A peer-reviewed version of this preprint was published in PeerJ on 24 August 2016.

Singer ML, Oreschak K, Rhinehart Z, Robison BD. 2016. Anxiolytic effects of fluoxetine and nicotine exposure on exploratory behavior in zebrafish. PeerJ 4:e2352 https://doi.org/10.7717/peerj.2352

Anxiolytic effects of fluoxetine and nicotine exposure on exploratory behavior in zebrafish

Matthew L Singer, Kris Oreschak, Zachariah Rhinehart, Barrie D Robison

Zebrafish (*Danio rerio*) have emerged as a popular model for studying the pharmacology and behavior of anxiety. While there have been numerous studies documenting the anxiolytic and anxiogenic effects of common drugs in zebrafish, many do not report or test for behavioral differences between the sexes. Previous studies have indicated that males and females differ in their baseline level of anxiety. In this study, we test for a sex interaction with fluoxetine and nicotine. We exposed fish to system water (control), 10 mg/L fluoxetine, or 1 mg/L nicotine for three minutes prior to being subjected to four minutes in an open-field drop test. Video recordings were tracked using ProAnalyst. Fish from both drug treatments reduced swimming speed, increased vertical position, and increased use of the top half of the open field when compared with the control, though fluoxetine had a larger effect on depth related behaviors while nicotine mostly affected swimming speed. a significant sex effect was observed where females swam at a slower and more constant speed than males, however neither drug produced a sex-dependent response.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

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

Matthew L. Singer[1], Kris Oreschak[2,1], Zachariah Rhinehart[1], and Barrie D. Robison[1]

[1]**Department of Biological Sciences, University of Idaho, Moscow, ID, United States**
[2]**Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Aurora, CO, United States**

ABSTRACT

Zebrafish (*Danio rerio*) have emerged as a popular model for studying the pharmacology and behavior of anxiety. While there have been numerous studies documenting the anxiolytic and anxiogenic effects of common drugs in zebrafish, many do not report or test for behavioral differences between the sexes. Previous studies have indicated that males and females differ in their baseline level of anxiety. In this study, we test for a sex interaction with fluoxetine and nicotine. We exposed fish to system water (control), 10 mg/L fluoxetine, or 1 mg/L nicotine for three minutes prior to being subjected to four minutes in an open-field drop test. Video recordings were tracked using ProAnalyst. Fish from both drug treatments reduced swimming speed, increased vertical position, and increased use of the top half of the open field when compared with the control, though fluoxetine had a larger effect on depth related behaviors while nicotine mostly affected swimming speed. A significant sex effect was observed where females swam at a slower and more constant speed than males, however neither drug produced a sex-dependent response.

Keywords: behavior, anxiety, fluoxetine, nicotine

INTRODUCTION

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

Anxiety-related behaviors are known to vary by sex in zebrafish and other model organisms. Male and female rats differed in their time spent in the center of an open field and a plus maze, though the nature of these differences were also dependent on the strain observed (Mehta et al., 2013). In zebrafish, females have been observed as less anxious or more bold than males when measuring locational preferences in the presence of a human observer (Benner et al., 2010; Oswald et al., 2013).

There is also evidence that sex may influence the behavioral response to drugs. Differential responses have been observed in humans utilizing Sertraline (an SSRI) where females showed an enhanced response compared to males. (Kornstein et al., 2000). Meanwhile, no differences were observed between the sexes in the effectiveness of the SSRI Fluoxetine (Quitkin et al., 2002). Evidence in rats suggest that nicotine's effects on stress and anxiety may also differ between the sexes with males exhibiting a greater anxiolytic effect. These differences depend on strain and dosage (Faraday et al., 1999).

In this experiment, we test the hypothesis that zebrafish exhibit sex-dependent responses to fluoxetine and nicotine, two drugs with known anxiolytic effects. Both drugs have been observed to reduce anxious behaviors in humans (Gilbert, 1979; Griffin and Mellon, 1999), rats (Cohen et al., 2009; Zhang et al., 2000) and zebrafish (Bencan and Levin, 2008; Bencan et al., 2009; Cachat et al., 2010; Levin et al., 2007).

METHODS

Subjects

Experimental fish were bred from adult Scientific Hatcheries strain that has been bred and maintained in our facility. Water in our Aquaneering Inc. (San Diego, CA) system was constantly circulating and kept at a temperature of 28.5 ^{o}C on a 14 hour light:10 hour dark cycle. The fish were fed a diet of brine shrimp twice and flake food (Tetramin) once for a total of three daily feedings. At the time of data collection, the fish were four months old and housed in three-liter tanks in groups of five. All aspects of this study were approved by the University of Idaho's Animal Care and Use Committee under protocol 2014-14.

