

**Strain differences in
novelty-evoked Stress
responses in zebrafish
(*Danio rerio*)**

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Introduction

Zebrafish (*Danio rerio*) have a long history of use as experimental animal models in biomedical research. These have predominantly been genetic and developmental studies (Zon and Peterson, 2005), but zebrafish are increasingly being used as a model in neurobehavioral research. Zebrafish are ideal for laboratory research because they are inexpensive, low-maintenance and can reproduce quickly and abundantly, as one may obtain 200-400 eggs from a single female zebrafish every other day (Gerlai et al., 2006).

Though quite a simple species as far as vertebrates go, zebrafish physiology is functionally and morphologically similar to that of humans, providing an opportunity for researchers to investigate mechanisms and pathways directly attributable to diseases and therapeutic treatments of humans: Unlike other inexpensive and commonly used animal models, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, zebrafish have all the same tissues commonly afflicted by human diseases, with the exception of lungs (Shin and Fishman, 2002; Lieschke and Currie, 2007). Of particular interest to neurobehavioral researchers is the fact that zebrafish possess all of the “classical” neurotransmitters of vertebrates (Mueller et al., 2004; Panula et al., 2006), and their hypothalamic-pituitary-interrenal axis closely parallels the human hypothalamic-pituitary-adrenal axis, responding robustly to stressful situations and stimuli (Alsop and Vijayan, 2008).

This fundamental physiological stress response manifests as anxious behaviors, and can be elicited by introduction to novel environments and aversive stimuli (Blaser and Gerlai, 2006). The novel tank diving test exploits this response, as zebrafish will dive to the bottom of an unfamiliar tank in an effort to avoid predation, and will stay there until comfortable enough to explore the new environment (Levin et al., 2007). As with other species, genetic differences in zebrafish may lead to differing behavioral phenotypes within the species, as well as different behavioral responses to

psychopharmacological agents. For example, chronic exposure to ethanol was found to decrease shoaling behaviors in wild-type zebrafish, but increase shoaling behaviors in long-fin striped mutants (Dlugos and Rabin, 2003). Though strain differences have been observed, little research has been done to qualitatively examine the differing behavioral phenotypes exhibited by mutants across this species. Our study examined the anxiety responses produced by four different mutant strains of zebrafish, with the goal of understanding the effects differing strains can have on data produced in anxiety-producing behavioral testing.

Methods

Animals and Housing: Adult zebrafish (3-5 months old, ~ 1:1 male to female) were obtained from a local commercial distributor (50 Fathoms, Metairie, LA). Strains included wild-type (n=14), long-fin (n=15), leopard (n=13) and albino (n=15) zebrafish (Figure 1). Each strain was housed separately. All fish were given at least 5 days to acclimate to the laboratory environment and housed in groups of no more than 20 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. Fish were kept on a 10/14 light-dark cycle (lights on at 9 am and off at 7 pm) with illumination provided by fluorescent lights from the ceiling. Fish were fed a mixture of ground flake food (Tetramin Tropical Flakes; Tetra USA, Blacksburg, VA). All fish were experimentally naïve.

Behavioral Testing: Individual zebrafish were transferred from their home tanks to a 1.5-L trapezoidal tank (approx. 15.2cm H x 27.9cm TW) maximally filled with aquarium treated tap water, with careful consideration to minimize net stress. Novel tanks were divided into equal horizontal portions, marked by a line on the outside walls. Tanks rested on a level, stable surface, with all environmental

