Zebrafish as a Model System for Research and Teaching
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Accumulating Evidence of Zebrafish's Response to Biogenic Amines

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Abstract - Zebrafish are an increasingly valuable model organism for pharmacologic and behavioral research. Zebrafish have well characterized behaviors that have been cataloged for use in assessment of spatial learning, anxiety, depression, and other cognitive functions. In addition, pharmaceutical treatment of zebrafish can be accomplished by immersion, a low-stress drug delivery method. While significant homology exists between zebrafish and humans, some differences exist that may limit their utility as a model for serotonergic signaling. Specifically, the serotonin transporter and an important autoreceptor ($5\text{-}HT_{1A}$) are duplicated in the zebrafish genome and the effect of this duplication on the serotonergic system is not entirely clear. The current studies are designed to add to the growing body of evidence concerning the behavioral effect of manipulating 5-HT and other biogenic amines. In order to accomplish this, zebrafish were treated with the SSRIs, citalopram, and escitalopram, along with the triple reuptake inhibitor, 5-APB, and the monoamine releasing agent, BMPEA. The fish were then evaluated for changes in spatial learning, movement, and anxiety by using a T-maze or novel dive tank. While there was no significant effect of SSRI treatment on spatial learning, there were significant decreases in movement and in body length for the SSRI treated groups. There were also significant decreases in movement and apparent anxiety in the 5-APB and BMPEA treated groups. These results are consistent with previous reports showing that increases in 5-HT that produce an anxiolytic effect both in zebrafish and in humans and support the value of continuing to cultivate this model.

Introduction

The zebrafish species *Danio rerio* (Hamilton) (aka zebra danio) has become a popular model organism for a wide range of research interests. Zebrafish have been used to study vertebrate developmental mechanisms, genome evolution, physiology, behavior, toxicology, disease (McClusky and Postlethwait 2015), pharmacology (Bailey et al. 2015), and complex brain disorders (Kalueff et al. 2015, Kalueff and Stewart 2015). This rise in popularity is due to their striking homology to humans, their low cost to maintain, and the availability of different phenotypes and mutants. In addition, the model organism zebrafish is ideal for experiments involving a variety of pharmaceutical treatments. Zebrafish have the unique ability to absorb these substances through their gills, eliminating any stress induced by the traditional method of injections (Magno et al. 2015). In response to these treatments, the central nervous system of the zebrafish responds similarly to the human nervous system, making them a useful model in the study of many human disorders. More specifically, the nervous and endocrine systems in zebrafish work together to elicit a stress response through the hypothalamus pituitary interrenal axis (HPI axis) analogous to the HPA axis in humans, which means zebrafish utilize similar endocrine system components such as hormones, receptors, and signaling cascades (Lohr and Hammerschmidt 2011, Tokarz et al. 2013). Similarly, serotonergic signaling has been linked to arousal, fear and anxiety in both zebrafish and in mammals (Herculano and Maximino 2014).

Importantly, zebrafish have well characterized behaviors like shoaling, exploration, feeding and overall movement that are affected by mood-altering compounds and can therefore be utilized as measures of depression or anxiety (Hall et al. 2014, Kalueff et al. 2013, Nguyen et al. 2014). Interestingly, these depression biomarkers show significant

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similarity to the behavioral changes that occur in rodent models. Because of these similarities, fields like psychology have turned to zebrafish as the subject for studying the development of complex disorders and possible pharmacological treatments. Studies into the mechanisms behind some common disorders (such as attention deficit hyperactivity disorder, post-traumatic stress disorder, depression, and substance abuse) have begun looking into the development of these disorders and possible treatment methods (Bailey et al. 2015, Stewart et al. 2014).

However, some differences have been revealed, particularly in genetic representation of serotonin signal transduction in teleost fish including zebrafish. Teleost fish underwent a whole genome duplication (WGD) over 300 million years ago, followed by massive gene loss and specialization (Glasauer and Neuhauss 2014, Magadum et al. 2013, Sato et al. 2009). Notably, genes associated with the neurotransmitter, serotonin, have been affected such that zebrafish now possess both paralogues and orthologues in that system. Specifically, the zebrafish genome includes twice as many genes encoding tryptophan hydroxylase (TPH) and serotonin transporter (SERT) and five additional serotonin receptor (5-HTR) genes but half the number of monoamine oxidase (MAO) genes as humans possess, calling into question the homology of that system to mammals (Herculano and Maximino 2014, Lillesaar et al. 2007, Norton et al. 2008). Explicitly, the existence of differences in genes that encode the synthesis machinery (TPH), and the uptake and metabolism machinery (SERT and MAO) suggest that there may be fundamental differences in the kinetics of neurotransmission in Zebrafish that are complex and therefore, crucial to characterize.

In addition, the relationship between anxiety and learning is a complex one that seems to relate to the nature of the learned task in addition to other factors. Holscher (1999) demonstrated that stressed rats were quicker to learn avoidance behaviors like a foot shock, but slower to learn spatial tasks like the Morris Water Maze. Other studies have examined the role of glucocorticoids in modulating memory formation and retrieval. Evidence from both human and non-human studies suggests that while glucocorticoids seem to enhance new memory consolidation (Beckner et al. 2006, Buchanan et al. 2006, McGaugh and Roozendaal 2002), they reduce memory retrieval (Gagnon and Wagner 2016, Larrosa et al. 2017). Furthermore, since patients with major depression often demonstrate anxiety, elevated cortisol levels and impaired memory, the interdependence of these factors should be explored along with the ability of anti-depressants to modulate all four.

