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Zebrafish Behavior in Novel Environments: Effects of Acute Exposure to Anxiolytic Compounds and Choice of *Danio rerio* Line

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Zebrafish (*Danio rerio*) associative responses are useful for pharmaceutical and toxicology screening, behavioral genetics, and discovering neural mechanisms involved in behavioral modulation. In novel environments, zebrafish swim to tank bottoms and dark backgrounds, behaviors attributed to anxiety associated with threat of predation. To examine possible genetic effects of inbreeding and segregation on this behavior, we compared Zebrafish International Resource Center (ZIRC) AB and WIK lines to zebrafish and GloFish® from a pet store (PETCO) in two qualitatively different novel environments: the dive tank and aquatic light/dark plus maze. Behavior was observed in the dive tank for 5 min, immediately followed by 5 min in the light/dark plus maze. Among strains, WIK spent more time in the dive tank top than AB (76 $\pm$ 30 vs. 17 $\pm$ 11 sec), and AB froze in the plus maze center for longer than PETCO or GloFish® (162 $\pm$ 61 vs. 72 $\pm$ 29 or 27 $\pm$ 27 sec). Further, behavior of zebrafish exposed for 3 min to 25 mg/L nicotine, desipramine, chlordiazepoxide, yohimbine, 100 mg/L citalopram, 0.05% DMSO, or 0.5% ethanol was compared to controls. Approximately 0.1% of drug is available in brain after such exposures. Desipramine or citalopram-exposed fish spent more time in the dive tank top, and both reuptake inhibitors bound to serotonin transporters in zebrafish brain with high affinity (K_i = 7 $\pm$ 5 and 9 $\pm$ 5 nM). In the plus maze, chlordiazepoxide, ethanol and DMSO-exposed fish crossed more lines and spent more time in white arms. Neither 25 mg/L nicotine nor yohimbine altered zebrafish behavior in novel environments, but nicotine was anxiolytic at higher doses. Overall, the light/dark plus maze and dive tank are distinct behavioral measures that are sensitive to treatment with anxiolytic compounds, but zebrafish line selection and solvents can influence baseline behavior in these tests.

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Animal models of anxiety, depression and other mood disorders are in demand for translational biomedical research. Zebrafish (*Danio rerio*) are gaining popularity in behavioral neuroscience, pharmacology and toxicology (e.g., Rubinstein, 2006; Ton, Lin, & Willett, 2006). As small, resilient vertebrates with mapped and malleable genomes, zebrafish are uniquely amenable to studies of genetic and neurophysiologic bases of behavior and cognitive function (Guo, 2004; Linney, Upchurch, & Donerly, 2004). While their nervous systems are simpler than those of mammals, zebrafish still perform complex behaviors that are similarly modulated by central neurotransmitter systems. Useful fish associative behaviors need to be reliably reproducible (Blaser & Gerlai, 2006), responsive to pharmaceutical treatments, and ideally, resemble aspects of human behavioral pathology.

Zebrafish for research in the United States can be obtained from commercial/retail suppliers, donor laboratories, or from the zebrafish international resource center (ZIRC). Progenitors of the AB line bred by George Streisinger in the 1970's came from a pet shop in Albany, Oregon. Since zebrafish research lines, such as AB, have been selected for high reproductive capacity and embryo survivorship (Spence, Gerlach, Lawrence, & Smith, 2008), natural selection for predator avoidance has ceased. We therefore postulated that innate predator vigilance in an unfamiliar environment could wane from these inbred populations. To test this hypothesis, we compared anxiety responses in two novel environments between ZIRC AB and WIK lines, the latter of which descends more recently from wild populations in India. Further, we compared anxiety responses in GloFish®, with added fluorescent protein genes, to standard zebrafish from PETCO pet stores to examine the influence of this heritable insertion on behavior in novel environments.

Anxiety disorders are prevalent psychiatric conditions that affect emotion and cognition; they exhibit > 50% co-morbidity with depression (Morilak & Frazer, 2004). Anxiety is associated with behavioral responses that are replicable in animal models and altered by anxiolytic drugs, such as the elevated plus maze for rodents (Lapiz-Bluhm, Bondi, Doyen, Rodriguez, Bédard-Arana, & Morilak, 2008). To model anxiety in fish, cues tied to predation threat such as predator odors, alarm pheromones or fleeing conspecifics are typically used (Bass & Gerlai, 2008; Ferrari, Messier, & Chivers 2008; Speedie & Gerlai, 2008). Such studies provide insight into physiological pathways and evolutionary pressures involved in fear, anxiety, or evasive strategies. However, exaggerated predator avoidance under circumstances posing no threat may be of greater clinical relevance, as exemplified by zebrafish thigmotaxic or light avoidance behaviors in novel environments.

Herein, we utilize two novel environment paradigms, the dive tank and light/dark plus maze, to compare initial responses to unfamiliar environments as indicators of zebrafish anxiety state. The dive tank anxiety test, used by Levin, et al. (2007), is based on the tendency of zebrafish to remain in side and bottom contact with solid boundaries (a thigmotaxic response) in a novel tank. The aquatic light/dark plus maze is based on the tendency of zebrafish to seek dark

backgrounds (or avoid light backgrounds) in unfamiliar environments (Guo, 2004; Serra, Medalha, & Mattioli, 1999). Both tests could enhance rapid drug or toxin screening and reveal two independent dimensions of instinctive zebrafish anti-predator anxiety responses.

In both novel environments we surveyed the acute effects of moderate doses of several water-soluble anxiolytic (chlordiazepoxide, ethanol, nicotine) or anxiogenic (yohimbine) compounds, as well as monoamine reuptake inhibitors (MRIs) desipramine and citalopram, on zebrafish exploratory vs. defensive behavior. Additionally we examined the effect of the solvent DMSO, as it is a commonly used *in vivo* vehicle for insoluble compounds. We traced acute aquatic uptake of [^{3}H] citalopram into zebrafish muscle and brain to quantify its bioavailability, which might be indicative of other compounds of similar size and molecular structure. Finally, since zebrafish serotonin transporters (SERTs) are target sites of MRIs, and SERT modulation may affect anxiety state, we characterized high-affinity SERT binding and its displacement by MRIs in zebrafish brain membrane homogenates.

In summary, the primary aim of this study was to compare innate anxiety responses in novel environments among zebrafish lines used in neurobehavioral research, and to assess the effects of compounds with anxiogenic and anxiolytic properties on this behavior. Other aims were to quantify drug uptake into zebrafish brain and explore the properties of ligand binding to their SERT, one of many target sites for drugs altering anxiety states in vertebrates.