Dosing

Fluoxetine (generic from Wal Mart) and nicotine (Sigma Aldrich) treatments were administered at concentrations of 10 mg/L for the fluoxetine and 1 mg/L for the nicotine. Each drug was dissolved in system water to make a working solution each morning of administration. A third treatment of only system water served as a control. Fish were netted from their home tank and immediately placed into a beaker containing 100mL of one of the three treatments. After three minutes of exposure to the drug dose, the fish were transferred to an open field test tank for behavioral recording. Treatment type and order were randomized.

Behavior Assay & Video tracking

The fish were placed in an empty rectangular tank measuring 25cm wide, 12 cm high (from water level to bottom), and 6 cm thick (front to back). The volume of water in the tank was approximately 2 L. Each fish was filmed for four minutes (240 seconds) at 25 frames/second beginning from the time that the subject entered the water. The camera and observer were hidden behind a blind during the recorded observation time. The tank was backlit with an opaque diffuser for the purposes of creating a silhouetted object for motion tracking. After the four-minute period, the fish was netted out of the test tank, placed into its own 1.5 L housing and returned to the main system. Observations were recorded over three days between the hours of 10:00 am and 2:00 pm. After all subjects had been recorded, weight and standard length measurements were obtained by first anesthetizing the individual in MS-222 solution and blotting excess water with a paper towel. At this time, we also recorded the sex of the individual.

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

Analysis

Freezing

Freezing time was defined as the time a subject spent motionless on the bottom of the tank. We defined motionless as maintaining a velocity of less than .01 cm/frame for more than 20 consecutive frames. Any short bursts of motion flanked by considerable freezing times were verified in the video to be true motion. If a time period of activity was less than 40 frames, it was recategorized as part of the freezing time. The freezing time was then calculated by counting the total number of frames marked as frozen.

Speed

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

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

Depth

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

Horizontal Place Preference

The width of the tank was divided into three equal sections and the proportion of time in the middle section calculated to differentiate preference to be located in the center versus the edge of the test environment. While we had clear expectations for location preference with respect to depth, it was unclear whether the middle or the edges represent a "safe" zone with respect to horizontal preference. Experiments with rodents have found that stressed individuals prefer the edges of their arenas (thigmotaxis), but that this behavior is analogous to stressed fish preferring the bottom (Levin et al., 2007).

Statistical Analysis

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

RESULTS

Multivariate

The full model Type-II MANOVA included the effects of weight, sex, drug treatment, and the sex by drug interaction on average depth, variance of depth, average speed, variance of speed, percent of time spent in the top half, number of crosses into the top half, latency to enter the top half, and proportion of time spent in the middle third horizontally (ie, away from the edges). There was a marginally significant effect of sex ($\Lambda = 0.17896$, $F_{8,73} = 1.9889, p = 0.05974$) and a significant effect of drug treatment ($\Lambda = 0.56646, F_{16,148} = 3.6551, p = 0.00001305$) on behavior, but no significant interaction. There was no significant effect of weight as a co-variate, and including weight in the model showed no improvement over removing it ($\Lambda = 0.95793, F_{5,76} = 0.66755, p = 0.6492$). With the reduced model, we observed a significant effect of sex ($\Lambda = 0.22404, F_{8,74=2.6707}, p = 0.01237$) and drug treatment ($\Lambda = 0.56659, F_{16,150} = 3.7057, p = 0.00001014$). Therefore, for all subsequent analyses we considered only the effects of sex, drug treatment, and the interaction term.

Individual components of behavior

We observed no significant interactions between sex and drug treatment in any of the individual behavior components (see Table 1), consistent with the results of the MANOVA above. All components indicated a significant effect of drug treatment ($p < 0.05$) except for freezing occurrence and freezing duration. The subsequent descriptions describe the results of the post-hoc pairwise comparisons of the drug treatments using the least-squared means and Tukey adjusted p-values based on 3 tests. We also observed a significant effect of sex with regard to average swimming speed ($F_{1,81} = 10.7178, p = 0.001562$) and consistency

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

	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.

(variance) of swimming speed ($F_{1,81} = 13.9196, p = 0.0003528$). Males were on average faster than females, but also exhibited less consistency in their swimming speeds. These were the only instances in which the sexes differed in their behavior.

