

**Video-aided analysis of zebrafish locomotion  
and anxiety-related behavioral responses**

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## **Abstract**

Due to physiological and anatomical similarities to other vertebrates, zebrafish are becoming a widely used model in neurobehavioral research. With the growing popularity of zebrafish as experimental subjects, it is important to develop tools that accurately record their behavioral phenotypes. Video-aided analysis of zebrafish behavior offers an increased spectrum of behavioral endpoints, some of which are not available using traditional (manual) observation. In addition, the use of computer software allows quantification of certain zebrafish behaviors that are otherwise highly subjective. This protocol describes method for fast, accurate and consistent video-aided measurements of zebrafish locomotion and anxiety-related behavior.

## **Introduction**

Zebrafish have long been utilized as an experimental animal model for biomedical research, particularly in developmental and genetic studies [12] and drug discovery [7]. Several characteristics contribute to the utility of zebrafish models. Being a constantly active animal that readily acclimates to new environments, zebrafish make an excellent species choice for behavioral experiments [11]. Additionally, zebrafish have a low maintenance cost, a robust reproductive cycle, rapid development and a large number of offspring [6].

Zebrafish are also becoming commonly used in neuroscience research [9]. Until recently, the recording of zebrafish behavior was performed manually, making it vulnerable to human error. Human errors and inter/intra-rater variability can lead to incorrect data interpretation, thereby reducing the validity of an experiment. Computerized video-tracking tools that record zebrafish movements provide standardized observation of behavioral endpoints and reduce human errors. Eliminating the discrepancies caused by manual observation of zebrafish behavior allows for a more regulated standard of data recording and promotes experimental soundness and reproducibility. Another benefit of using the video-tracking program is that instead of relying on a single chance to manually observe every behavioral endpoint, videos can be stored, replayed and reanalyzed.

The setup of the video-tracking system is essential for recording the zebrafish movements. For example, variations in lighting may hinder the program's ability to detect and analyze the fish. It is, therefore, important to determine an appropriate background for video-tracking with adequate light and that these conditions are standardized for all subjects. It should be noted that the video-tracking system is less reliable in analyzing erratic movements (Table 1). A methodological problem can also arise if the video-tracking system fails to record the fish for an unknown reason (such as multiple shadows or spastic water movement, which interfere with the programs' ability to recognize the fish),

as this may skew the results. The present protocol outlines the video-tracking approach to analysis of zebrafish behavioral phenotypes.

**Materials:** Adult zebrafish (purchased from a commercial distributor) must be experimentally naïve, and given at least ten days to adapt to the laboratory environment (e.g., the room and water temperature maintained at 25-27°C with illumination provided by ceiling-mounted fluorescent light tubes). The video-tracking programs used here to record zebrafish movements are TopScan (TopView Animal Behavior Analyzing System) from CleverSys Inc. (Reston, VA) and Ethovision® XT from Noldus Information Technology (Netherlands). However, other video-tracking programs may also be used in zebrafish neurobehavioral research.

**Experimental setup:** After pre-treatment, zebrafish are placed individually in a 1.5-L trapezoidal tank (e.g., 15.2 height×27.9 top×22.5 bottom×7.1 width cm; Aquatic Habitats, Apopka, FL) maximally filled with aquarium treated water. Novel tanks rest on a level, stable surface and are divided into two equal virtual horizontal portions, marked by a dividing line on the outside walls. Once zebrafish are relocated to novel tanks, swimming behavior is recorded by two trained observers (inter-rater reliability >0.85) and by video-tracking system over a 6-min period (Fig. 1).

**Behavioral Endpoints:** During the novel tank diving test, video-tracking programs can analyze the number of times the zebrafish entered the top of the novel tank, duration in the top/bottom, duration proportion in top/bottom, distance (m) traveled in top/bottom, latency to enter the top (s), velocity (m/s), total distance traveled and information about erratic movements and freezing bouts (frequency, duration). Detailed definition of each endpoint can be found in Table 1. Once all the data have been collected and analyzed, comparison of the control and experimental groups can be performed (if necessary, they may also be paralleled with physiological (e.g., endocrine) analyses; see chapter on cortisol assay in this book).

**Time Requirement:** The time required for the protocol varies depending on the number of animals per group and the number of experimental groups, and is based on zebrafish locomotor activity levels. In general, zebrafish behavior assessment will last 6 min per animal. Depending on the amount of behavioral data collected, analysis may take between 2 and 4 days.

**Data Analysis:** To analyze the data, researchers may use the Mann-Whitney U-test for comparing two groups (parametric Student's *t*-test may be used if data are more normally distributed) or an analysis of variance (ANOVA) for multiple groups, followed by a post hoc test. More complex designs, such as a one-way ANOVA with repeated measures (time) or *n*-way ANOVA (additional factors: time, treatment, genotype, stress, sex, etc.), can also be used in zebrafish behavioral studies.

