# The Utility of Zebrafish in Neuroscience Research

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**Introduction**

Zebrafish (*Danio rerio*) are small cyprinids native to rivers of Pakistan, India, and Bangladesh (Mortez, Martins et al. 2007). Zebrafish are a genetically diploid vertebrate, with a good balance of genetic understanding and behavioral simplicity. They are small animals, measuring 3-5 centimeters in length (Figure 1) (Guo 2004).

*Figure 1 - Adult wild type zebrafish* ( Danio Rerio)

Zebrafish have a low maintenance cost, a robust reproduction cycle, a rapid life cycle and a large number of offspring (Bertram) which is beneficial for usage in the lab. These beneficial properties, among many others, make it easy to maintain a large number of Zebrafish in a relatively small area, which is important for large-scale genetic and behavioral studies (Guo 2004). Zebrafish have long been utilized as an experimental animal model for biomedical research, particularly in developmental and genetic studies (Zon and Peterson 2005). However, the potential of the Zebrafish as a model in neurobehavioral research is gradually developing only now.

Years of studying zebrafish, by developmental biologists have provided us with a comprehensive toolkit of genetic techniques, including a multitude of distinct artificially selected strains and a complete genome sequence. This makes the zebrafish an ideal species for studying the genetic basis of behavioral syndromes (Mortez, Martins et al. 2007). Researchers have discovered a massive amount of information on hundreds of zebrafish genetic mutations and how they affect the living organism and have recorded that information into databases such as the one found at Zebrafish International Resource Center (ZIRC). Mutations that disturb basic developmental processes have also been documented, including mutations that affect the development of embryo shape , generation of germ layers, complex organ systems, the organization of distinct brain regions, the vascular architecture and the establishment of defined neural circuitry(Grunwald and Eisen 2002). Genetic analyses in zebrafish have the potential to provide important insights onto the relationship between genes, neuronal circuits and behavior in normal as well as diseased states (GUO 2004).

Aminergic neurotransmitters (dopamine, noradrenaline, serotonin and histamine) are involved in important physiological mechanisms in the brain. Many of these neurotransmitters are also involved in brain diseases such Parkinson's, Schizophrenia, Alzheimer's, and Depression (Panula and Sallinen et al.2006). These behavioral disorders are among the most widespread and costly brain diseases in modern societies. Our understanding of the molecular networks underlying these disorders is still limited, there by effectively preventing adequate therapies (Ninkovie and Bally-Cuif 2006). Although these complex diseases are difficult to reproduce in zebrafish, it is feasible that many of the underlying basic phenomena can be better explained using zebrafish models, provided that genetics, behavior and alterations in the brain can be reliably monitored (Mueller and Vernier et al. 2004).

When compared to Drosophila and C. elegans, the nervous system of zebrafish is more analogous to that of humans (Guo 2004). Recent extensive research has shown that both basic and complex brain phenomena as well as endocrine mechanisms, share a substantial homology between zebrafish and mammals such as mice and men (Miklosi and Andrew 2006). Zebrafish brain aminergic systems share many structural properties with the mammalian systems (including humans). All systems are homologous and, different only in that the dopaminergic system shows a lack of dopamine in the mesencephalon (Mueller, Vernier et al. 2004). Besides this difference, zebrafish are neurobiologically very similar to mammals, and therefore may represent a promising alternative to these models.

A number of animal models have been used to investigate the role of different brain structures in disease development. However, the identification of the genes involved has been difficult both because of the complexity of the disorder, and because of a limited number of identifiable candidate genes (Ninkovie and Bally-Cuif 2006). Genetic effects on brain development are so well understood in Zebrafish, providing invaluable comparative material for birds and mammals (Miklosi and Andrew 2006) .

As neurological models, rodents have two main disadvantages against zebrafish. They require that the genes of interest be known and very well characterized for experiment, and the production of an adequate sample size is costly (Ninkovie and Bally-Cuif 2006). In contrast, zebrafish are more amenable to genetic and behavioral studies, which is known to be difficult and costly to perform in mice (Guo 2004).