Freezing behavior

Freezing behavior is a commonly observed anxiety related behavior in zebrafish (Egan et al., 2009). Of the 87 individuals observed, 52 exhibited freezing behavior. Though males tend to be more likely to freeze than females on average, this difference was only marginally significant ($\chi^2 = 3.7866, p = 0.05167$). We also failed to observe a significant effect of drug treatment on freezing occurrence ($\chi^2 = 3.7964, p = 0.14983$) as well as a sex by drug interaction ($\chi^2 = 0.3949, p = 0.82083$). For freezing duration, or latency to explore, we only included the 52 individuals that exhibited freezing behavior. This improved the assumptions of normality required for the ANOVA. Results of the type II ANOVA suggest that neither sex nor drug treatment have any significant effect on freezing duration (Sex: $F_{1,46} = 1.9604, p = 0.1682$; Drug: $F_{2,46} = 1.3707, p = 0.2641$). Figure 1 shows the results of freezing behaviors.

Speed

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

Depth

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

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

Horizontal Place Preference

All subjects spent most of their time near the edges avoiding the center (figure 4), consistent with the concept of thigmotaxis. However, subjects dosed with fluoxetine spent less time in the center and more time near the edges than subjects in the control and nicotine treatments ($t = 3.257, p = 0.0046$).

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

DISCUSSION

Differences in fluoxetine and nicotine behavioral responses

Small prey fish such as zebrafish tend to behave in such a way as to reduce risk. When placed in a novel open field, such behavioral strategies include diving to the bottom and remaining motionless (Egan et al., 2009; Wong et al., 2010), and avoiding potentially risky locations such as the surface of the water (Bencan et al., 2009; Egan et al., 2009). Exposure to anxiogenic drugs alters these behaviors in ways that may indicate an association between anxiety related behaviors and risk management. We observed a decrease in bottom dwelling and an increase in time spent in the top half of the tank in fish exposed to fluoxetine (figures 3 and 4). This is consistent with patterns observed by Egan et al. (2009) who also report an increased use of the top of the water column by zebrafish exposed to fluoxetine. However, the study by Egan et al. (2009) also reports a reduction in freezing bouts and freezing time, a pattern we failed to observe. One explanation for this discrepancy could be differing effects of chronic and acute dosing. Fluoxetine is metabolized into norfluoxetine, its active metabolite, in the liver by cytochrome P450 enzymes (Rasmussen et al., 1995). It then travels through the bloodstream to the brain where it blocks the reuptake of serotonin (Beasley et al., 1992). We have shown that similar behavioral changes can occur with just a single acute dose of the same concentration. Acute exposure to fluoxetine has also been shown to reduce cortisol levels of zebrafish exposed to a stressful environment (de Abreu et al., 2014), suggesting that the behaviors we observed may be due to a reduction in stress resulting from exposure to the drugs.

Nicotine is a paradoxical substance with regard to anxiety. In low doses, it has been shown to have an anxiolytic effect, but at high doses, the effect changes to be anxiogenic File et al. (1998). We observed changes in swimming speed, average depth, and latency to enter the top in fish exposed to nicotine. Fish exposed to nicotine were quicker to enter the top and swam higher in the water column on average compared to control fish. This is consistent with a reduction in anxiety related behaviors as seen in the Fluoxetine treatment group. By contrast, exposure to nicotine appeared to decrease swimming speed while increasing the consistency at which the fish swam. The increased consistency (reduction of individual variance) might be explained by a reduction in anxiety, where individuals that are calm should move at a fairly normal and constant pace, while anxious individuals may constantly alter their swimming speeds in an erratic fashion Gerlai et al. (2009). Egan et al. (2009) reported an increase in average swim speed with exposure to fluoxetine, which contrasts with our observations of slower average swim speeds with exposure to either fluoxetine or nicotine. Sackerman et al. (2010) suggests that nicotine may have sedating effects which could account for the slower swim speeds. However, we also observe slower average swim speeds in the fluoxetine treatment, and though the difference is not statistically different from the control, it is also not different from the nicotine effect. We observed a similar pattern in the nicotine treatment with respect to the time spent at the top and the variation in depth use, where the nicotine treatment was statistically indistinguishable from both the control and the fluoxetine treatments. In these two instances, it is possible that the nicotine is having an anxiogenic effect, but that we used too low of a dose to observe an effect that is different from the control. Sackerman et al. (2010) also failed to observe an effect of nicotine on swim depth using a low dose of 25 mg/L, but noted that higher doses such as 50 mg/L and 100 mg/L do produce a significant effect (Levin et al., 2007). Our dose of 1 mg/L is noticeably lower than other studies of nicotine in adult Zebrafish, but we also used pure nicotine liquid while the other studies used a nicotine tartrate salt (Levin et al., 2007; Sackerman et al., 2010).