**General procedure:** Zebrafish are transported individually from their home tank to the novel tank with careful handling to reduce net-stress. Recording starts and continues for a period of 6 min. Following the testing period, the animals are removed from the novel tank and can be reintroduced to their original tank for further experimentation or dissected for tissue harvesting and collection of biological data. Each zebrafish is given a subject number and the recorded video file name is changed accordingly, to correlate to that zebrafish number (Note: The recorded video file must be in MPEG format for video-tracking software to analyze it). The recorded videos are then imported into TopScan or EthoVision to be analyzed.

**TopScan:** TopScan is able to provide several important endpoints unavailable to human observation, such as total distance traveled, distance traveled in the top/bottom portion, velocity, and a traceable path of the subject's swimming pattern.

- The first protocol step required for TopScan video analysis is to open the selected video for analysis.
- Next, a background frame must be set, a required prerequisite for video analysis, which can be achieved by finding the "Background" tab and clicking on "Set this frame as background".

- The protocol then requires the setting of the arenas/parameters. Go to “Design” to set the top and bottom arenas by using the “polygon” tool under the “Arena Design Tools”.
- After setting the arenas, the protocol demands activation of the arenas. The investigator goes to the “Area” section of the “Event” and clicks on the top and bottom arenas (one at a time) to activate it.
- The final step in the protocol requires the “Analyze” box to be checked for the analysis. The “Analyze” function calculates every movement that the zebrafish made.
- After the analysis is performed, all data are exported to Microsoft Excel, to be compared and statistically evaluated. More detailed information about TopScan can be obtained from <http://www.cleversysinc.com>.

**EthoVision® XT:** EthoVision® XT provides tracking and analysis of parameters such as path and distance traveled, velocity, meandering, and angular velocity (Table 1). This is an established user-friendly program that facilitates observation and analysis of behavioral endpoints while minimizing human error. The protocol for this program is as follows.

- Open EthoVision and click “New Experiment.” The experiment should be named with an appropriate description.
- Desired detection features and units should be selected. Video files are moved into the “Media Files” sub-folder in a newly-created experimental folder. A “Trial List” is created with the following variables: fish group, group ID, and fish ID. Next, the arena settings are adjusted.
- To set the background, capture the image prior to introduction of the fish into the novel tank. Then use a square or rectangle tool to define the entire novel tank as Arena 1. Divide this Arena at the midline into defined Top and Bottom Zones.
- Calibration and validation of arena settings are then performed. Detection settings should be adjusted ensuring that the subject is darker than the background image.

- Save the settings and check the box for “Track Smoothing”. Next, press “Play”.
- Videos are then analyzed. When analyses are complete, enter independent variables (Group, GroupID, FishID) for trial and collected data.
- To export data into Excel for statistical analysis and further comparison, go to the “Export” menu and select “Analysis Data.” The Settings screen will appear. Type the name of the appropriate destination folder in the field for “File name prefix”. Under “File Type” select Excel and click “Start Export”. More information on EthoVision® XT can be obtained from [www.noldus.com](http://www.noldus.com).

### **Anticipated/typical results**

**Observation comparability:** We anticipate the comparisons of data produced by the video-tracking system with those produced by manual observation to show a significant correlation between the two approaches. Indeed, our own recent data (Fig. 2) demonstrate high (>80-90%) correlation between the two methods [5] for most of the major parameters assessed, confirming that the video-tracking system is a reliable tool for zebrafish neurobehavioral research.

**The novel tank diving test:** The novel tank diving test exploits the stress response and allows comparison of anxiety induced behavior in experimental versus control groups. Figure 1 illustrates how this test is employed in an experimental design. When the zebrafish is exposed to a novel environment, it initially dives to the bottom, and then gradually explores the top. Inhibited exploratory movement, reduced speed, and increased frequency of escape-like erratic behaviors are associated with higher levels of anxiety elicited by different stressors [3, 8, 10] (Table 1). These behaviors are highly sensitive to pharmacological treatment, as zebrafish exploration is increased after treatment with anxiolytic drug, including benzodiazepines, SSRIs, nicotine, and ethanol [1, 3, 4, 8]. Conversely, stressful stimuli (e.g., predator exposure or alarm pheromone, anxiogenic drugs, and drug withdrawal) have been shown to increase anxiety-like behavior in this paradigm, leading to longer latency to

explore the upper half of the novel tank, less time in the top, more erratic movements, and longer/more frequent freeze bouts [2, 3, 10]. Figure 3 illustrates typical results observed in the novel tank diving test after exposure to anxiogenic acute caffeine. This simple yet high-throughput test can be used as an approach in quickly and accurately identifying the biomarkers linked to a disorder and in screening the efficacy of different pharmacological treatments.