Methods

Experimental subjects: zebrafish

Adult zebrafish (*Danio rerio*) were obtained from one of four sources. PETCO Animal Suppliers Inc., retail branch in Paramus, NJ (corporate headquarters in San Diego, CA) was the source for "PETCO" and GloFish® lines. The AB and WIK lines were obtained from the Zebrafish International Resource Center (ZIRC) at the University of Oregon, Eugene, OR. These were used in experiments conducted at William Paterson University, Wayne, NJ in 2007-2008. The third source of zebrafish was Aquatic Eco-Systems (Apopka, FL) and the fourth was a PETCO branch in San Antonio, TX; both were used in binding studies and a second round of behavioral experiments conducted at the University of Texas Health Science Center at San Antonio in 2008-2009. All zebrafish were housed in either 3 L or 10 L tanks in a benchtop aquatic habitat with re-circulating filtered de-ionized tap water supplemented with 200 mg/L Instant Ocean salts (Aquatic Eco-Systems, Apopka, FL). Zebrafish were fed twice daily a diet of brine shrimp (*Artemia franciscana*, GSL Brine Shrimp, Ogden, UT) and flake fish food (Wardley Total Tropical, Hartz Mountain, Secaucus, NJ). Fish were maintained on a 14-hour light/10-hour dark cycle with lights on at 0700 h, and all behavioral assays were run during the light cycle. In all experimental groups, mature zebrafish males and females were present in roughly equal numbers. All animals were maintained and procedures were performed in accordance with the Institutional Animal Care and Use Committees of William Paterson University and the University of Texas Health Science Center at San Antonio, following the Institute for Laboratory Animal Research (ILAR) and/or the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

General procedure for drug exposures and behavioral tests in novel environments

Zebrafish behavior in novel environments was tested following acute exposure to dissolved drugs or solvents at one or more concentrations in the range of 25 – 100 mg/L, in a 1L beaker filled with 500 mL water from their aquatic habitat. Drug and solvents included citalopram (Forrest Laboratories, Jersey City, NJ), nicotine, desipramine, chloridazepoxide, yohimbine, 0.05% DMSO (Sigma, St. Louis, MO), and 0.5% ethanol (Fisher Scientific, USA). Zebrafish were transferred by net from home tanks into the beaker containing 500 mL of tank water with dissolved test compound (no additive for controls) for 3 - 4 min. Behavioral testing consisted of a 5 min session in the dive tank immediately followed by netting and transfer to a 5 min session in the light-dark plus-maze. All fish were drug and environment naïve. Tests were conducted between 0900 and 1700 h. Test time from drug exposure to completion was 14-15 min per fish. Water filling the novel environments was refreshed with habitat water after each fish's trial.

Novel dive tank. The dive tank was a transparent, triangular 4 L fish tank (Aquascene 1, TopFin, Phoenix, AZ) filled to a depth of 18 cm with 3.5 L of home tank water (Figure 1). Lines dividing the tank into thirds were drawn on the outside with marker to aid observation. The tank sat on a black countertop, with a 24 cm x 22 cm white board against its back wall to enhance contrast for video recording by digital camera (HP Photosmart R742, OfficeMax, USA). Fish in the dive tank were observed and digitally recorded for 5 min to determine the amount of time spent in the top 2/3 vs. bottom 1/3 of the tank. This dive tank test was based on Levin and colleagues (2007).

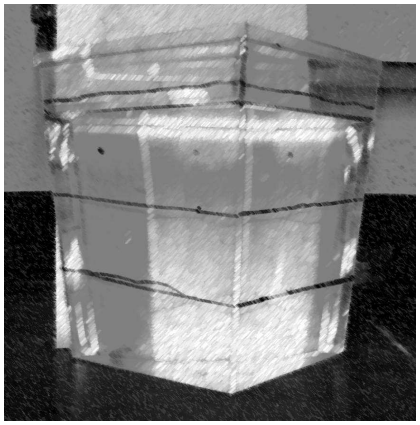


Figure 1. The novel dive tank. Upon introduction to the dive tank, untreated controls initially dwell in the bottom third of the tank before venturing up to the top 2/3 of the tank (per Levin et al., 2007). Observations are made over 5 min.

Novel light-dark plus maze. The aquatic plus maze test was performed in the 71 cm (H) x 51 cm (W) X 10 cm (D) offset cross maze (Ezra Scientific, San Antonio, TX), subdivisible into 10 x 10 cm square (10 cm^2) units by drop-in doors. The plus maze module employs the 10 cm^2 center section and surrounding four 10 cm^2 arms (Figure 2). The clear acrylic maze was filled to a depth of 4 cm. Two opposite arms were lined with black polyethylene 10 cm^2 squares and the other two were lined with white polyethylene 10 cm^2 squares cut from folders and secured to walls with binder clips. The grey background of the copy stand (Kaiser RS1, B&H Photo, New York, NY) showed through the middle 10 cm^2 section of the maze. A lit 60 W desk lamp was situated on the copy stand above the maze and behind the digital camera (HP Photosmart R742) during testing.

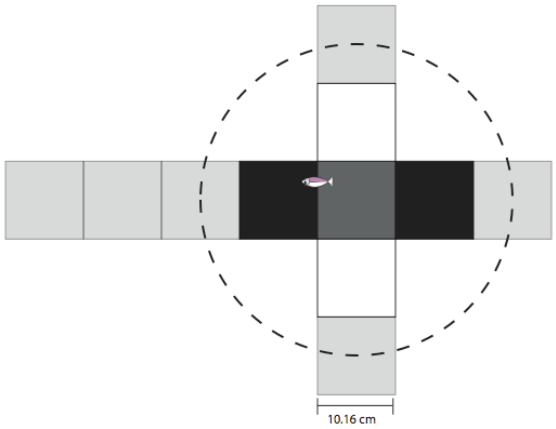


Figure 2. The novel aquatic light/dark plus maze. Untreated fish tend to freeze in the center and initially enter black arms when first introduced into the plus maze. After several minutes they begin to explore the maze. Observations are made over 5 min.

Fish netted from the dive tank were released into the center section of the plus maze for 5 minutes of observed and digitally recorded testing. During this trial, the amount of time the fish spent in white arms, the number of crosses into white or black arms, and the amount of time that fish spent motionless in the center section upon introduction (initially frozen) were recorded by two cognizant observers (one recording time, the other recording arm entries), as per scoring of rats in the elevated plus maze (Lapiz-Bluhm et al., 2008). Videorecordings from both the dive tank and plus maze tests were subsequently reviewed by a treatment-blind observer to confirm real time data collection of times and line crosses.