A disadvantage faced with using the zebrafish arises from its genome. The zebrafish has a diploid genome composed of 25 chromosome pairs. Zebrafish have an ancient genome duplication process that results in a redundancy in the duplication of genes. Having 2 loci for the same gene can be seen as a disadvantage, because the increase loci can complicate experimental interpretation. The redundancy and compensation that arises from its diploid genome prevents measurable phenotypes to be induced (Con 104).

Importantly zebrafish are very well suited for behavior based drug discovery (Kokel and Peterson 2008). Readily acclimatizing to new environments, being constantly active and little disturbed by the presence of observers make zebrafish a excellent specie choice for behavioral experiments (Spence, Geralch et al. 2008). The drug delivery system in rodents proves to be stressful for them because rodents must be handled by a humans to be injected (intramuscular, intraperitoneally ect.) with drugs and as a result the method can stress the rodent and provide inaccurate data for behavioral studies (Adamec, et al. 1991). In contrast, zebrafish stress due to drug administration is minimal because the mechanism of drug induction is delivered by adding the selected drugs to their water. Limited handling stress is very beneficial in accurate readings in the zebrafish, especially for studies requiring chronic treatment.

Another characteristic a zebrafish has is its ability to learn through classical conditioning. Conditioned responses can develop after a single trial and a response can be obtained even when there is a time delay of several minutes between the presentation of the unconditioned and conditioned stimuli (Spence, Geralch et al. 2008). Conditioned responses can also be passed on to naïve fish in a process known as social facilitation. The naïve fish will display a conditioned response in the presence of a previously conditioned fish, and will retain this learned response when solitary or in the company of a new group of naïve fish (Spence, Geralch et al. 2008). Fish can remember training for up to a 10 day period (Spence, Geralch et al. 2008). Learned preferences are mediated by olfactory as well as visual cues. Zebrafish can differentiate between familiar and unfamiliar conspecifics on the basis of odor and thus appear capable of individual recognition (Spence, Geralch et al. 2008)

Several studies have examined anxiety in zebrafish using anxiolytic agents such as nicotine (Levin, Bencan et al. 2007), α-fluromethylhistidine (Peitsaro, Kaslin et al. 2003), ethanol (Gerlai, Lee et al. 2006) and diazepam (Lopez-Patino, Yu et al. 2008). Drug-evoked anxiety has also been reported in zebrafish using the benzodiazepine inverse agonist FG-7142 or following abrupt cessation of chronic cocaine administration (Lopez-Patino, Yu et al. 2008).

Physiological endpoints, such as stress hormone levels, can also be used to parallel behavioral data (Alsop and Vijayan 2008). The hypothalamus-pituitary-interrenal axis is fundamental to stress responses in zebrafish and involves a cascade of hormones from corticotrophin releasing hormone (CRH) to adrenocorticotropic hormone (ACTH) and cortisol. A recent analysis illustrates how whole-body cortisol levels can be differentially affected as a function of predator exposure (Barcellos 2007).

Our study examined zebrafish as a model of anxiety by comparing behavioral and physiological phenotypes produced by various environmental and pharmacological anxiolytic or anxiogenic manipulations.

**Methods**

Animals and Housing: Adult zebrafish (90 days old; ~ 50/50 male, female) were obtained from a local commercial distributer (50 Fathoms, Metaire, LA). All fish were given at least 10 days to acclimate to the laboratory environment and housed in groups of 20-30 fish per ~40L tank. All tanks were filled with deionized water treated with Prime Freshwater & Saltwater Concentrated Conditioner (Seachem Laboratories, Inc., Madison, GA). The room and water temperatures were maintained at 25-27°C. Illumination was provided by fluorescent light tubes from the ceiling with lights turned on at 9 am and off at 7 pm. Fish were fed a mixture of ground flake food (Tetramin Tropical Flakes; Tetra USA, Blacksburg, VA). All fish used in this study were experimentally naïve.