In order to add to the growing body of data on biogenic amines, the following studies employed pharmaceutical manipulations of related neurotransmitter systems. The pharmacologic agents included the selective serotonin reuptake inhibitors (SSRI), citalopram and escitalopram, which block removal of serotonin (5-HT) from the synaptic cleft. Both acute and chronic administration of SSRI's were tested because the mechanism of action has been demonstrated to increase serotonergic signaling immediately but transiently. The serotonergic neurons then undergo adaptation which reduces signal transduction for days or weeks before desensitization of the inhibitory 5-HT_{1A} autoreceptors promotes increased serotonin signaling (Stahl 1998, Volle et al. 2018). As a result of this adaptation, both acute and sufficiently long chronic SSRI treatments are expected to produce similar results. A second type of substance tested, 5-(2-aminopropyl)-benzofuran (5-APB), is a serotonin–norepinephrine–dopamine reuptake inhibitor (SNDRI), or triple reuptake inhibitor, meaning that 5-APB acts as a reuptake inhibitor for the monoamine neurotransmitters serotonin, norepinephrine, and dopamine, by blocking the action of the serotonin transporter, norepinephrine transporter, and dopamine transporter, respectively (Rickli et al. 2015). Another study suggested that 5-APB, was able to activate serotonin receptors 5-HT_{2A} and 5-HT_{2B} in addition to blocking neurotransmitter transporters (Dawson et al. 2014). Finally, beta-

methylphenethylamine (BMPEA) was tested. Phenethylamines are monoamine-releasing agents that specifically stimulate the release of serotonin, norepinephrine and dopamine (Xie and Miller 2008). BMPEA was selected because of its inclusion in some supplements despite limited data available on potency and toxicity (Cohen et al. 2015).

The novel dive tank paradigm has been developed as a means for testing fear response and anxiety in zebrafish. When placed into a novel situation (a new tank) zebrafish will dive to the bottom, presumably to avoid predators. Over time, the fish will begin to explore the new environment. Fish that begin exploring sooner, swim faster and explore more extensively are considered to be less anxious, while fish that spend more time in the bottom of the novel tank, freeze more often and swim more slowly are considered to be more anxious. Pretreatment with anxiogenic or anxiolytic substances supports this interpretation. (Bencan and Levin 2008, Bencan et al. 2009, Egan et al. 2009, Wong et al. 2010). Thus, placement of zebrafish into a novel tank following treatment with pharmacologic agents and then recording the time spent in the bottom third, swimming speed and bouts of freezing, provides evidence of the effects of the treatment on anxiety in the fish.

A T-maze or plus maze has been demonstrated to provide a setting for spatial learning. Colwill et al. (2005) demonstrated that zebrafish trained using a T-maze were able to successfully interpret color and achromatic pattern cues and to reverse those cues with training in subsequent experiments. Al-Imari and Gerlai (2008) utilized a plus maze to demonstrate spatial learning by pairing a visual cue using conspecifics as a reward while Sison and Gerlai (2010) utilized a plus maze to demonstrate spatial learning by pairing a visual cue with a food reward. The popularity of this paradigm is evident from its inclusion in *Methods of Behavior Analysis in Neuroscience*, 2nd Edition (Levin and Cerutti 2009) and *Zebrafish Neurobehavioral Protocols* (Gould 2011). The current studies utilize a T-maze with achromatic visual cues and conspecifics as a reward to determine the effect of citalopram, 5-APB and BMPEA on memory in treated zebrafish. These data are paired with assessments of anxiety-like behavior measured using the Novel dive tank paradigm to provide a more comprehensive picture of the relationship between these factors in this model organism.

Methods

Zebrafish husbandry

This research was conducted using 25 to 50 wildtype zebrafish in each group. All fish were housed in a Pentair/Aquatic Habitats (Apopka, FL) Z-hab multi-tank system, kept on a 14-10 L/D cycle and fed brine shrimp once per day. All zebrafish experiments were compliant with Belmont University's animal welfare policies and monitored by the Laboratory Oversight Committee. The zebrafish were treated and tested individually.

Treatment

For acute treatments, each fish was placed in a 400 mL beaker containing 200 mL of system water and the appropriate concentration of chemical for 10 minutes for 5-APB (5 micrograms) and the BMPEA (50 micrograms), 15 minutes for citalopram (0 to 50µM final concentration) and escitalopram (0.5µM or 1µM final concentration). Control fish were incubated in system water only. Following treatment, each fish was transferred to a rest beaker containing system water only for 15 (citalopram and escitalopram) or 20 (5-APB and BMPEA) minutes to allow for the treatment to take effect. This resulted in 30 minutes total time elapsed between initial exposure to the treatment and initiation of testing.

The escitalopram chronic treatment was administered by housing those fish in a separate 40L aquarium containing 1.5 micrograms of escitalopram per liter. 10% (4 L) of water was

removed daily and replaced with fresh escitalopram solution to ensure continued treatment. 5-APB and BMPEA were obtained from Sigma-Aldrich (St. Louis, MO). Citalopram and escitalopram were obtained from Tocris Bioscience (Bristol, UK).

Anxiety

At the end of the treatment period, the fish were observed for 10 minutes in a novel dive tank for signs of stress per the method of Egan et al. (2009). Briefly, when placed into a novel situation (a new tank) zebrafish will dive to the bottom. Over time, the fish will begin to explore the new environment. Fish that begin exploring sooner, swim faster and explore more extensively are considered to be less anxious, while fish that spend more time in the bottom of the novel tank, freeze more often and swim more slowly are considered to be more anxious. The novel dive tank utilized for testing anxiety was a 1L flow-through tank from the Aquatic Habitats System (Apopka, FL), filled with system water and divided into thirds vertically which were labeled upper, middle and lower zones. A digital camera was used to monitor fish in the tank to avoid human interference effects. Noldus Ethovision XT 11 (Leesburg, VA) analysis of the digital recordings was used to determine time in each zone, distance traveled, velocity and mobility. Mobility was calculated by determining the percentage of changed pixels of the subject fish between the current sample and the previous sample. These percentages were then used to define the fish's mobility state as highly mobile (>60% mobility) mobile (20%–60% mobility) or immobile (<20% mobility) (see Supplemental Figure S1, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-025-McGrew-s1.pdf). The data were analyzed using a t-test or a one-way ANOVA with Tukey post-hoc test, depending on the number of treatment groups.