Multivariate analysis of variance (ANOVA) of behavioral data was performed using *Statistica* for the Macintosh (Statsoft, Tulsa, OK). Comparisons among strain and drug exposures were made for the following behavioral measures: seconds spent in top 2/3 of dive tank, number of line crossings in plus maze, % white of total line crossings, time spent in white arms and initial time frozen (introduction immobility) in the middle of the plus maze.

Uptake of [^{3}H] citalopram from water into zebrafish muscle and brain. Adult zebrafish were exposed in 25 mL beakers filled with habitat water to either 75 nM or 35 nM of the selective serotonin reuptake inhibitor radiologand [^{3}H] citalopram (79 Ci/mmol, Perkin-Elmer, Boston, MA) for 3 min. Fish were removed from radioligand baths with forceps and rapidly decapitated with a scalpel. [^{3}H] citalopram labeled zebrafish brains and a square segment of lateral muscle were removed, weighed and placed in 1.5 mL microcentrifuge tubes containing 200 µL scintillation cocktail (Ecolume, Fisher Scientific, USA). Labeled brains and muscles were homogenized with a small plastic pestle in the microcentrifuge tubes and then transferred to 8 ml scintillation vials (Beckman Mini Poly-Q, Fisher Scientific, USA), to which 5 mL of scintillation cocktail (Ecolume, Fisher Scientific, USA) was added. Tissue homogenates in vials were vortexed, and tritium label (DPM) was measured on a Packard 1900 TR liquid scintillation counter (Packard Instrument Co., Downers Grove, IL) with an efficiency of 40%.

[^{3}H] Citalopram saturation and displacement binding in brain membrane homogenates. Radioligand binding to zebrafish serotonin transporters (SERTs) in whole brain homogenates was performed as in previous studies (Gould, Brooks, & Frazer, 2007). Whole brains pooled from 10-12 adult zebrafish of mixed gender (Aquatic Eco-Systems, Apopka, FL) were homogenized in 25 mL of 4°C 50 mM Tris, 120 mM NaCl, 5 mM KCl buffer, pH 7.4 at 26°C, for 15 sec on a Polytron

homogenizer (Brinkman, Westbury, NY). The homogenate was centrifuged for 10 min at 30,600 x G at 4°C. The supernate was discarded and the pellet re-suspended with a Potter Elrehijem homogenizer into 25 mL 4°C buffer and centrifuged. The final pellet was suspended in a 12 mL buffer and protein concentration was determined using Bradford reagent (Sigma), BSA standards and a spectrophotometer (DU 640, Beckman, USA).

Incubation with [^{3}H] citalopram was carried out in triplicate for 1 h at 26°C in pH 7.4 Tris-HCl, NaCl, KCl buffer. Each tube contained 100 µL of brain homogenate, in a total volume of 250 µL. The radioligand concentration for saturation assays ranged from 0.1-10 nM, for which non-specific binding was defined with 20 µM fluoxetine (Eli Lilly & Co., Indianapolis, IN), or was 2.5 nM [^{3}H] citalopram for displacement assays. The serotonin, norepinephrine, and dopamine reuptake inhibitors desipramine (Sigma), sertraline (Pfizer, Groton CT), and GBR12909 (Sigma) were used as displacing agents. [^{3}H] citalopram incubation was terminated by addition of 4 mL of pH 7.4 at 4°C buffer. Labeled homogenates were captured by filtration under vacuum onto glass fiber filters (Schleicher and Schuell, Keene, NH) pre-soaked in 5% polyethyleneimine (Sigma) with a Brandel tissue harvester (Gaithersburg, MD). Filters were washed twice more with 4 mL of buffer. [^{3}H] Radioactivity trapped by the filters was measured on a scintillation counter (1900 TR, Packard Instrument Co., Downers Grove, IL) with 40% efficiency. Binding data were analyzed by non-linear regression using DeltaGraph (Red Rock, Salt Lake City, UT) to determine the equilibrium dissociation constant (K_D) and estimate maximal binding (Bmax), and Cheng and Prusoff (1973) correction was used to determine inhibition constant (Ki) values for competition curves.

Results

Effects of line and compound exposures on vertical location of zebrafish in the dive tank

In the dive tank, untreated WIK zebrafish spent significantly more time in the top of the tank (76 $\pm$ 30 sec) than AB zebrafish (17 $\pm$ 11 sec) (ANOVA $F_{(3,42)}$ = 2.88, Tukey's HSD $p < 0.05$). There was no significant anxiolytic effect of nicotine exposure at 25 mg/L in any zebrafish line ($F_{(1,42)}$ = 1.58, p = 0.22). An additional group of PETCO zebrafish was exposed to nicotine at 125 mg/L for 3 min (N = 4), but spent only 4 $\pm$ 4 sec as compared to the 11 $\pm$ 7 sec control mean at the dive tank top. Results of the four-strain comparison and 25 mg/L nicotine exposure on fish performance in the dive tank are shown in Figure 3. In a follow-up experiment conducted at UTHSCSA the next year, we found a trend ($F_{(2,14)}$ = 3.01, Tukey HSD p = 0.08, N = 5 - 6) toward nicotine exposure increasing time spent in the tank top 2/3 at 50 mg/L (73 $\pm$ 35 sec), but not at 100 mg/L (1 $\pm$ 1 sec), over untreated PETCO zebrafish controls (17 $\pm$ 10 sec).

After 3 min exposure of PETCO zebrafish to 25 mg/L of the norepinephrine reuptake inhibitor desipramine, fish spent significantly more time in the top of the dive tank than untreated fish (ANOVA $F_{(4,35)}$ = 2.57, Tukey HSD $p < 0.05$). There was a non-significant trend toward zebrafish spending more time in the top 2/3 of the tank after exposure to 0.5% ethanol than controls (Fisher's LSD p = 0.08). The solvent DMSO had no significant anxiolytic effect on the time zebrafish spent in the top 2/3 of the tank. These data are shown in Figure 4.

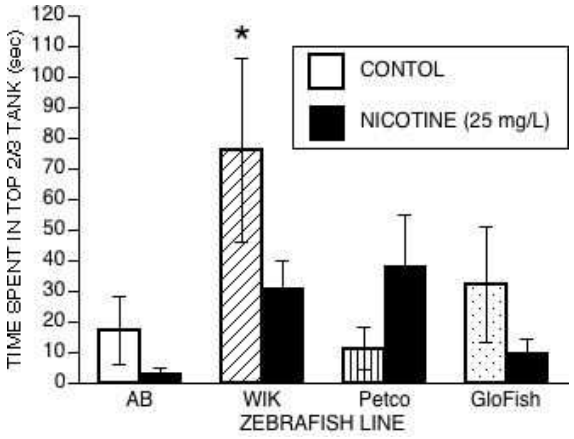


Figure 3. Behavior of zebrafish lines and lack of nicotine effect at 25mg/L in dive tank. WIK line zebrafish spent more time in the top 2/3 of the novel dive tank than AB fish, and nicotine treatment had no significant effect. Mean ± S.E.M. are shown. Sample sizes (N) for AB and WIK = 6, GloFish® = 5, and PETCO zebrafish = 8, both for untreated and nicotine treated fish. An * indicates significantly more time in the top of the tank than the AB strain ($p < 0.05$).