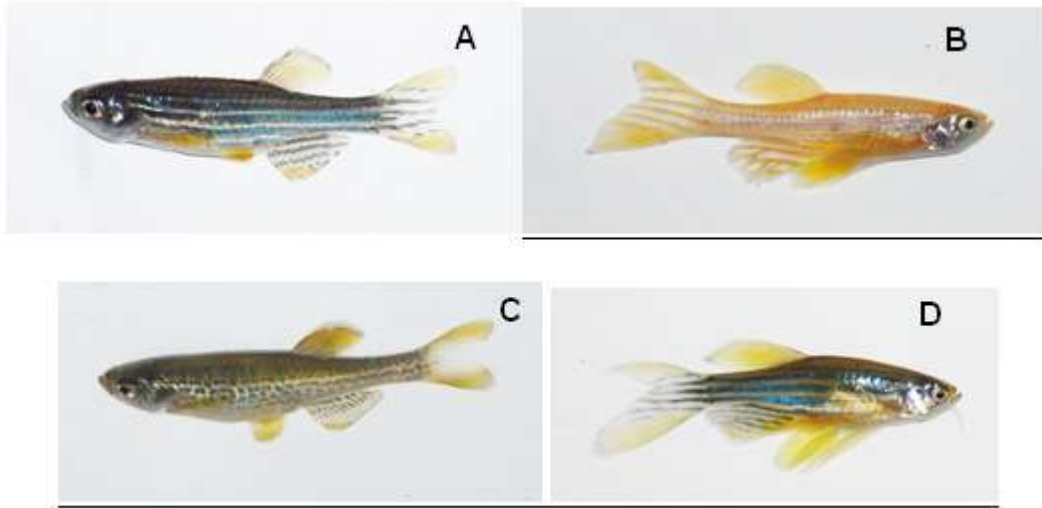


Figure 1: (A) Wild-type (short fin), (B) Albino, (C) Leopard, and (D) Long-fin strains of zebrafish

disruptions minimized. Swimming behavior trials in novel tanks were recorded by using a USB webcam and Dell laptop over a 6-min period (Figure 2). Subsequent analysis was performed using LocoScan software (CleverSys Inc., Reston, VA). By establishing “top” and “bottom” registration parameters within the software, we were able to measure behavioral endpoints such as 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, distance traveled in the upper portion of the tank, and swimming velocity in the upper portion of the tank. Significant decreases in exploration behaviors, including longer latency to reach the top, fewer entries to the top, or less time spent in the top region, represent stress responses indicative of a high-anxiety phenotype.

Statistical Analysis: Analyses of experimental data were performed using a two sample (Mann-Whitney) U-Test for significance between control and experimental groups. Data are expressed as \pm S.E.M. Significance was determined to be at $p < .05$.



Figure 2: Zebrafish novel tank trial recording apparatus. Zebrafish are introduced to novel tank (right) for 6-min trial. Behavior was recorded on laptop (left) via webcam (center) for later analysis.

Results

Overall, robust strain differences were observed in latency to top half entry, with WT zebrafish exhibiting the lowest value. Other strains demonstrated longer latencies, with albino and long-fin showing roughly equal latencies and leopard strain exhibiting by far the longest latency (Figure 3). Though all three mutant strains showed longer latencies to enter the top half of the novel tank, the difference between these and the WT group was only significant in the leopard mutant ($p=0.0036$), suggesting the leopard strain shows higher anxiety and is less motivated to explore the new environment.

The total number of entries into the upper half of the novel tank was lower for all mutant strains compared to WT controls (Figure 4). As can be seen in the figure, each mutant cohort exhibited approximately the same number of entries into the top. Though the control group appeared to show many more entries, the differences were not significant for any group with respect to this behavioral endpoint.

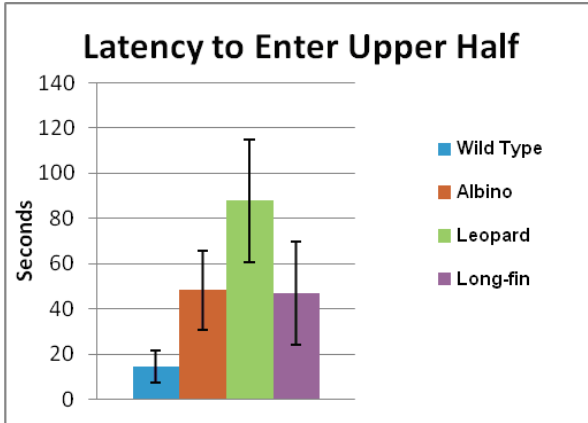


Figure 3: Average latency from novel tank introduction to entrance into upper portion. Data are represented as mean \pm S.E.M.