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

The effect of sex on behavior and drug exposure

We found no evidence that sex interacts with the effects of nicotine or fluoxetine on anxiety-related behavior in zebrafish, which is consistent with the results of Quitkin et al. (2002). However, we did find consistent differences in behavior among the sexes independent of the drugs, particularly in behaviors related to swimming speed. These differences may be consistent with patterns observed in other exper-

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

iments that suggest females are more bold under stress than males Benner et al. (2010); Oswald et al. (2013).

Conclusions

We have confirmed that fluoxetine and nicotine do affect exploratory behaviors that are often used as indicators of anxiety, and that the effects are consistent with a reduction in anxiety. However, our data do not support the hypothesis that the effects of these drugs are sex dependent. One caveat in this experiment is that we only used a single strain. Zebrafish exhibit a high degree of genetic diversity (Parichy, 2015), which can affect the efficacy of drugs as well as the base behaviors among different laboratory populations. Even sex-drug interactions may be strain specific. For example, Faraday et al. (1999) found sex-specific effects of nicotine on startle responses in rats, but only in one strain and not another. If confirmed in other strains of zebrafish, the lack of a sex dependent response to these drugs may support the continuing expansion of the zebrafish as a pharmacological model organism.

ACKNOWLEDGMENTS

We would like to thank Dr. Craig McGowan for the use of his equipment and ProAnalyst license in order to track the videos used in this experiment.

REFERENCES

Barcellos, L. J. G., Ritter, F., Kreutz, L. C., Quevedo, R. M., da Silva, L. B., Bedin, A. C., Finco, J., and Cericato, L. (2007). Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, Danio rerio. *Aquaculture*, 272(1-4):774–778.

Barros, T. P., Alderton, W. K., Reynolds, H. M., Roach, a. G., and Berghmans, S. (2008). Zebrafish: an emerging technology for in vivo pharmacological assessment to identify potential safety liabilities in early drug discovery. *Br. J. Pharmacol.*, 154(7):1400–13.

Beasley, C. M., Masica, D. N., and Potvin, J. H. (1992). Fluoxetine: a review of receptor and functional effects and their clinical implications. *Psychopharmacology (Berl).*, 107(1):1–10.

Bencan, Z. and Levin, E. D. (2008). The role of alpha7 and alpha4beta2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish. *Physiol. Behav.*, 95(3):408–12.

Bencan, Z., Sledge, D., and Levin, E. D. (2009). Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacol. Biochem. Behav.*, 94(1):75–80.

Benner, M. J., Drew, R. E., Hardy, R. W., and Robison, B. D. (2010). Zebrafish (Danio rerio) vary by strain and sex in their behavioral and transcriptional responses to selenium supplementation. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.*, 157(4):310–318.

Buske, C. and Gerlai, R. (2014). Diving deeper into Zebrafish development of social behavior: analyzing high resolution data. *J. Neurosci. Methods*, 234:66–72.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., Dileo, J., Frank, K., Chang, K., Utterback, E., Hart, P., and Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nat. Protoc.*, 5(11):1786–1799.

Cohen, A., Young, R. W., Velazquez, M. a., Groysman, M., Noorbehesht, K., Ben-Shahar, O. M., and Ettenberg, A. (2009). Anxiolytic effects of nicotine in a rodent test of approach-avoidance conflict. *Psychopharmacology (Berl).*, 204(3):541–9.

de Abreu, M., Koakoski, G., and Ferreira, D. (2014). Diazepam and Fluoxetine Decrease the Stress Response in Zebrafish. *PLoS One*, 9(7):1–5.

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., Elkhayat, S. I., Bartels, B. K., Tien, A. K., Tien, D. H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., and Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav. Brain Res.*, 205(1):38–44.

Faraday, M. M., O'Donoghue, V. a., and Grunberg, N. E. (1999). Effects of nicotine and stress on startle amplitude and sensory gating depend on rat strain and sex. *Pharmacol. Biochem. Behav.*, 62(2):273–284.

File, S. E., Kenny, P. J., and Ouagazzal, a. M. (1998). Bimodal modulation by nicotine of anxiety in the social interaction test: role of the dorsal hippocampus. *Behav. Neurosci.*, 112(6):1423–1429.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

Gerlai, R. (2014). Social behavior of zebrafish: from synthetic images to biological mechanisms of shoaling. *J. Neurosci. Methods*, 234:59–65.