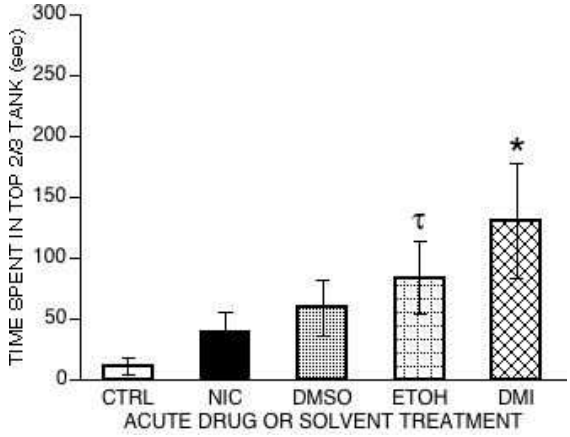


Figure 4. Drug and solvent effects on dive tank exploration. Zebrafish treated for 3 min with 25mg/L desipramine (DMI) spent significantly more time in the top 2/3 of the dive tank than controls (CTRL). Mean ± S.E.M. are shown, N = 8. Fish were obtained from PETCO, Paramus, NJ. An * indicates significantly more time in top 2/3 of tank than controls ($p < 0.05$). There was a non-significant trend for zebrafish exposed to 0.5% ethanol (EtOH) to spend more time in the top 2/3 of the tank ($p = 0.08$), indicated by τ. Neither 25 mg/L nicotine (NIC) nor 0.05% DMSO affected behavior in the dive tank.

In a subsequent experiment, zebrafish from Aquatic Eco-Systems (Apopka, FL) were used as subjects. These zebrafish spent significantly more time in the top 2/3 of the tank after 3 min exposure to 100 mg/L of the serotonin reuptake inhibitor citalopram than unexposed controls (ANOVA $F_{(3,32)}$ = 23.3, Tukey HSD $p < 0.001$). Treatment of zebrafish with 25 mg/l of either the α-noradrenergic antagonist yohimbine, or the $GABA_A$ benzodiazepine site receptor agonist chlordiazepoxide resulted in mean times spent in the dive tank top 2/3 that did not differ from untreated control fish. These data are shown in Figure 5.

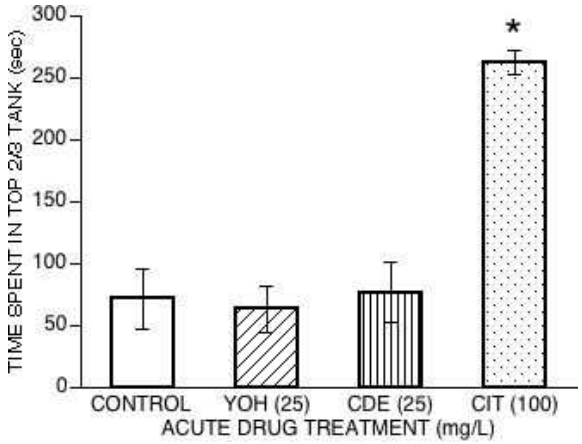


Figure 5. Anxiolytic, but not anxiogenic drug effects on zebrafish behavior in the dive tank. Citalopram exposure for 3 min at 100mg/L resulted in zebrafish spending significantly more time than controls in the top 2/3 of the dive tank. Mean ± S.E.M. are shown, sample size = 9. Fish were obtained from Aquatic-Ecosystems. An * indicates significantly more time in top of tank than controls or other treatment groups ($p < 0.001$). Exposure to 25 mg/L yohimbine (YOH) or chlordiazepoxide (CDE) did not affect zebrafish vertical localization within the dive tank.

Effects of zebrafish line and compound exposure on behavior in the light/dark plus-maze

In the aquatic plus maze, among AB, WIK, PETCO and GloFish, there was no effect of zebrafish line or exposure to 25 mg/L nicotine in total number of arm entries, which averaged 34 ± 12 entries for all groups combined (ANOVA $F_{strain\ (3,44)} = 0.7$, p =0.54; $F_{nicotine\ (1,44)} = 0.13$, $p = 0.71$). There was also no effect of line or nicotine on % white arm entries of total entries, which averaged 17 ± 5 % for all groups ($F_{strain\ (3,44)} = 1.48$, $p = 0.23$; $F_{nicotine\ (1,44)} = 1.26$, $p = 0.27$), nor was there an effect on the amount of time spent in white arms, for which the pooled average was 49 ± 23 sec ($F_{strain\ (3,44)} = 0.44$, $p = 0.72$; $F_{nicotine\ (1,44)} = 0.32$, $p = 0.58$). However, AB line fish spent significantly more time initially frozen (immobile upon introduction) in the center of the maze before entering any arm than either PETCO zebrafish or GloFish, but not in comparison to the WIK line ($F_{strain\ (3,44)}$ =

3.10, $p < 0.05$, Tukey's HSD $p < 0.05$). Zebrafish line effect on initial time frozen in the center is shown in Figure 6.

Exposure of PETCO zebrafish to a higher dose of nicotine (125 mg/L, N= 4) had no significant effect on arm entries (30 $\pm$ 9 entries), % of white entries (25 $\pm$ 3 %), total time spent in white arms (56 $\pm$ 24 s), or time frozen in the middle (57 $\pm$ 17 s) as compared to untreated PETCO zebrafish. There were no significant interactions between nicotine treatment and strain for any parameter. In zebrafish from a PETCO in San Antonio, TX doses of 50 and 100 mg/L of nicotine had no effect on white arm entries or time spent in white arms ($F_{(2,14)} = 0.13$ or 0.84, $p = 0.88$ or $p = 0.45$) in the light-dark plus maze. However, zebrafish administered 100 mg/L nicotine spent significantly more time ($F_{(2,14)} = 5.91$, Tukey's HSD $p < 0.005$) frozen in the center of the maze (198 $\pm$ 46 sec) than zebrafish administered 50 mg/L nicotine (9 $\pm$ 5 s), but not controls (81 $\pm$ 48 s); N = 5 - 6.