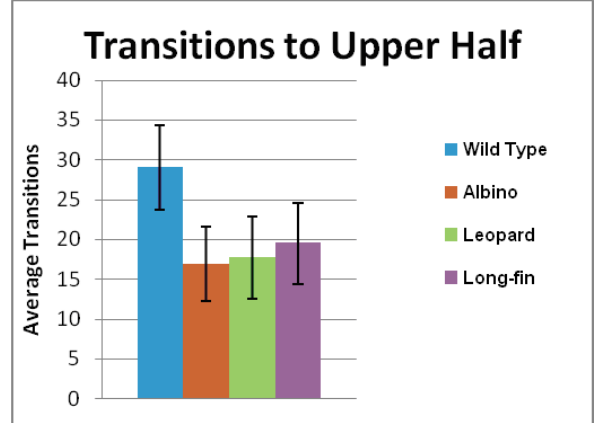


Figure 4: Average number of transitions from bottom portion to top portion during testing trial; Legend as in Fig. 3.

Total time spent in the top half of the testing tank proved to provide very robust differences between the control and experimental groups (Figure 5). Significance was found across all three mutant cohorts, with the leopard and long-fin mutant strains spending an especially small amount of time in the upper half and the WT spending a comparatively great amount of time in the top region. This suggests increased anxiety responses in all mutant cohorts compared to WT, with the leopard and long-fin strains particularly susceptible to this.

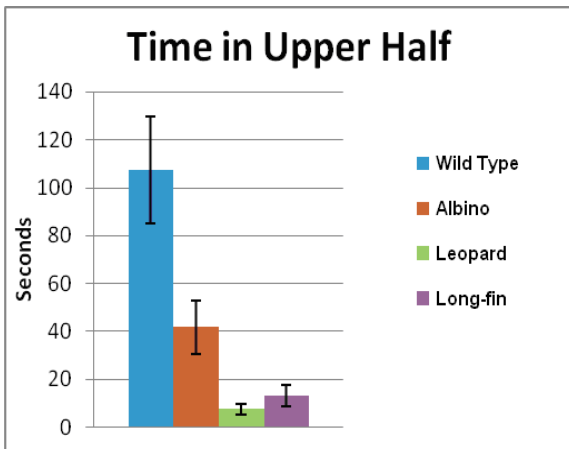


Figure 5: Average time spent in upper half of novel tank per strain; Legend as in Fig. 3.

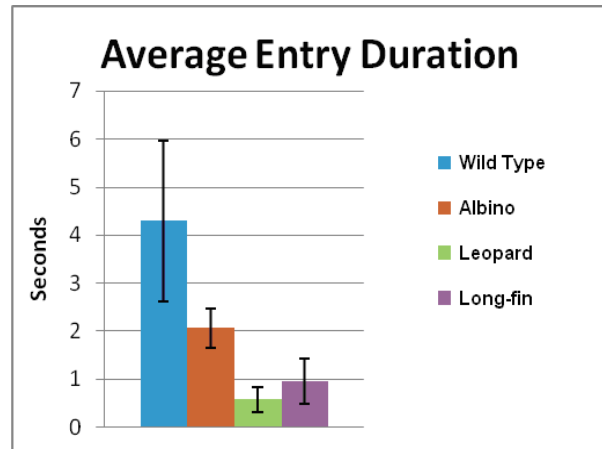


Figure 6: Average entry duration into top half of novel tank per strain; Legend as in Fig. 3.

Figure 6 shows data for average duration of entry into the top portion of the tank. Overall, the WT strain spent a much longer time in the top each entry compared to other cohorts, although greater variability was observed within the strain as well, as indicated by the S.E.M. bar. Both leopard and long-fin strains showed greatly reduced duration of entry on average, and these findings are highly significant (for leopard, $p=0.0022$; for long-fin, $p=0.0052$). The albino strain tended to display decreased top entry duration compared to controls, but these findings were not significant. These data suggest a marked decrease in exploratory behavior for the leopard and long-fin strains, implying higher baseline anxiety levels compared to WT. This observation is in line with findings from the total time spent in the upper half, with the albino strain representing a behavioral phenotype somewhere between these groups.