Gerlai, R. (2015). Zebrafish phenomics: behavioral screens and phenotyping of mutagenized fish. *Curr. Opin. Behav. Sci.*, 2:21–27.

Gerlai, R., Fernandes, Y., and Pereira, T. (2009). Zebrafish (Danio rerio) responds to the animated image of a predator: Towards the development of an automated aversive task. *Behav. Brain Res.*, 201(2):318–324.

Gilbert, D. (1979). Paradoxical Tranquilizing and Emotion-Reducing Effects of Nicotine. *Psychol. Bull.*, 86(4):643–661.

Griffin, L. and Mellon, S. (1999). Selective serotonin reuptake inhibitors directly alter activity of neurosteroidogenic enzymes. *Proc. Natl. Acad. Sci.*, 96(23):13512–13517.

Kornstein, S. G., Schatzberg, a. F., Thase, M. E., Yonkers, K. a., McCullough, J. P., Keitner, G. I., Gelenberg, a. J., Davis, S. M., Harrison, W. M., and Keller, M. B. (2000). Gender differences in treatment response to sertraline versus imipramine in chronic depression. *Am. J. Psychiatry*, 157:1445–1452.

Langheinrich, U. (2003). Zebrafish: a new model on the pharmaceutical catwalk. *Bioessays*, 25(9):904–12.

Levin, E. D., Bencan, Z., and Cerutti, D. T. (2007). Anxiolytic effects of nicotine in zebrafish. *Physiol. Behav.*, 90(1):54–8.

Mathur, P. and Guo, S. (2010). Use of zebrafish as a model to understand mechanisms of addiction and complex neurobehavioral phenotypes. *Neurobiol. Dis.*, 40(1):66–72.

Maximino, C., de Brito, T. M., Colmanetti, R., Pontes, A. A. A., de Castro, H. M., de Lacerda, R. I. T., Morato, S., and Gouveia, A. (2010). Parametric analyses of anxiety in zebrafish scototaxis. *Behav. Brain Res.*, 210(1):1–7.

Mehta, N. S., Wang, L., and Redei, E. E. (2013). Sex differences in depressive, anxious behaviors and hippocampal transcript levels in a genetic rat model. *Genes, Brain Behav.*, 12:695–704.

Oswald, M. E., Drew, R. E., Racine, M., Murdoch, G. K., and Robison, B. D. (2013). Is Behavioral Variation along the Bold-Shy Continuum Associated with Variation in the Stress Axis in Zebrafish? *Physiol. Biochem. Zool.*, 85(6):718–28.

Parichy, D. M. (2015). Advancing biology through a deeper understanding of zebrafish ecology and evolution. *Elife*, 4:1–11.

Quitkin, F. M., Stewart, J. W., McGrath, P. J., Taylor, B. P., Tisminetzky, M. S., Petkova, E., Chen, Y., Ma, G., and Klein, D. F. (2002). Are there differences between women's and men's antidepressant responses? *Am. J. Psychiatry*, 159(November):1848–1854.

Rasmussen, B. B., Menp, J., Pelkonen, O., Loft, S., Poulsen, H. E., Lykkesfeldt, J., and Brfsen, K. (1995). Selective serotonin reuptake inhibitors and theophylline metabolism in human liver microsomes: potent inhibition by fluvoxamine. *Br. J. Clin. Pharmacol.*, 39:151–159.

Sackerman, J., Donegan, J. J., Cunningham, C. S., Nguyen, N. N., Lawless, K., Long, A., Benno, R. H., and Gould, G. G. (2010). Zebrafish Behavior in Novel Environments: Effects of Acute Exposure to Anxiolytic Compounds and Choice of Danio rerio Line. *Int. J. Comp. Psychol.*, 23(1):43–61.

Shin, J. T. and Fishman, M. C. (2002). From Zebrafish to human: modular medical models. *Annu. Rev. Genomics Hum. Genet.*, 3(24):311–40.

Sison, M. and Gerlai, R. (2010). Associative learning in zebrafish (Danio rerio) in the plus maze. *Behav. Brain Res.*, 207(1):99–104.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K. M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J., and Kalueff, A. V. (2010). Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behav. Brain Res.*, 208(2):450–7.