Spatial learning

Zebrafish were trained in a T-maze (see Supplemental Figure S2, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-025-McGrew-s2.pdf) using a protocol adapted from Colwill et al. (2005). Following treatment, fish were transferred into the start box of the T-maze. Following one minute of acclimation in the start box, the plastic gate was removed and the fish was allowed to make a decision. For training purposes, the circle was deemed the correct side while the plus sign represented the incorrect side. If a fish chose correctly, it was rewarded with conspecifics for two minutes (Al-Imari and Gerlai 2008). If the fish chose incorrectly, it was punished with a confined space for swimming for two minutes. Training was conducted once per day for five consecutive days. A digital camera was used to monitor fish in the tank to avoid human interference effects. Noldus Ethovision XT 11 (Leesburg, VA) analysis of the digital recordings was used to determine time in each zone, distance traveled, velocity and mobility. Mobility was calculated by determining the percentage of changed pixels of the subject fish between the current sample and the previous sample. These percentages were then used to define the fish's mobility state as highly mobile (>60% mobility) mobile (20%–60% mobility) or immobile (<20% mobility). The data were analyzed using a t-test or a one-way ANOVA with Tukey post-hoc test, depending on the number of treatment groups.

Results

Citalopram had a biphasic effect on choice accuracy in the zebrafish trained to solve the T-maze (Fig. 1). The 25 µM group performed significantly better than control animals ($p = 0.04$) while the 50 µM group showed a significant decrease in accuracy ($p = 0.03$). There was also a significant decrease in both the mean velocity and the freezing bouts for the fish treated with 50 µM citalopram (Figs. 2A and B). Mean velocity of the 50 µM citalopram

treated fish decreased almost 50% to just over 13 cm/s when compared to control fish that traveled at over 25 cm/s on average ($p = 0.002$). In addition, the 50 µM citalopram treated fish spent more than 70% of the trial time immobile compared to only 28% immobility for control fish ($p = 0.04$). Lower doses of citalopram had no significant effect on mean velocity, but the percentage of time spent in the highly mobile state decreased significantly in the 10, 20, 25 and 30 µM citalopram treated groups. Acute treatment with escitalopram demonstrated similar results. Spatial learning was impaired by the higher dose of escitalopram (Fig. 3) while periods of immobility increased significantly (Fig. 4).

Chronic exposure to low dose escitalopram significantly decreased total distance traveled (Fig. 5) as well as time elapsed prior to first entry into the middle and upper zones of the novel dive tank (Fig. 6A). Time spent in the mobile state was also significantly reduced in escitalopram treated zebrafish (Fig. 6B). In addition, escitalopram treatment resulted in an 11% reduction in overall length of the treated fish when compared to their untreated age-matched controls, although mass was not significantly different (Table 1).

Similarly, both the 5-APB and BMPEA treatments resulted in a significant decrease in movement (Fig. 7). The 5-APB treatment produced a significant difference in the time spent in lower and upper zones of the novel dive tank. (Fig. 8A) while the BMPEA treat-

Table 1: Chronic administration of escitalopram reduces growth in zebrafish. Following treatment with 1.5µg/L of escitalopram, zebrafish were euthanized so that their length and mass could be accurately determined. *$p < 0.05$ compared to control.

	Control	Treated	Number of fish
Body length (cm)	3.63 ±0.06	3.23 ± 0.05*	25
Body mass (mg)	305 ±11	301±9	25