Importantly, swimming velocities of these strains in the top half of the tank showed no differences between cohorts (Figure 7). No significant difference was found between any of these groups, suggesting that behavioral measurements in this study were not affected by motor physiological differences related to swimming abilities between these strains.

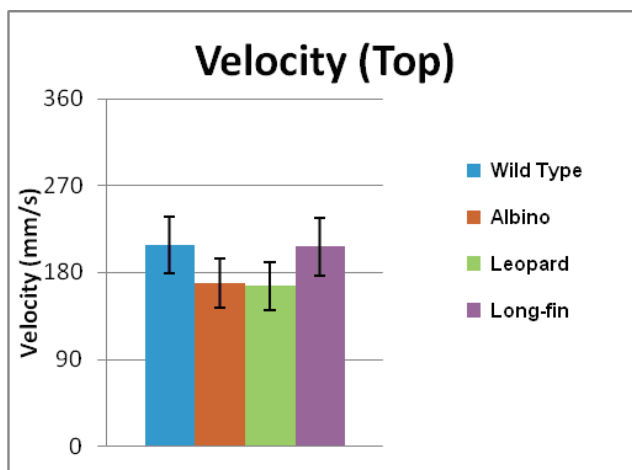


Figure 7: Average velocity exhibited in upper half of novel tank by strain, as measured by LocoScan software; Legend as in Fig. 3.

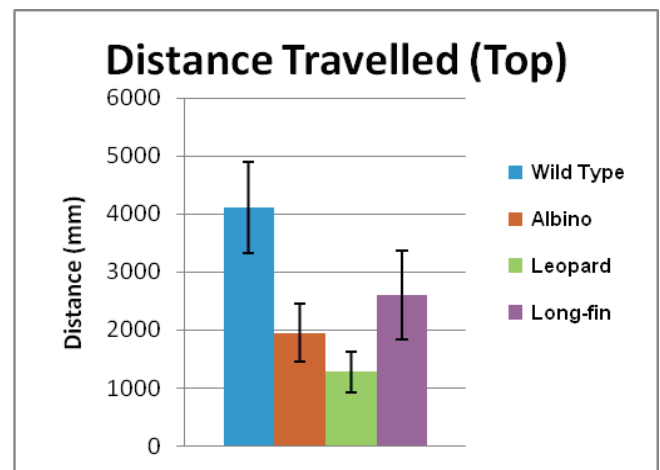


Figure 8: Average distance of path travelled in upper portion of novel tank by strain, as measured by LocoScan software; Legend as in Fig. 3.

Figure 8 shows the total distance travelled in the upper portion of the novel tank during the testing period. Once again, the WT group exhibited greater exploratory behavior compared to the other strains, as indicated by a greater distance travelled in the upper half. Significance was found in the travel paths of albino and leopard strains compared to the control group, but not in the long-fin mutants.

Discussion

Data generated through the novel tank diving test shows the wild-type zebrafish to exhibit greater exploratory behavior all around compared to the three mutant strains tested here. This difference is especially apparent in the leopard strain, a cohort that showed particularly pronounced anxiousness across all behavioral endpoints tested. The lessened exploratory behavior exhibited by the mutant strains denotes a greater anxiety response to perceived threats, suggesting higher baseline anxiety levels in the mutants than the wild-type. This finding is particularly valid as no pharmacological manipulations were made.

