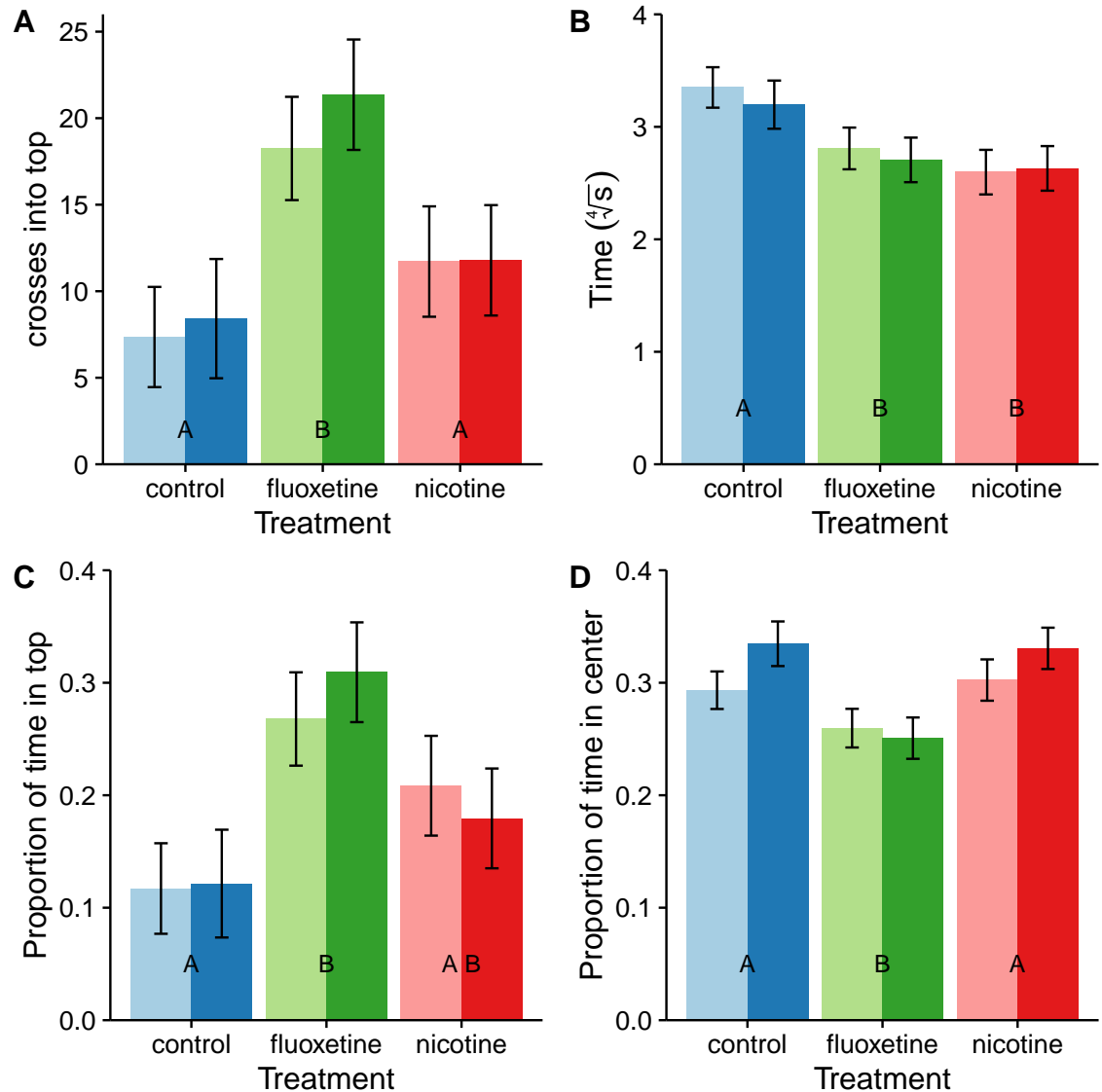


Figure 4. Average number of entries into the top half (A), latency to enter the top half (B), proportion of time spent in the top half (C), and proportion of time spent in center (D). Means \pm SE are reported and the results of the Tukey pairwise comparisons of drug treatment groups are delineated with letter groupings where similar letters represent a non-significant difference between treatments ($p > 0.05$). Sex is distinguished by females with light bars and males with dark bars. Latency to enter the top half is transformed using a fourth root transformation in order to meet the assumption of normality in the ANOVA.