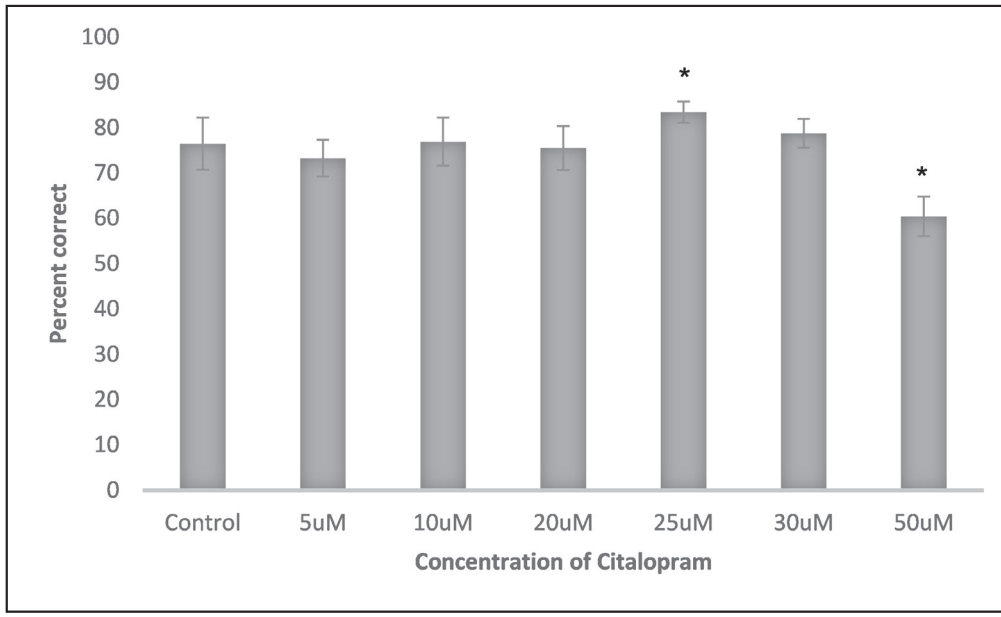

Figure 1. Spatial learning is improved by low doses of citalopram and impaired by high doses of citalopram based on the proportion of zebrafish that chose the correct arm of the T-maze. Zebrafish were treated with the appropriate concentration of citalopram (0 to 50 micromolar) in system water. Following treatment, the fish were trained for five consecutive days in a T-maze. The fish were then allowed to rest for 5 days before being tested in the T-maze for ability to recall the correct arm. N = 50 for each treatment group. *$p < 0.05$ compared to control using Tukey post-hoc test for significant ANOVA. Error bars indicate SEM.

ment produced a significant difference in the time spent in the mobile and immobile states (Fig. 8B). 5-APB-treated fish demonstrated a 35% decrease in movement from 2004 cm to 1321 cm ($p = 0.02$) while BMPEA treated fish showed a 36% decrease to 1286 cm traveled on average ($p = 0.01$). The time that 5-APB treated fish spent in the lowest zone decreased significantly to 162 seconds compared to 354 seconds that control fish spent at that depth ($p = 0.004$). Time that BMPEA fish spent in the immobile state decreased significantly from 364 seconds for control fish to 152 seconds ($p = 0.005$).

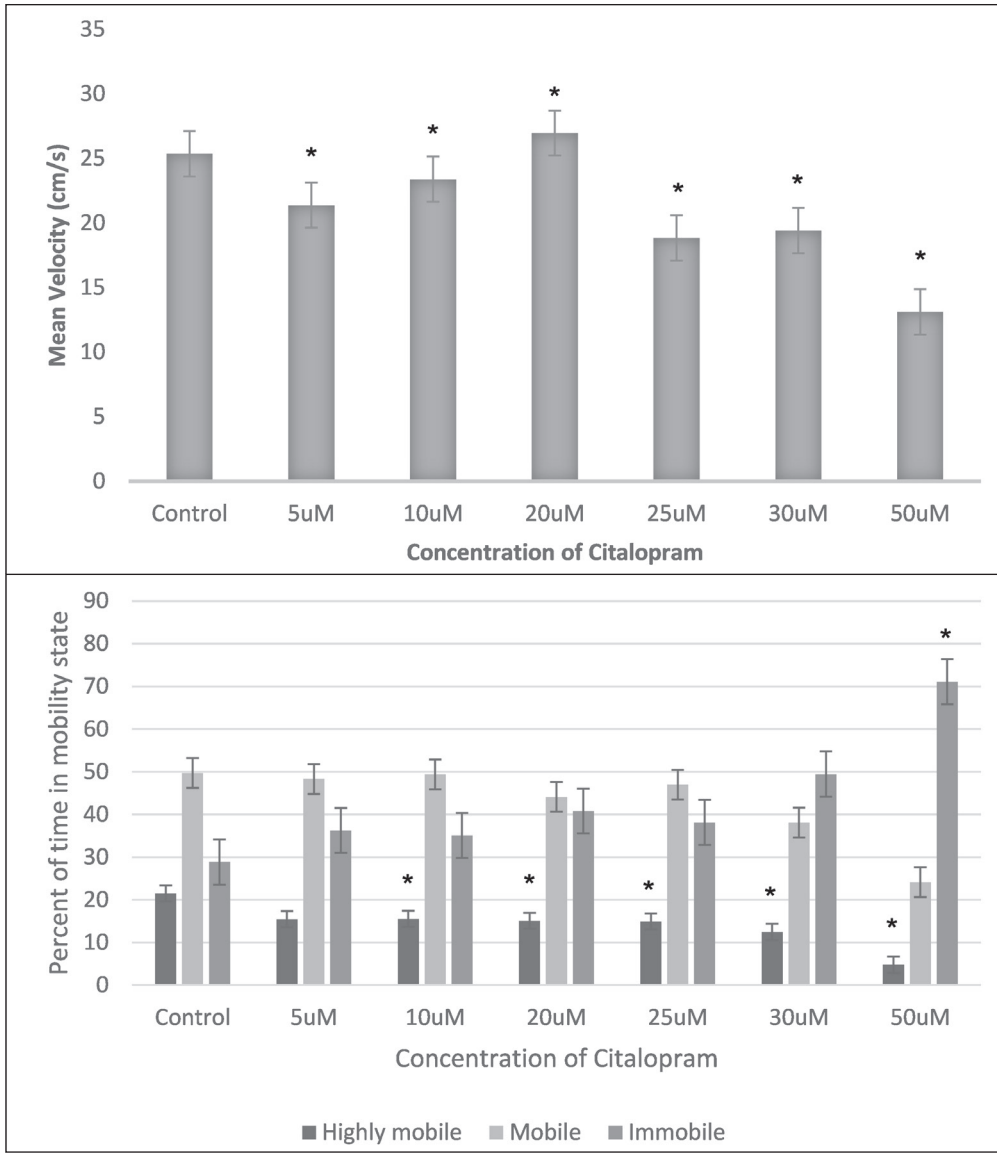

Figure 2. A) Mean velocity is decreased while B) freezing bouts are increased by high doses of citalopram. N = 50 for each group. Zebrafish were treated once per day for five days with the appropriate concentration of citalopram (0 to 50 micromolar) in system water. Following treatment, the fish were trained for five consecutive days in a T-maze. The fish were then allowed to rest for 5 days before being tested. Analysis of the recorded trials was used to determine mean velocity (A) and mobility state (B). N = 50. *$p < 0.05$ compared to control using Tukey post-hoc test for significant ANOVA. Error bars indicate SEM.