Zhang, Y., Raap, D. K., Garcia, F., Serres, F., Ma, Q., Battaglia, G., and Van de Kar, L. D. (2000). Long-term fluoxetine produces behavioral anxiolytic effects without inhibiting neuroendocrine responses to conditioned stress in rats. *Brain Res.*, 855(1):58–66.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

FIGURES

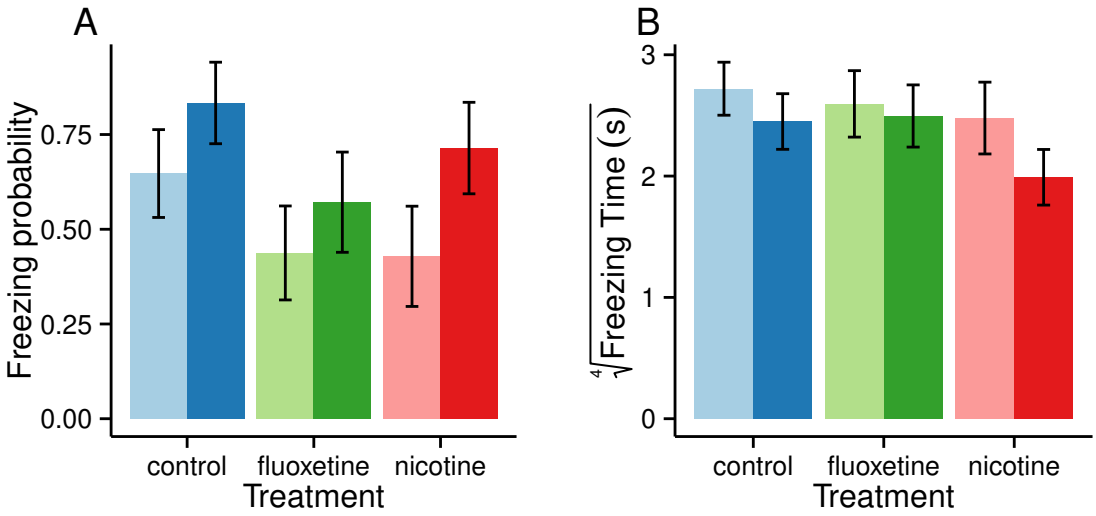

Figure 1. Freezing behaviors (motionless at the bottom of the tank) appear not to be affected by exposure to fluoxetine or nicotine. These graphs show the probability of freezing $\pm$ SE. (A) and the mean time spent frozen $\pm$ SE (B) for both sexes in each drug treatment group. Females are represented as light bars and males as dark bars.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