The contrasting behavioral phenotypes found here have several important implications to researchers. First, researchers must take into consideration the effects of using or comparing data gathered from different strains within this species. Secondly, the differing anxiety responses elicited here raises the possibility of expanding this animal model to account for population differences in humans. Experiments utilizing different mutations of zebrafish could be used to represent differences in humans that are different in their susceptibility to stressors. This is particularly relevant in psychopharmacological research. Additionally, strain selection could have great applications in experimental design. As some genotypes seem to exhibit higher baseline anxiety levels, selection of a certain strain in the testing of the behavioral effects of anxiolytic or anxiogenic compounds could yield more robust results. If a certain strain, like the leopard mutant, shows greater baseline anxiety,

behavioral manipulation by anxiolytic drugs could yield more distinct effects (mediated by dose, time exposed, etc.) as compared to using anxiogenic drugs in the same strain.

Conclusion

Behavioral pharmacological research is currently bottlenecked by the use of mammalian models which require a lot of space, exhibit complex behaviors that have proven to be quantitatively difficult, and are expensive to maintain (Zon and Peterson, 2005). Zebrafish show great potential as an animal model in reducing such limiting factors, particularly in conjunction with computer-aided tracking systems. Behavioral assessment by computer has already shown evidence of promisingly high validity in results. Our lab has shown, in preliminary testing, high correlation between automated behavioral assessment to manual observation and testing. Computerized testing also eliminates the effects of inter-rater variability and allows for the assessment of behavioral endpoints not previously attainable, such as velocity, traveled path tracing (Figure 9) and total distance traveled.

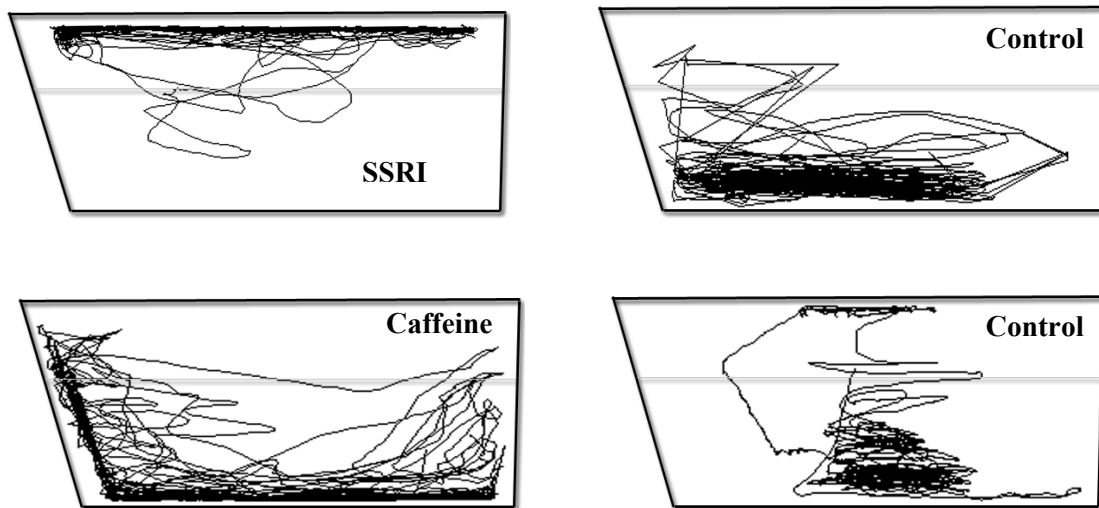


Figure 9: Swimming paths during novel tank testing trial of Zebrafish exposed to SSRI fluoxetine (top) and caffeine (bottom). Path tracings generated by LocoScan software.

This new method also allows for reduced time spent in experimentation and analysis, allowing for overall high-throughput, high-validity research to be done. We are currently applying these

approaches to analyze differences between the strains of zebrafish used in the present study. Zebrafish appear to have enormous potential as an animal model. However, much remains to be investigated. As our results revealed differing baseline anxiety levels between genotypic mutants, investigation of physiological correlates to this phenomenon, such as baseline and stress-induced cortisol levels, must be performed. Significant genetic homology has been found between zebrafish and mammals (Inohara and Nuñez, 2000), allowing for the possible identification of genes related to stress responses in mammals through genetic investigation of zebrafish homologs. The current study is a preliminary one, necessitating further research of behavioral phenotypes in the strains presented here and others not currently examined.

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