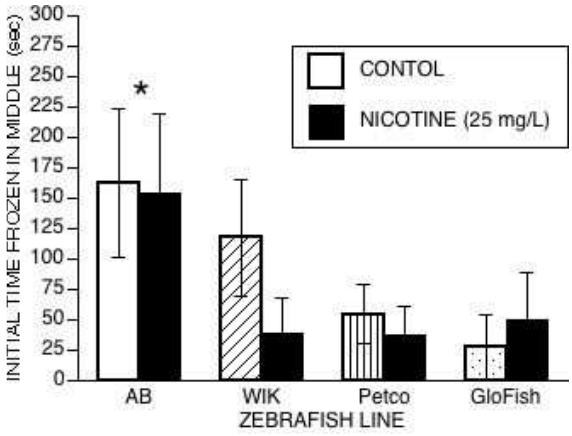


Figure 6. AB line zebrafish exhibited significantly greater latency to enter an arm than PETCO or GloFish zebrafish, but not WIK line zebrafish. Nicotine exposure had no influence on this parameter. Mean + S.E.M. are shown. Sample sizes for AB, WIK and GloFish® = 6, and PETCO zebrafish = 8. An * indicates significantly more time initially frozen in the middle of the plus-maze than GloFish or PETCO zebrafish (ANOVA and Tukey's HSD post-hoc, $p < 0.05$).

Exposure of PETCO zebrafish to 0.5% ethanol or 0.05% DMSO resulted in a significant increase in total arm entries (ANOVA $F_{(4,34)} = 3.6$, $p = 0.01$, Fisher's LSD $p < 0.05$). Neither nicotine nor desipramine exposure had any effect on arm entries (see Figure 7a). There was no difference between untreated controls and any of the drug or solvent treatments in the % white of total arm entries ($F_{(4,34)} = 1.99$ $p = 0.12$); the pooled group average was 21 $\pm$ 4 entries. However, ethanol-exposed fish spent significantly more time in white arms (126 $\pm$ 20 s) than untreated controls (41 $\pm$ 17 s) ($F_{(4,34)} = 2.86$, $p < 0.05$, Fisher's LSD $p < 0.01$), as shown in Figure 7b. There was no significant difference among controls and drug or solvent treatment groups in initial time frozen in the middle ($F_{(4,34)} = 1.30$, $p = 0.29$). One male ethanol-exposed zebrafish was injured during transfer

from dive tank to plus-maze, so data from this fish was dropped from the analysis (ethanol N = 7, all other drug/solvent groups N = 8).

a.

b.

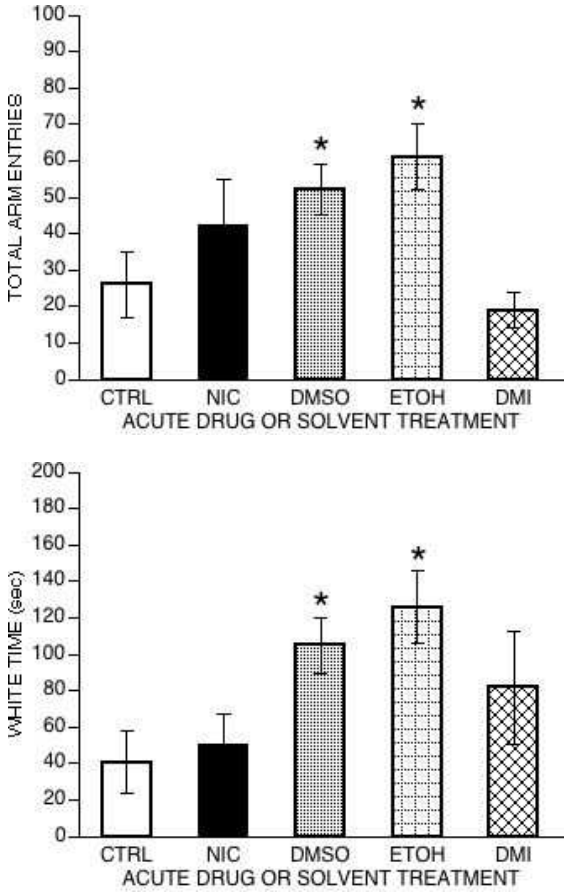
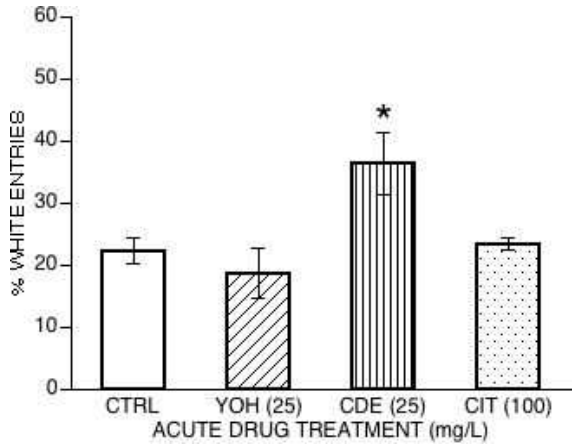


Figure 7 (a) Zebrafish from PETCO exposed to ethanol (0.5%) or DMSO (0.05%) entered significantly more arms in the aquatic light/dark plus-maze than controls (ANOVA and Fisher's LSD post-hoc, $p < 0.05$). Mean $\pm$ S.E.M. are shown, sample size = 8, except N = 7 for the EtOH group in which one fish was injured on transfer. Nicotine (NIC) and desipramine (DMI) treatment had no effect on this measure. (b) White time: percent time spent in white arms. PETCO zebrafish exposed to 0.5% ethanol (EtOH) or 0.05% DMSO spent significantly more time in white arms than untreated controls (ANOVA and Fisher's LSD post-hoc, $p < 0.05$).

Zebrafish from Aquatic Eco-Systems treated with either 25 mg/L yohimbine, chlordiazepoxide or 100 mg/L citalopram did not differ in total arm entries, which averaged 36 $\pm$ 7 entries across groups (ANOVA $F_{(3,32)} = 0.19$, $p = 0.9$). Chlordiazepoxide-treated fish entered white arms more frequently (36 $\pm$ 5% of total entries) than untreated fish (22 $\pm$ 2%) ($F_{(3,32)} = 4.91$, Fisher's LSD $p < 0.01$) and spent more time in the white arms than controls, 124 $\pm$ 25 s, vs. 60 $\pm$ 10 s

($F_{(3,32)}$ = 3.05, p < 0.05). Neither chlordiazepoxide nor citalopram-treated fish froze in the middle of the maze upon introduction, but this response was not significantly different from the behavior of yohimbine-treated or untreated controls ($F_{(3,32)}$ = 1.69, p = 0.19). Figure 8a shows the effects of these drugs on % white of total entries, and 8b. shows drug effects on time in white arms.

a.



b.