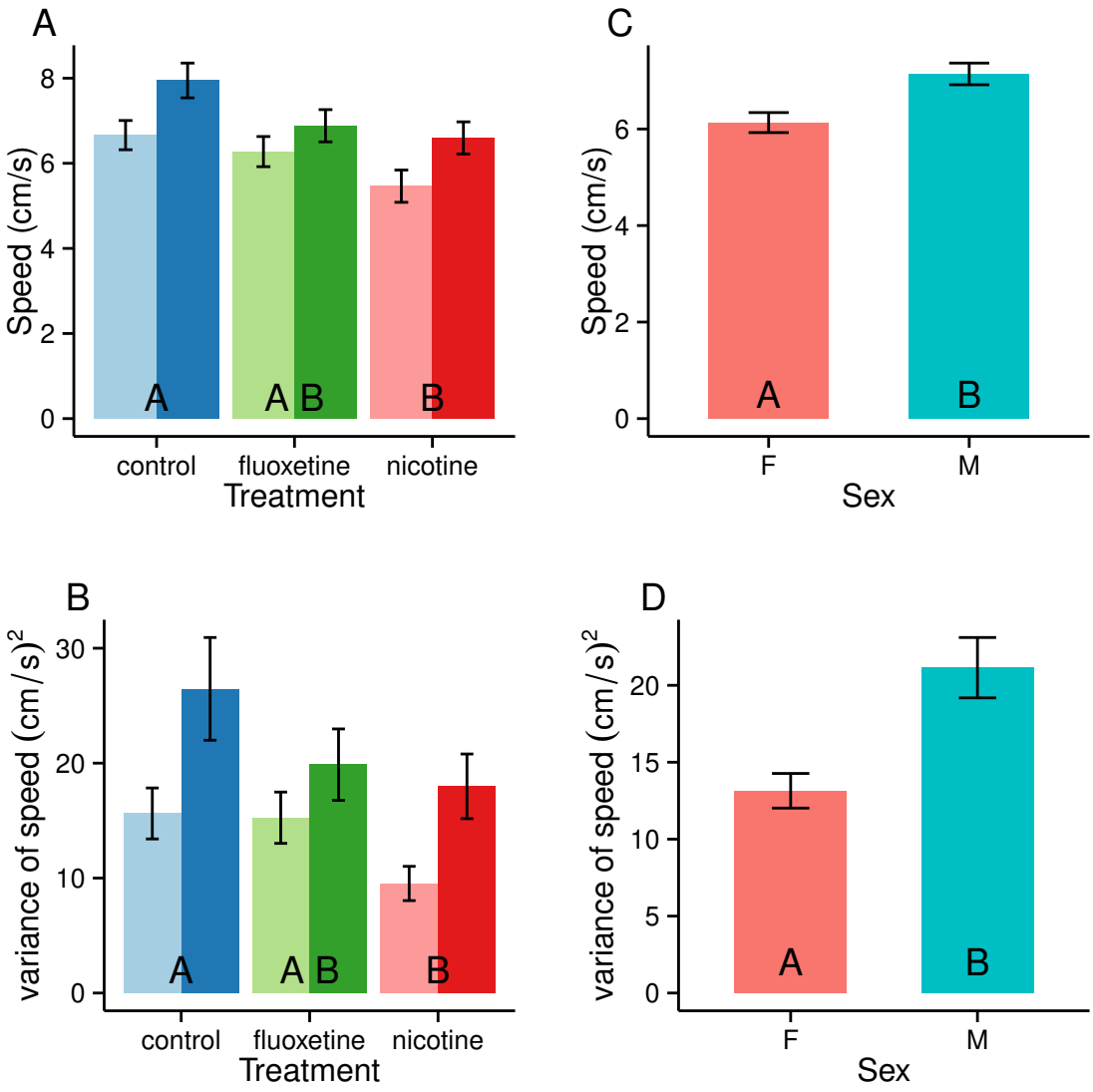

Figure 2. Average swimming speed (top) and variance of swimming speed (bottom) are affected by fluoxetine and nicotine (A & B) as well as by sex (C & D). The fluoxetine treatment is not statistically different from the control, but is also not different from the nicotine treatment. Means ± SE are reported and results of the Tukey pairwise comparisons are delineated with letter groupings. In panels A & B, females are represented with light bars and males with dark bars.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

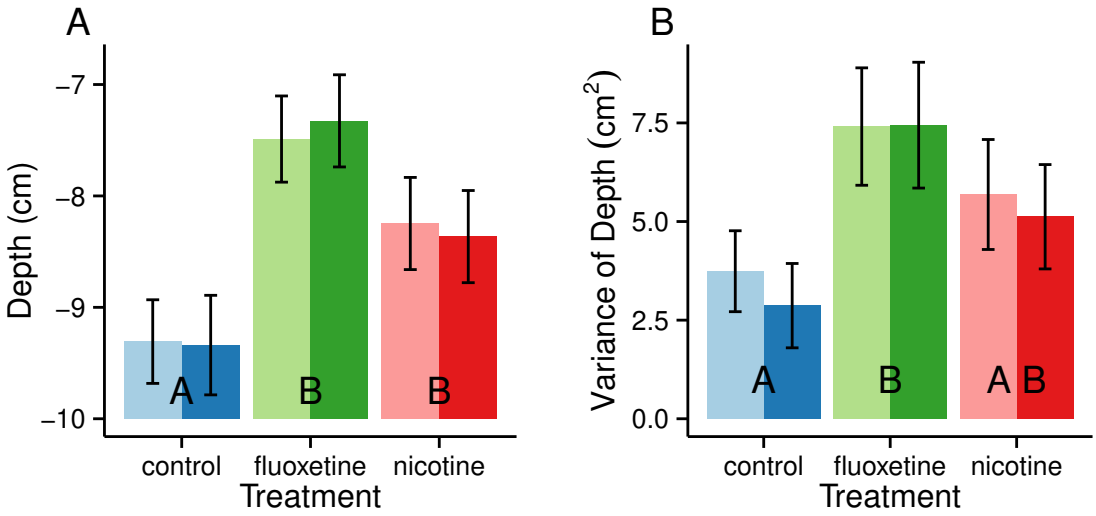

Figure 3. Average swimming depth (A) and variance of vertical usage (B) are affected by fluoxetine and nicotine. The nicotine treatment was not significantly different than the control with depth variance, but was also not different from the fluoxetine treatment. Means ± SE are reported and the results of the Tukey pairwise comparisons are delineated with letter groupings. Sex is distinguished by females with light bars and males with dark bars.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016