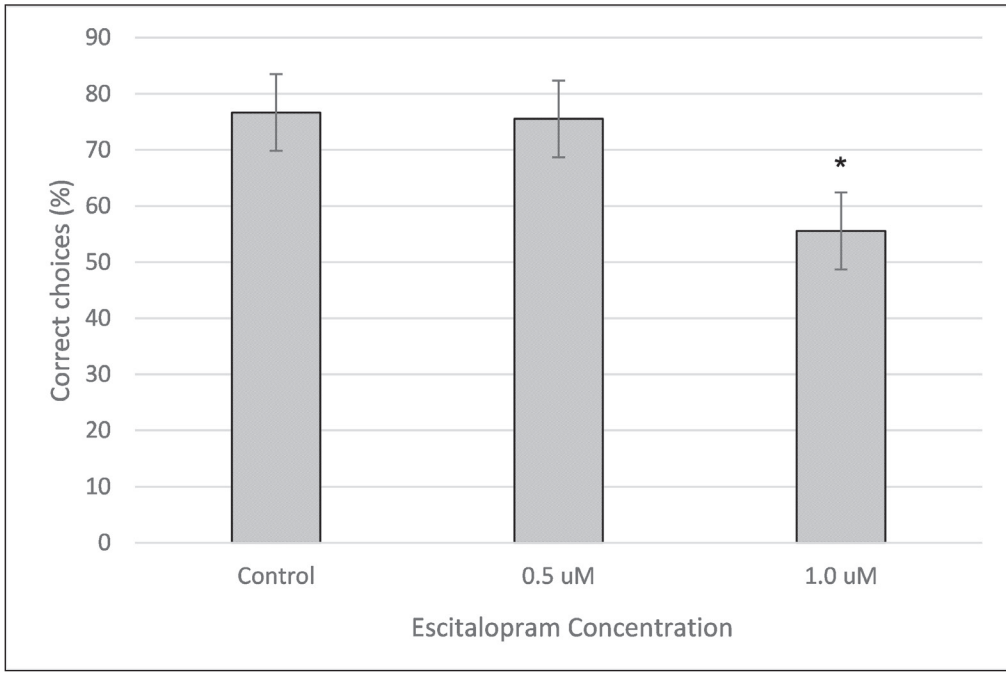

Figure 3. Spatial learning is impaired by acute high doses of escitalopram based on the proportion of zebrafish that chose the correct arm of the T-maze. Zebrafish were treated with the appropriate concentration of escitalopram (0.5 or 1.0 micromolar) in system water. Following treatment, the fish were trained for five consecutive days in a T-maze. The fish were then allowed to rest for 5 days before being tested in the T-maze for ability to recall the correct arm. N = 25 for each treatment group. *p < 0.05 compared to control using Tukey post-hoc test for significant ANOVA. Error bars indicate SEM.

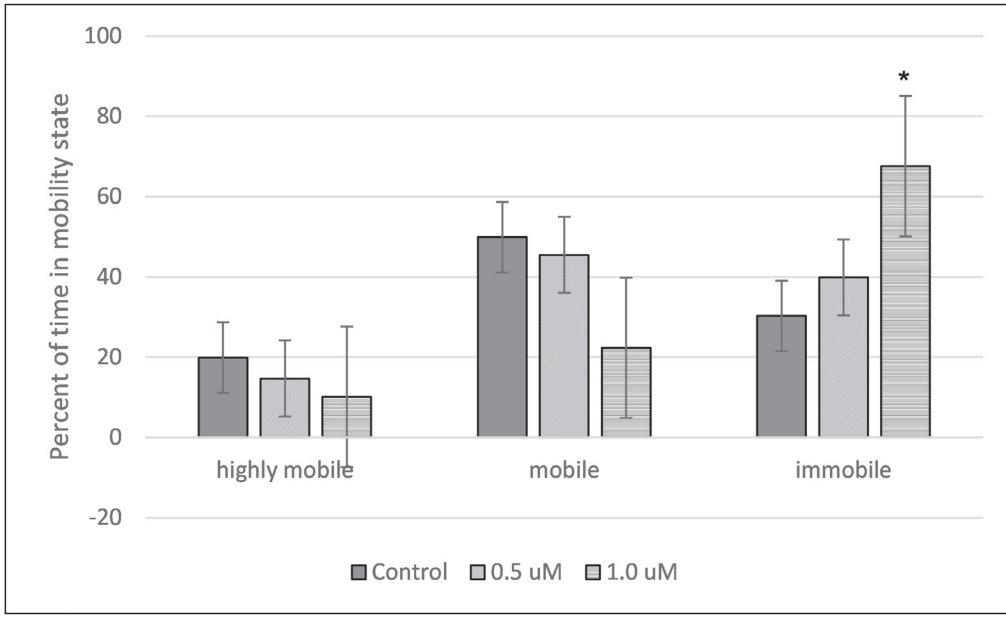

Figure 4. Mobility state is altered in zebrafish following acute treatment with escitalopram. Zebrafish were treated with the appropriate concentration of citalopram (0.5 or 1.0 micromolar) in system water. Following treatment, the fish were trained for five consecutive days in a T-maze. The fish were then allowed to rest for 5 days before being tested in the T-maze for ability to recall the correct arm. N = 25 for each treatment group. *p < 0.05 compared to control using Tukey post-hoc test for significant ANOVA. Error bars indicate SEM.