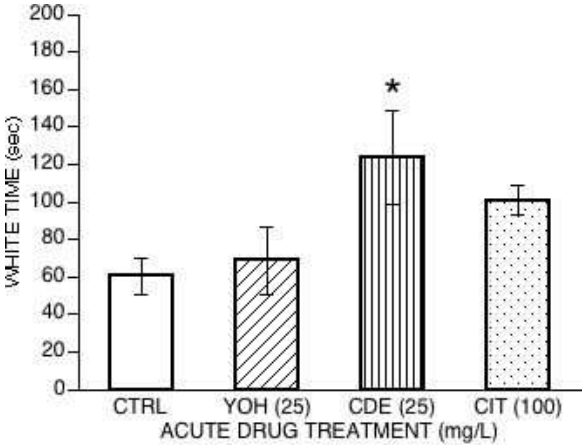
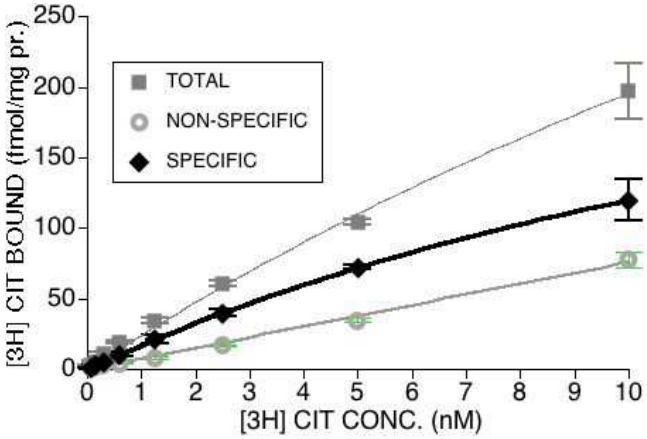


Figure 8. Effects of drugs targeting excitatory and inhibitory neurotransmitter systems on behavior of zebrafish from Aquatic Eco-Systems in the light/dark plus-maze. Mean + S.E.M. are shown, sample size = 9, CTRL = control, YOH = yohimbine 25 mg/L, CDE = chlordiazepoxide 25 mg/L; CIT: citalopram 100 mg/L. Total arm entries did not differ significantly among drug treatment groups, yet (a) chlordiazepoxide-treated zebrafish had proportionally more white arm entries, and (b) spent more time in white arms than untreated controls (ANOVA and Fisher's LSD post-hoc, p < 0.05).

a.



b.

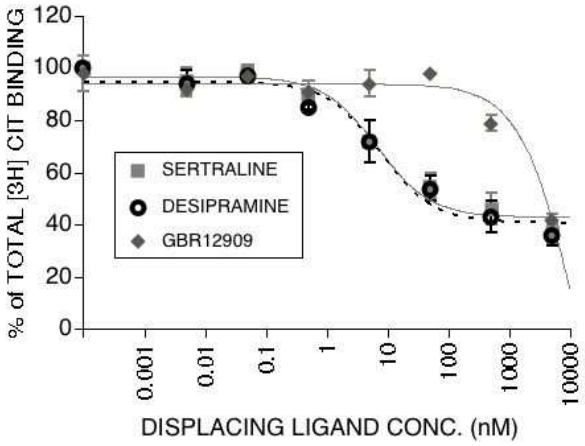


Figure 9 (a) Saturation binding of [^{3}H] citalopram to serotonin transporters (SERTs) in zebrafish whole brain membranes by non-linear regression (N = 3 assays). Membranes pooled from 10-12 adult mixed-sex zebrafish were incubated with concentrations of [^{3}H] citalopram ranging from 0.1 to 10 nM. Non-specific binding was defined by 20 μM fluoxetine. The K_D of zebrafish brain SERT binding sites is 15.6 $\pm$ 5 nM and Bmax is 278 $\pm$ 70 fmol/mg protein. (b) Displacement of 2.5 nM [^{3}H] citalopram from zebrafish whole brain membranes by sertraline, desipramine and GBR 12909 (N = 3 assays). Sertraline (Ki = 9 $\pm$ 5 nM) and desipramine (Ki = 7 $\pm$ 5 nM) exhibit similar high affinities for zebrafish SERT binding sites, while GBR 12909 (Ki > 1000 nM) exhibits negligible affinity for them.

[^{3}H] Citalopram uptake into zebrafish tissues from water

Zebrafish exposed to 75 nM [^{3}H] citalopram (24.3 μg/L) for 3 min took up 115 $\pm$ 37 ng/g wet weight in brain, and 193 $\pm$ 33 ng/g wet weight in muscle tissue (N = 6). With bath exposure to 35 nM (11 μg/L) [^{3}H] citalopram, zebrafish brain

took up 100 ± 13 ng/g, and muscle took up 70 ± 4 ng/g wet weight. Assuming a linear relationship between fish tissue uptake and bath concentration of citalopram, exposure to 100 mg/L would result in citalopram concentrations of 116 μg/g in wet brain, and 949 μg/g in wet muscle tissue. Hence [^{3}H] citalopram acute uptake in zebrafish brain and muscle is approximately 1/1000 and 1/100 of bath solution concentration.

Zebrafish brain SERT binding properties

Specific [^{3}H] citalopram saturation binding to zebrafish serotonin transporters (SERTs) was fit by single site non-linear regression ($r^2 \geq 0.99$). Addition of a second binding site to the model did not improve the r^2 value of the curve fit. [^{3}H] citalopram binds to zebrafish SERT(s) with a $K_D = 15.6 \pm 5.0$ nM and $B_{max} = 278 \pm 70$ fmol/mg protein. A plot of [^{3}H] citalopram saturation binding is shown in Figure 9a. Both desipramine and sertraline have high affinity for zebrafish SERT(s), with $K_i = 7 \pm 5$ nM and 9 ± 5 nM, as determined by their displacement of [^{3}H] citalopram. In contrast, GBR 12909 has negligible affinity for zebrafish SERTs, its $K_i = 1714 \pm 500$ nM. Figure 9b shows [^{3}H] citalopram displacement from zebrafish SERT by monoamine reuptake inhibitors.