Novel Tank Diving Test: Zebrafish were placed individually in a 1.5-L trapezoidal tank (approx. 15.2cm H x 27.9cm TW) maximally filled with aquarium treated tap water. As shown in figure 2 the tank was divided into two equal virtual horizontal portions, marked by a rubber band surrounding the outside walls.

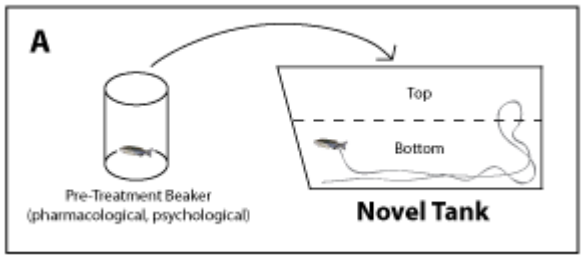



*Figure 2: A Novel Tank*

Tanks rested on a level, stable surface, and all environmental disruptions were minimized. Zebrafish were relocated from home-tanks to novel tanks, with careful consideration to reduce net-stress. Swimming behavior was quantified by human observation, recording endpoints over a six minute period of latency to reach the upper portion of the tank, time spent in the upper portion of the tank, number of entries into the upper portion of the tank, erratic movements, freezing bouts, and freezing duration. Erratic movements were defined as sharp changes in direction and velocity, and repeated rapid darting behaviors. Freezing was defined as a total absence of movement, with the exception of gills and eyes. A significant decrease in exploration, including: longer latency to reach the top, fewer entries to the top, longer freezing duration, or elevated erratic movements represent stress responses indicative of a high-anxiety phenotypes.

For the chronic ethanol exposure experiments, home tank water was treated with ethanol (.03%) for one week. Tank water was replaced every two days, and ethanol was administered before introducing subjects back into the home tank. Similarly, in the ethanol withdrawal experiment, zebrafish were exposed to ethanol for one

week prior to discontinuation of treatment. To provoke withdrawal symptoms, zebrafish were relocated to an identical tank without ethanol treatment for 12 hours before performing the novel tank diving test.

To investigate the anxiolytic effects of chronic antidepressant administration, the selective serotonin reuptake inhibitor fluoxetine (5µg/L) (Brooks, Smith et al. 2003) was administered daily for 2 or 4 weeks. In each experiment, the fish were then individually tested in the novel tank paradigm, euthanized in Tricane (5%), and immediately dissected and frozen at -80*C for further physiological assessments.

The cortisol extraction procedure was adopted from Alsop and Vijayan (2008). Individual fish body samples obtained from behavioral cohorts were homogenized in 750µL of ice cold 1X PBS (buffer). The homogenizing rotor blade was then washed with an additional 250µL of ice cold 1X PBS and collected into the 2mL tube containing the homogenate. Samples were then transferred to glass extract-O tubes and cortisol was extracted 2 times (on consecutive days) with 5mL of Ether (per extraction). After allowing the Ether to evaporate over night (under a fume hood), the cortisol was reconstituted in 1mL of 1X PBS. After each sample was homogenized, the rotor blade and rotor shaft were triple washed in dH2O, EtOH, and dH2O, then wiped with a Kimwipe. Between groups, the rotor blade and shaft were autoclaved, to prevent any possible contamination. During this process all samples were kept on ice.

ELISA was performed to quantify cortisol levels using a human salivary cortisol assay kit (Salimetrics LLC, State College, PA). ELISA plates were read in a plate reader using the WALLAC plate reader and the manufacturer's software package. Absorbencies were normalized using a 4-parameter sigmoid minus curve fit and total body cortisol levels were analyzed based on the generalized concentrations derived from these calculations. Cortisol levels were further normalized based on the weight of the initial body sample and are reported as absolute cortisol concentrations (µg/dL).