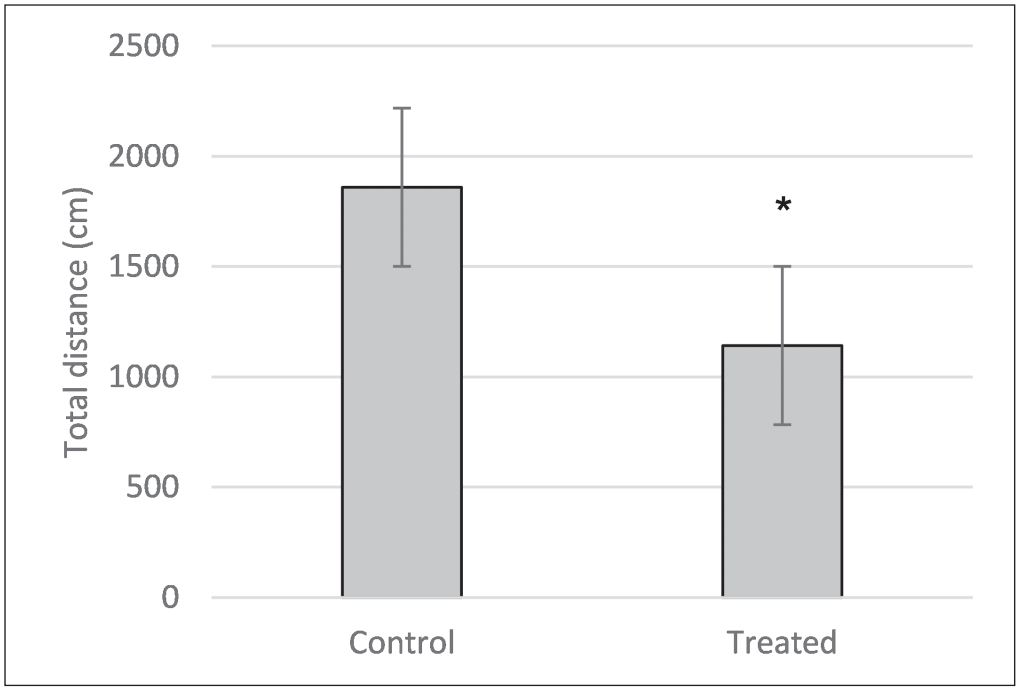

Figure 5. Total distance traveled by zebrafish is decreased by chronic low doses of escitalopram. N = 25 for each group. Zebrafish were maintained in system water containing 1.5 micrograms per liter (approx. 3.6 nM) of escitalopram oxalate. Following three weeks of treatment, the fish were tested for anxiety using a novel dive tank. Analysis of recorded trials was utilized to determine total distance traveled. *$p < 0.05$ compared to control using Student's t statistic. Error bars indicate SEM.