Discussion

Herein we examined the effects of genetic lineage and several anxiety-state altering drugs (including monoamine reuptake inhibitors (MRIs)) on zebrafish behavior in two novel environments:the dive tank and light/dark plus maze, and we characterized the binding properties of the zebrafish serotonin transporter, an MRI target, the status of which can affect anxiety states in zebrafish. Zebrafish behavior in novel environments is essentially the time-dependent outcome of a trade-off between anxious (anti-predator or defensive) vs. exploratory (food-gathering, territorial or social) instincts. Individual zebrafish introduced into novel tanks initially swim close to the walls and bottom, a thigmotaxic response that may evade detection by predators (Peitsaro, Kaslin, Anichtchik, & Panula, 2003). After several minutes with no predator cues, individual zebrafish begin to explore the novel environment more extensively. We anticipated that the AB line and GloFish® would exhibit a muted predator "anxiety" or avoidance response as compared to outbred WIK or standard PETCO zebrafish lines. However, the outbred WIK line spent significantly more time in the top 2/3 of the dive tank. Fluorescent GloFish® did not behave differently from either standard PETCO or AB, all three lines spent the majority of the test time in the bottom of the dive tank. In the wild, zebrafish are found in shallow ponds and rice paddies that are generally free of large predators, and only occur in rivers and streams due to monsoons, during which time they dwell at the outer edges but occupy the entire height of the water column (Spence, Gerlach, Lawrence, & Smith, 2008). Hence the apparent lack of anxiety associated with a novel environment in WIK, in contrast to retail or AB zebrafish lines, might be due to

absence of predation pressure in Indian wild populations, compounded by the common experience of netting and transfer in tank-raised zebrafish.

In our initial line x nicotine study, 3 min of exposure to 25 mg/L of nicotine failed to alter the amount of time spent in the top 2/3 of the tank by any zebrafish line, and particularly of interest, exposure to 125 mg/L had no effect. Yet in previous studies, nicotine at 50 and 100 mg/L doses reduced zebrafish bottom dwelling at earlier time points in novel dive tanks (Levin et al., 2007). In a follow-up study we exposed zebrafish to 50 and 100 mg/L nicotine and found that the 50 mg/L dose dramatically increased time spent in the top 2/3 of our dive tank, but the 100 mg/L dose failed to do so. As zebrafish exposed to 100 or 125 mg/L were relatively less mobile than controls or fish exposed to lower doses, it is possible that nicotine has sedative effects or otherwise induces immobility at the higher doses. We did not impose a 5 min delay between the 3 min exposure and testing in the dive tank, as was done in prior studies (Levin et al., 2007; Bencan & Levin, 2008). By testing the fish 5 min earlier we could have missed the maximal effect of the 100 mg/L dose, but this seems unlikely since our 50 mg/L dose of nicotine dramatically increased time spent by zebrafish at the top of the tank. Differences in the shape or dimensions of the dive tank aquaria (trapezoidal vs. triangular), and different housing conditions may have contributed to increased bottom dwelling in our 100 mg/L nicotine exposed zebrafish compared to those of Levin and colleagues (2007).

As observed previously (Gerlai, Lahav, Guo, & Rosenthal, 2000), 0.5% ethanol tended to increase zebrafish top dwelling and locomotor activity in novel dive tanks, but this trend was not significant in the present study, perhaps due to our 3 min vs. their 60 min ethanol exposure. In another study, buspirone and diazepam exposures reduced bottom dwelling by zebrafish, but chlordiazepoxide did not (Bencan, Sledge, & Levin, 2009). We also found that chlordiazepoxide failed to increase zebrafish dwelling at the top of the tank. Yohimbine (25 mg/L) was expected to exhibit anxiogenic properties, yet it did not increase bottom dwelling by zebrafish under these exposure conditions. Exposure to 25 mg/L of the norepinephrine reuptake inhibitor desipramine or 100 mg/L of the serotonin reuptake inhibitor citalopram significantly increased the amount of time spent by zebrafish in the upper sections of the dive tank in the present study, which suggests that both drugs exhibit anxiolytic properties in this paradigm. However, zebrafish swim bladders have cholinergic, adrenergic and serotonergic innervation, so fish buoyancy is potentially sensitive to ligands targeting these systems (Finney, Robertson, McGee, Smith, & Croll, 2006), and could confound assessment of fish anxiety levels by vertical localization.

An alternative novel environment test is the light-dark plus-maze for zebrafish. The aquatic light/dark plus maze test is predicated on the observed innate preference of zebrafish for dark backgrounds (Serra et al.,1999). Analogous to the rodent elevated plus maze, entry into white arms parallels exploration in opened arms, and dwelling in black arms or closed arms is a defensive (or anxious) response. Most naive fish initially freeze or enter only black arms before exploring the white arms minutes later. This behavior appears driven by a telencephalic fear

response to white or pale backgrounds rather than a preference for dark (Lau, Gould, & Guo, 2010). Exposure to ethanol or anxiolytic drugs reduces this propensity for light avoidance (Guo, 2004).

We examined zebrafish behavior in the plus maze immediately after the dive tank so response to drug exposure in both tests could be compared directly within their previously demonstrated 20 min window of maximum effect (Levin et al., 2007). As both novel environments are distinct in appearance, the tests are brief (5 min each) and there is an intervening netting and transfer, acclimation to the novel light/dark plus maze due to prior dive tank exposure is unlikely to occur. For example, two or more trials must transpire before zebrafish respond in a stereotypical manner to a single aversive stimuli such as an advancing net (Arthur & Levin, 2001). In this study naïve zebrafish exposed to tank water or a dose of compound were used to generate the dive tank and plus maze data such that each fish was the subject in one tandem test trial run.

In the aquatic light/dark plus maze, there was no difference among AB, WIK, PETCO or GloFish® lines in the number of line crosses, white line crosses, or time spent in white arms. However time spent immobile or frozen in the middle square upon introduction to the plus maze was significantly greater for the AB strain than any other strain aside from WIK. As observed with rodents in the elevated plus maze, time frozen (immobile) in the maze center can be interpreted either as an anxiety response of greater magnitude, a risk assessment state, a sedative-hypnotic effect or as locomotor impairment (Carobrez & Bertoglio, 2005; Lapiz-Bluhm et al., 2008). In this context, we interpret AB freezing to be either an anxiety response or a state of risk assessment, as no drugs were administered to half of the WIK fish and their locomotor activity in home tanks was not impaired. Nicotine at 25 mg/L and doses $\geq$ 100 mg/L had no significant effect on zebrafish behavior in the plus maze, but exposure to 50 mg/L significantly reduced freezing in the maze middle relative to the 100 mg/L dose, but not relative to controls.

Exposure to 0.5% ethanol or 0.05% DMSO significantly increased total arm entries by zebrafish in the light/dark plus maze, which is consistent with their increased swimming activity, but neither compound increased white arm entries. However, both ethanol and DMSO-exposed zebrafish spent significantly more time in white arms. Hence 0.5% ethanol exposure produced robust effects in both novel environments, while DMSO did so only in the plus maze. Thus, if ethanol or DMSO are used as solvent vehicles in bath exposures to otherwise insoluble drugs, researchers should anticipate and measure potential anxiolytic effects on baseline response. Such an effect, if reversible, could enhance the detection of anxiogenic drug properties by raising control baseline values for time spent in the top 2/3 of the dive tank or time and entries into white arms in the plus maze. For example, we failed to observe increased anxiety response with 25 mg/L yohimbine exposure, which could be due to either a true lack of anxiogenic properties of yohimbine in zebrafish, an inappropriate dose selection or a “floor effect” of exploratory behavior in both the dive tank and the plus maze. Further studies are needed to clarify the dose-response profile of anxiolytic compounds for zebrafish

behavior in novel environments and their interactions with DMSO or other non-polar solvents.