Statistical Analysis: Experimental data were analyzed with a two sample (Mann-Whitney) U-Test for significance between control and experimental groups. Data are expressed as ±S.E.M. Significance was set at $p<.05$.

**Results:**

**Ethanol withdrawal**

Zebrafish experiencing ethanol withdrawal showed overall trends of anxiety-like behavior . As compared to control fish, the withdrawal cohort displayed less transitions to the upper half of the novel tank, had significantly more freezing bouts and were frozen for a greater duration. These behavioral endpoints are an indicative of elevated stress levels. Moreover, this overall anxiety-like state is confirmed by physiological

measures of average whole-body cortisol concentrations (p=.0135). Data is expressed as Mean ± S.E.M, *p<0.05, **p<0.005, ***p<0.0005

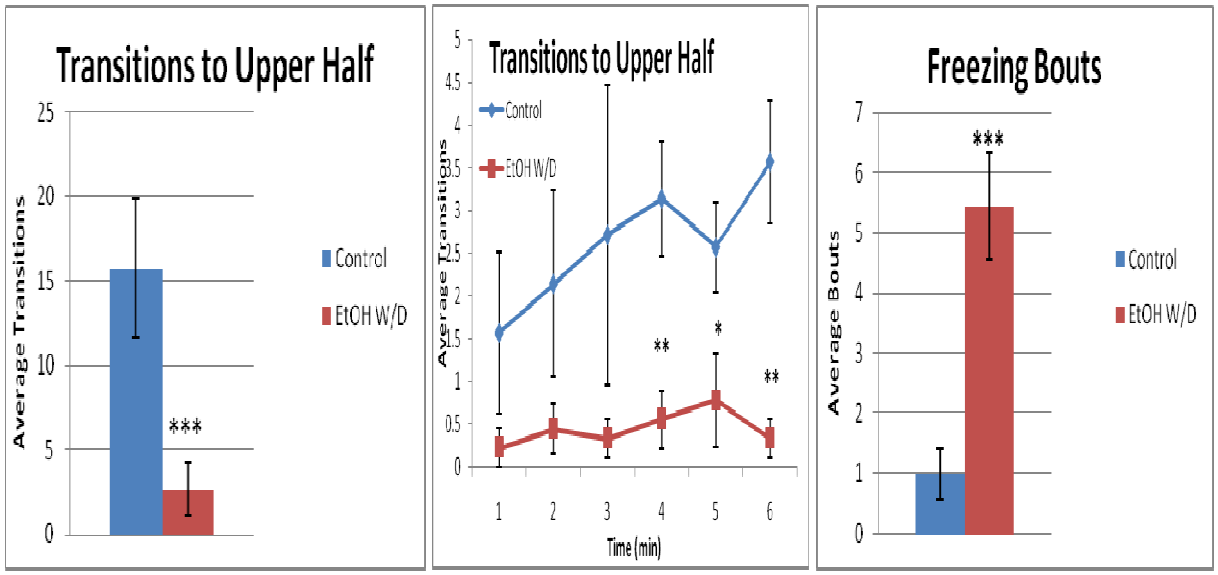
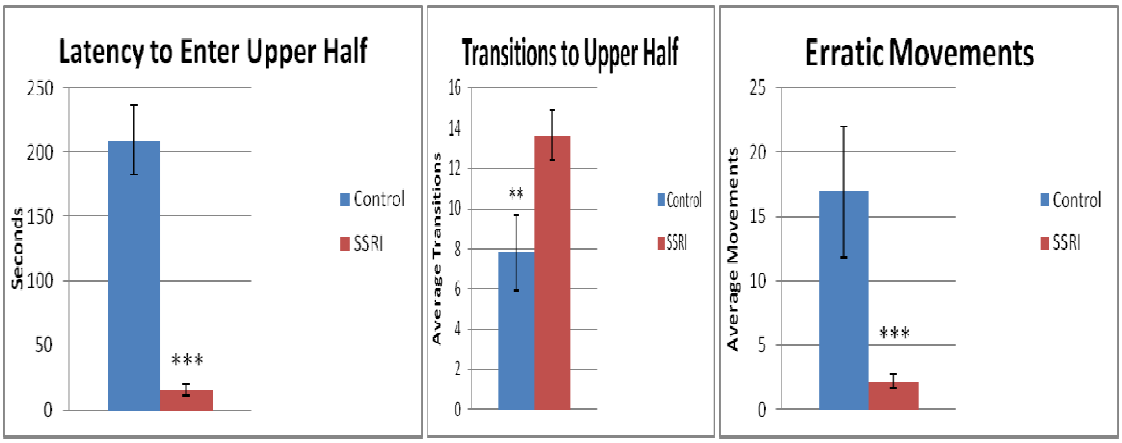