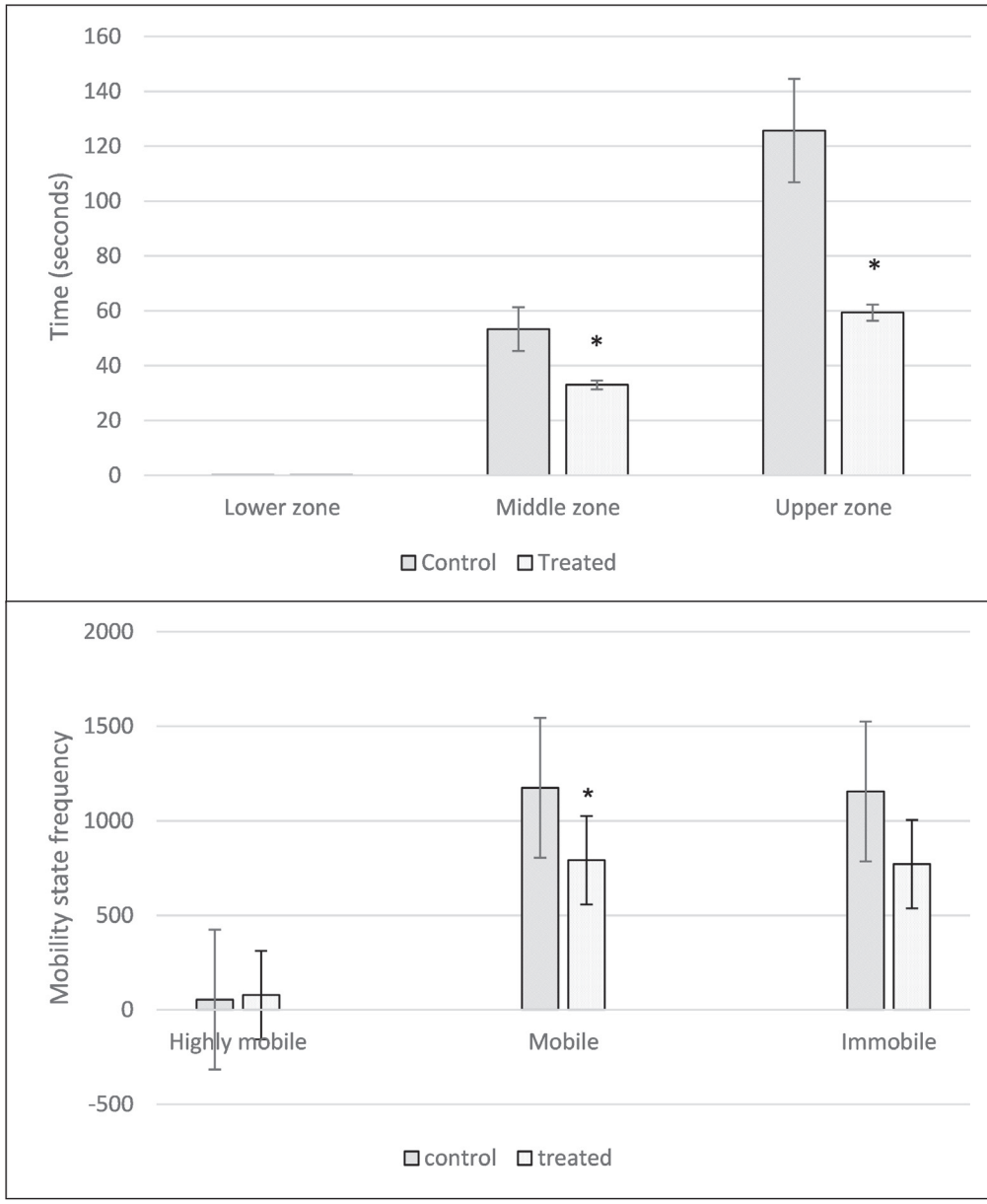

Figure 6. Anxiety is reduced in zebrafish exposed to chronic low doses of escitalopram. N = 25 for each group. Zebrafish were maintained in system water containing 1.5 micrograms per liter (approx. 3.6 nM) of escitalopram oxalate. Following three weeks of treatment, the fish were tested for anxiety using a novel dive tank. Analysis of recorded trials was used to determine time elapsed prior to first entry into lower, middle and upper zones (A) and mobility state (B). *$p < 0.05$ compared to control using Student's t statistic. Error bars indicate SEM

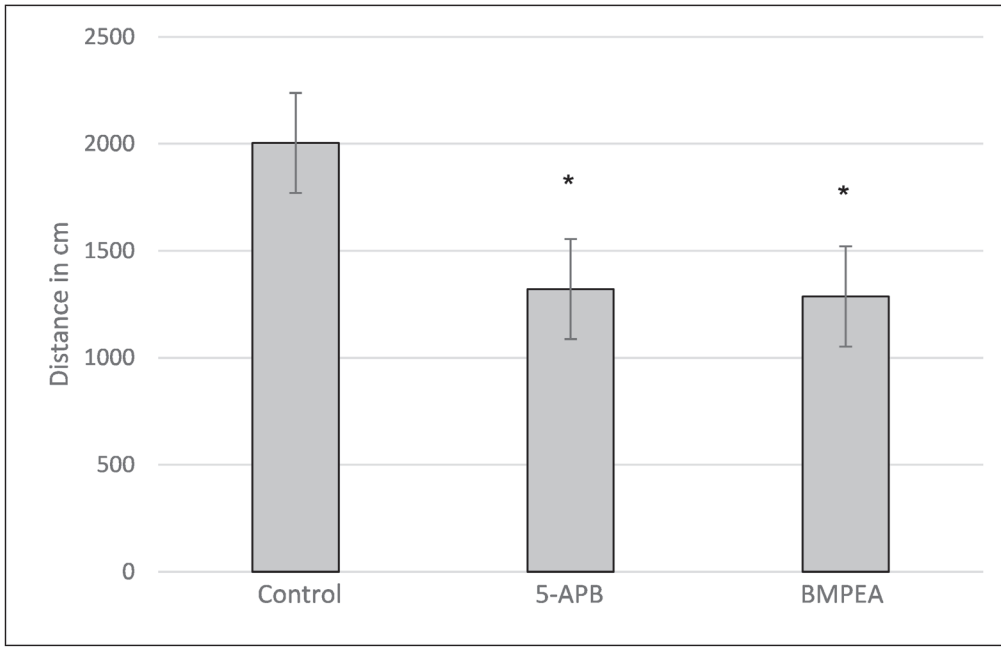

Figure 7. Total distance traveled by zebrafish is decreased by treatment with 5-APB and BMPEA. N = 25 for each group. Zebrafish were treated with 25 microgram per liter 5-APB or 250 micrograms per liter of BMPEA for 10 minutes. Following a 20 minute rest in system water, the fish were tracked in the novel dive tank for 10 minutes. Analysis of recorded trials was used to determine total distance traveled by each fish. * $p < 0.05$ compared to untreated control fish using Tukey post-hoc test for significant ANOVA. Error bars indicate SEM.