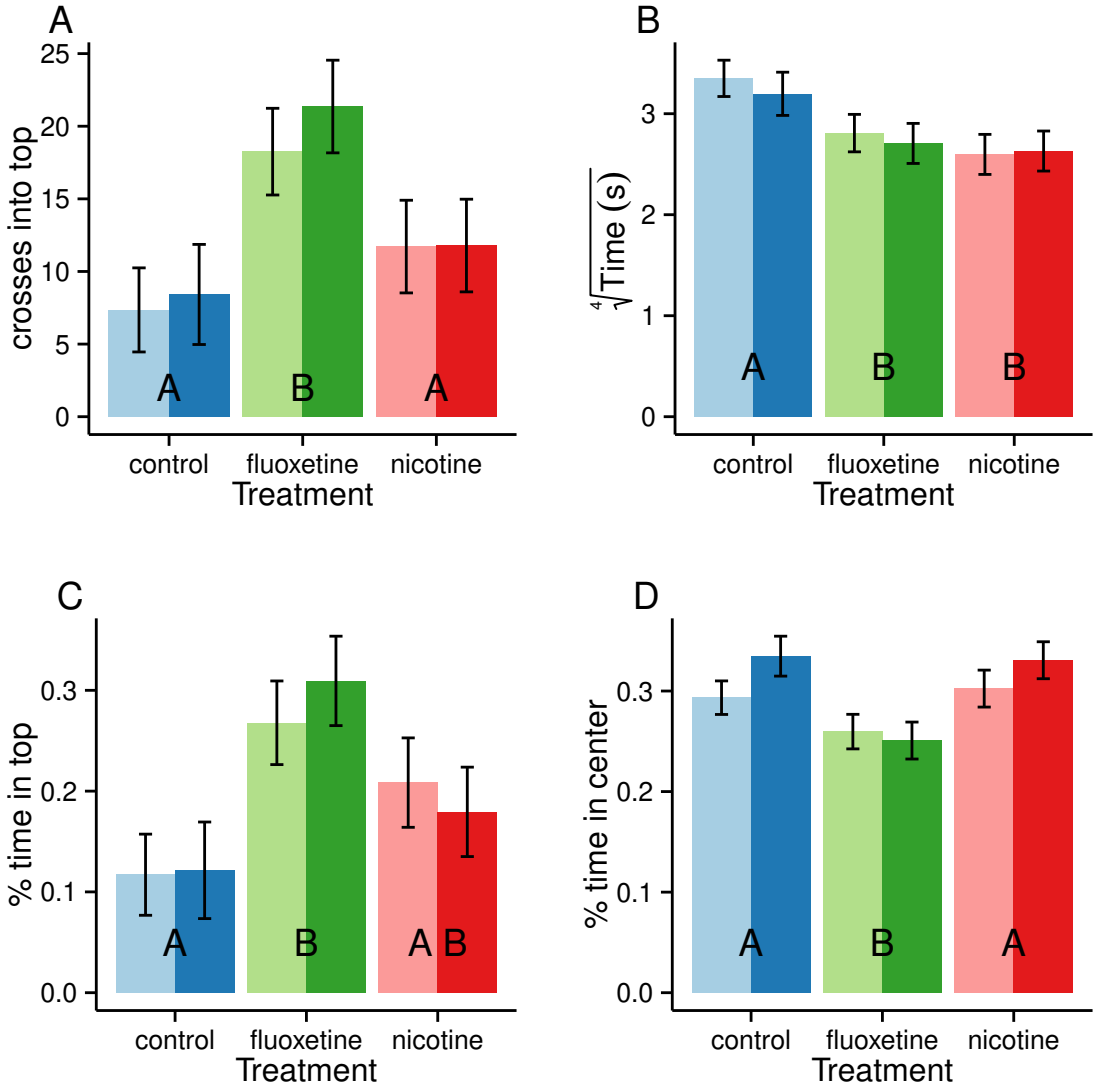

Figure 4. Average number of entries into the top half (A), latency to enter the top half (B), proportion of time spent in the top half (C), and proportion of time spent in center (D). Means $\pm$ SE are reported and the results of the Tukey pairwise comparisons are delineated with letter groupings. Sex is distinguished by females with light bars and males with dark bars.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1718v1 | CC-BY 4.0 Open Access | rec: 6 Feb 2016, publ: 6 Feb 2016