Chlordiazepoxide significantly increased both white arm entries and time spent in white arms by zebrafish, in contrast to its failure to decrease bottom dwelling in the dive tank (present study and Bencan, Sledge, & Levin, 2009). Chlordiazepoxide also increased the amount of time zebrafish spent on the light side of a tank in a light avoidance test (Lau et al., , 2010). All GABAergic receptors are functionally conserved among mammals and zebrafish, and exhibit similar pharmacological properties (Renier et al., 2007). Chlordiazepoxide displaced [^{3}H] flunitrazepam binding (K_D = 1.5 + 0.4 nM) in zebrafish whole brain membranes with an inhibition constant K_i =143 $\pm$ 55 nM, indicative of a lower affinity for the benzodiazepine site than diazepam, with a K_i = 23 $\pm$ 4 nM (Lau et al., 2010). It is plausible that greater affinities for the benzodiazepine site, such as those of diazepam, are required to reduce bottom dwelling in the dive tank, as observed by Bencan and colleagues (2009). $GABA_A$ agonism at benzodiazepine binding sites generally reduces anxiety at low doses and sedates at higher doses. However, the anxiolytic effects of benzodiazepines in zebrafish may be limited to reducing the light avoidance response and may not impact vertical localization in the dive tank.

In contrast, neither desipramine nor citalopram produced any anxiolytic behaviors in the plus maze, while both increased time spent in the dive tank top. The effect of these monoamine reuptake inhibitors on vertical localization in the dive tank may selectively reduce thigmotaxis and have no affect on light avoidance. Alternatively the response in the dive tank could be mediated by increased systemic serotonin levels stimulating swim bladder inflation due to blockade of plasma SERT activity (Finney et al., 2006). Both doses of these drugs should have produced substantial occupancy of the zebrafish SERT, given that 100 mg/L citalopram exposure is anticipated to reach levels > 100 μg/g, and citalopram $K_D \approx$ 16 nM and desipamine Ki ≈ 7 nM in the zebrafish brain. Selective serotonin reuptake inhibitors produced anxiogenic effects in rats tested on the elevated plus aze (rats spent less time in opened arms and entered them less frequently);, benzodiazepines produced anxiolytic effects, and desipramine had no effect (Drapier et al., 2007). Hence, similar to their lack of effect in mammalian anxiety tests, monoamine reuptake inhibiting antidepressants may not exhibit anxiolytic properties in zebrafish.

Water-soluble compounds, with similar chemical properties to the serotonin reuptake inhibitor citalopram that are acutely administered to zebrafish via submersion in bath solution, are likely to be taken up into brain at roughly 1/1000 and into muscles at 1/100 of bath concentrations after 3 minutes, if this process approximates a linear relationship at doses extending from μg/L into the mg/L range. Our findings of a 1:1000 brain: bath solution ratio for acute aquatic uptake of drug is consistent with previous findings in zebrafish for exposure to morphine sulfate as assessed by LC tandem MS chromatography (Lau, Bretaud, Huang, Lin, & Guo, 2006). Effects of longer exposure times, nonlinear uptake, distinct chemical properties, and timing of maximal effect after bath administration

of water-soluble drugs to zebrafish certainly warrant further pharmacokinetic investigations. However, the present finding for citalopram provides an estimate the relationship between bath uptake and brain availability of citalopram and other drugs administered in this study over 3 min prior to the novel environment behavioral assay.

The pharmacological properties of the zebrafish brain serotonin transporter(s), or SERTs, differ somewhat from mammalian SERTs, as was reported previously in other minnow lineages (Gould et al., 2007). The K_D of [^{3}H] citalopram in zebrafish brain was an order of magnitude higher (15.6 $\pm$ 5 nM) than it is for mammalian SERT, indicating a relatively lower affinity for the zebrafish SERT. Further, we found that desipramine binds with high apparent affinity (Ki = 7 $\pm$ 5 nM) to zebrafish SERTs, similar findings of the imipramine derivatives desipramine and didesmethylimipramine having high affinity for zebrafish SERTs in transfected HEK cells have been reported (Severinsen, Sinning, Muller, & Wiborg, 2008). In comparison, desipramine exhibits affinity for SERT that is an order of magnitude lower than that of sertraline in the rat brain (Owens, Morgan, Plott, & Nemeroff, 1997). While two SERTs (SERTa and SERTb) have been cloned from zebrafish brain, SERTa is more homologous to mammalian SERTs, is more widely distributed throughout brain, and binds with high affinity to serotonin reuptake inhibitors. In contrast, SERTb is limited to the medulla and retina, and is therefore unlikely to contribute substantially to high affinity binding in the present study (Norton, Folchert, & Bally-Cuif, 2008; Wang, Takai, Yoshioka, & Shirabe, 2006).

In conclusion, both zebrafish novel environment assays can enhance rapid drug or toxin screening for anxiolytic properties, because they reveal two independent dimensions of novel environment induced anxiety states. Further studies are necessary to characterize anxiogenic responses to drugs in the zebrafish light/dark plus maze and dive tank, independent of the pharmacological blockade of the behavioral effects of anxiolytic drugs. Uptake of water-soluble drugs into zebrafish brain through bath exposure produces brain drug concentrations that are roughly 1/1000 of bath concentrations. Further, while the pharmacological properties of neurotransmitter and drug binding sites are largely conserved among zebrafish and mammals, as exemplified by $GABA_A$ receptors, evolutionary divergence has also rendered some differences, as evidenced by slightly lower affinity for citalopram and greatly enhanced affinity for desipramine in zebrafish as compared to mammalian SERTs. Moreover, because variability among the zebrafish lines surveyed in this study influenced exploratory behavior in the dive tank as well as freezing behavior in the light/dark plus maze, the selection of *Danio rerio* line for similar behavioral studies should be considered carefully, pilot tested, and should be specified in publications of behavioral studies in which they have been used. Overall, zebrafish at adult as well as embryonic life-stages, are well suited for simultaneous examination of gene, drug and environmental factor effects on neurotransmission, neurophysiology, and behavior. Findings from studies employing their associative behavioral responses, such as anxiety in novel

environments may greatly advance translational drug development and toxicology research.

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