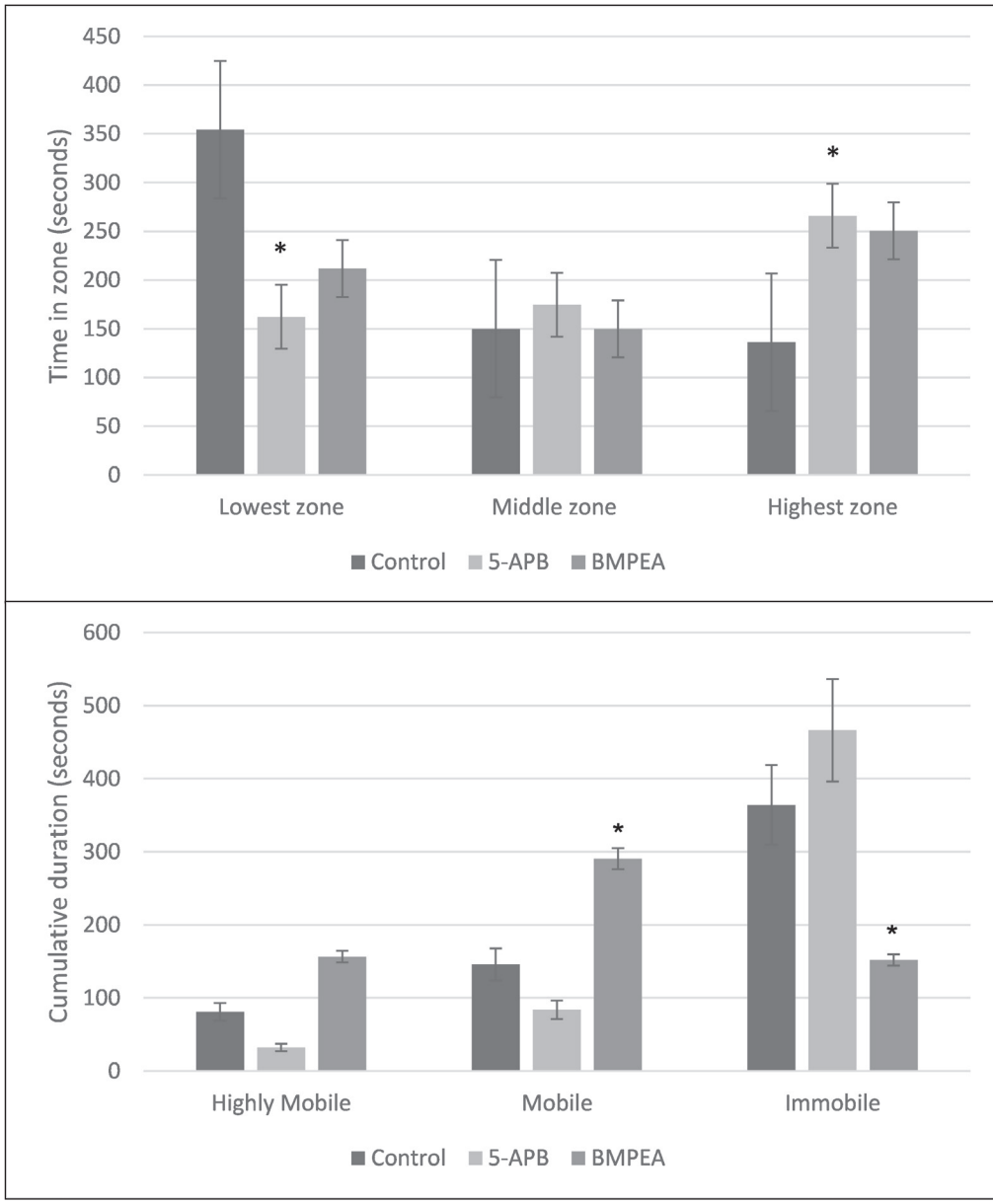

Figure 8. Anxiety is reduced in zebrafish exposed to 5-APB. N= 25 for each group. Zebrafish were treated with 25 microgram per liter 5-APB or 250 micrograms per liter of BMPEA for 10 minutes. Following a 10 minute rest in system water, the fish were tracked in the novel dive tank for 10 minutes. Analysis of recorded trials was used to determine (A) total time spent in each zone and (B) mobility state. * $p < 0.05$ compared to untreated control fish using Tukey post-hoc test for significant ANOVA. Error bars indicate SEM.

Discussion

Collectively, the current studies suggest that moderate SSRI doses administered chronically are able to mildly reduce anxiety in zebrafish. These results are consistent with those of Connors (2013) and his team who found that the treated fish were bolder and less anxious as concentration increased among others (Egan et al. 2009, Maximino 2013). Similar results were obtained in fish exposed to escitalopram at a much lower concentration (Nielsen et al. 2018). This reduction in anxiety may result in decreased total movement by reducing agitation in the treated fish. Furthermore, high doses of SSRI's produce the opposite effect—increased periods of immobility punctuated by bouts of nonproductive movement—which may correspond to the agitation that is reported by some SSRI consumers (Bouchard et al. 1987). The effect on learning similarly shows an improvement at lower doses and impairment at higher doses suggesting a correlation between anxiety and spatial memory recall. Specifically, Zebrafish demonstrated an improved performance at 25μM of citalopram as well as a decrease in performance at the highest dose, 50 μM of citalopram or 1.0 μM of escitalopram. These effects may be attributed to the changes in anxiety observed in the treated groups and, again, mirror behavioral changes seen in humans (Grignaschi et al. 1998, Soczynska et al. 2014). In addition, the change in growth rate of treated fish, while troubling from an ecological perspective, is consistent with changes observed in toxicology studies (Kalichak et al. 2016).

In addition to decreased distance traveled, the 5-APB treated fish also demonstrated a significant decrease in the time spent in the lowest zone and a corresponding increase in the time spent in the highest zone compared to control fish during the novel dive tank test. BMPEA treated fish demonstrated a decrease percent of time that they were immobile (freezing) with a corresponding increase in time that they were mobile. This is indicative of a decrease in anxiety, contrary to the response typically reported in humans (Jebadurai et al. 2013). However, this decrease in apparent anxiety is consistent with increased 5-HT neurotransmission as seen in the SSRI-treated zebrafish. The present studies did not address whether dopamine or norepinephrine levels were affected by the treatment, although future studies should address that issue. It should also be noted that single concentrations of 5-APB and the BMPEA were tested. Additional studies with higher concentrations may produce different results. If these compounds elicit robust increases in 5-HT, dopamine or norepinephrine at higher doses, zebrafish may respond with increased anxiety and movement, which would be consistent with the effects of higher doses of SSRIs and with the reports of human 5-APB and BMPEA users.

As a whole, despite reported differences in 5-HT receptor genetics, there are strong correlations that support continued use of zebrafish for modeling behaviors influenced by serotonergic signaling. Furthermore, cataloging these homologous behaviors can be an excellent medium in which to train undergraduate student researchers.

Acknowledgements

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