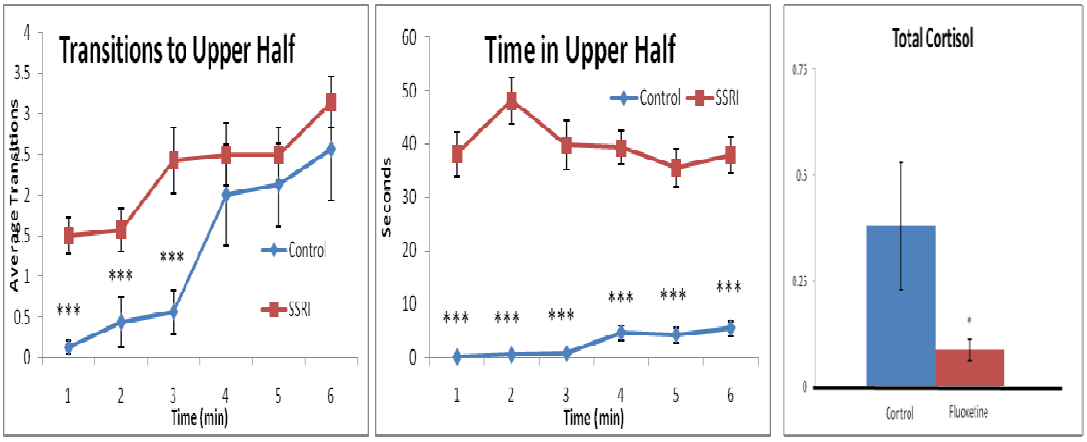

*Figure 3: Anxiogenic effects of ethanol withdrawal in zebrafish*

**SSRI Treatment**

Zebrafish pretreated with fluoxetine showed overall decreases in anxiety-like behavior. The experimental cohort took significantly less time to enter the upper half of the novel tank, in fact all fish almost immediately ventured into the top half. As compared to control fish, the SSRI treated fish also displayed significantly more transitions to the upper half of the novel tank, spend more time per upper entry and had significantly less erratic movements. Moreover, the anxiolytic effects of fluoxetine are reflected by whole-body cortisol levels, in which experimental fish on average had significantly less. The video tracking software (CleverSys) was able to provide the trace images, that eloquently reflect the general differences in swimming behavior between SSRI and control cohorts.




*Figures(from left to right) 4a,4b, and 4c: The graphical results of the SSRI Treatments*




*Figures(from left to right) 5a,5b and 5c: The graphical results of the SSRI Treatments*

**Conclusion**

The attributes that give the zebrafish its power for genetics, drug discovery and developmental research, make this species an ideal subject for cost-effective models of disease pathogenesis, including stress and anxiety. By comparing the robust behavioral phenotypes seen here with the physiological evidence from cortisol assays, we were able to demonstrate the consistency and reliability of this species as an experimental model for stress and anxiety. Both manual and automatic registration of zebrafish behavior were able to distinguish differences in acute drug treatment and withdrawal paradigms. In future studies, we plan to further explore the effects of anxiolytics and anxiogenics on zebrafish behavior, physiology and eventually genetic expression.

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