**Strain differences:** Using the video-tracking approach, we found robust observable strain differences in the novel tank diving test. As can be seen in Fig. 4, the wild-type zebrafish exhibited greater exploratory behavior (compared to the leopard mutant strain), suggesting higher baseline anxiety levels in the mutants than the wild-type. Due to the behavioral differences amongst zebrafish strains, strain selection must be taken into consideration when choosing and comparing zebrafish for experimentation.

**Troubleshooting** (the following generally applies to all video-tracking software programs)

**1. Software not detecting fish:** The problem with detection of the object (fish) by software can be resolved by altering one or several settings: detection setting, lighting, and background. For example, if the software detects the glare or another object as the experimental object, changing the contrast between the object and the background in Detection Setting can offer a solution. If the problem still persists, consider using another detection method available (e.g., Differencing, Dynamic subtraction, Gray scaling, or Static subtraction). If different detection methods have been employed, but the software still cannot detect the fish, this may be a video-related problem. Adequate lighting is necessary. If the video is too dim or too bright, the lighting of the setup must be adjusted prior to recording. Too dim or too bright lighting will make it harder for the system to differentiate the subject from the background, and hence, the subject may remain undetectable during the analysis. Using a solid color as a background will also help decrease the chance of misdetection of the subject.



**2. Software losing fish in the middle of the video:** The software may detect something else as the fish in the middle of the video. For example, this is usually caused by a glare in the video. A simple adjustment in detection setting, such as contrast, will likely resolve this problem.

**3. Behavioral endpoints collected do not reflect actual behavior:** Make sure that the arenas and the zones were properly defined, calibration is accurate, and the endpoint's "rules" were identified clearly. For example, for zone transition, make sure the endpoint is set from "bottom" to "top" instead of "top" to "bottom", if the endpoint is measuring how many times the subject enters the top.

**4. An error occurred during the acquisition of a trial:** During acquisition, unexpected errors may occur. For example, the software may lose detection of the fish, or the experimenter may use a wrong video for the trial. Fortunately, reanalysis of the video is possible. However, if the video was recorded with another program, a new trial must be added. Simply delete the error/unwanted trial, and add a new one to start over.

**5. The video program is running very slow:** Typically, a hardware problem. Make sure that the computer meets the minimum program requirements. Also, turn off tracing option during playback/analysis of data.

**6. Unable to define zones:** Zone definition (i.e., top half vs. bottom half, or center zone vs. border zone) may be undefined or defined incorrectly according to the video-tracking program. The solution for this problem is to investigate the arena setting and calibrations (found under the "Setup" tab) to see if they are correct or need to be readjusted.

**7. Video-tracking program unable to play video:** Video-tracking programs analyze videos under specific formats. The file must be converted into the specific required (e.g., MPEG) file type.

**8. Calculation of "Distance to zone" in the analysis yields either 0.00, or no results at all:** The calculation of "distance to zone" is found under the analysis tab. Calculations that yield 0.00 are

specifically due to miss calibration of the zones. To fix this problem, simply recalibrate the zones and also check that the arena itself is not selected, instead of a specified zone.

**9. Role of memory and conditioned responses:** Zebrafish demonstrate good learning and memory phenotypes. For example, 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 [11]. 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 [11]. Likewise, fish can recall training for up to a 10 day period [11]. Collectively, this implies a good memory capability for this animal. Therefore, re-testing zebrafish in the novel tank should be avoided. If this is not possible, a longer interval (e.g. >3 weeks) between trials must be allowed in order to minimize potentially confounding data.

**10. Zebrafish display abnormally high or low levels of locomotion:** It may be a strain-specific phenomenon, and the researchers may need to re-assess the strain's suitability for the experiment. Ameliorating the environmental and testing conditions would also aid in normalizing zebrafish behaviors. This includes proper handling, the use of fewer and/or less stressful tests, and improving husbandry. If locomotor activity remains too low, extending the test for 6-12 more minutes may be a good practical solution, as it minimizes the initial anxiety and disinhibits zebrafish behavioral activity.

**11. Zebrafish display aberrant behavioral phenotypes:** Factors such as altered pain sensitivity, vestibular deficits or motor/coordination impairments may non-specifically alter animal behavior in a way that could be misinterpreted as altered anxiety phenotype. In addition, peculiar "rare" forms of zebrafish behavior may be present, and need a more careful in depth behavioral assessment. For example, zebrafish treated with hallucinogenic or opioid drugs may exhibit trance-like

passive swimming or epilepsy-like states that will confound analysis by video-tracking software. To address this possibility and rule out all non-specific factors, a careful examination of zebrafish neurological and sensory phenotypes is recommended.

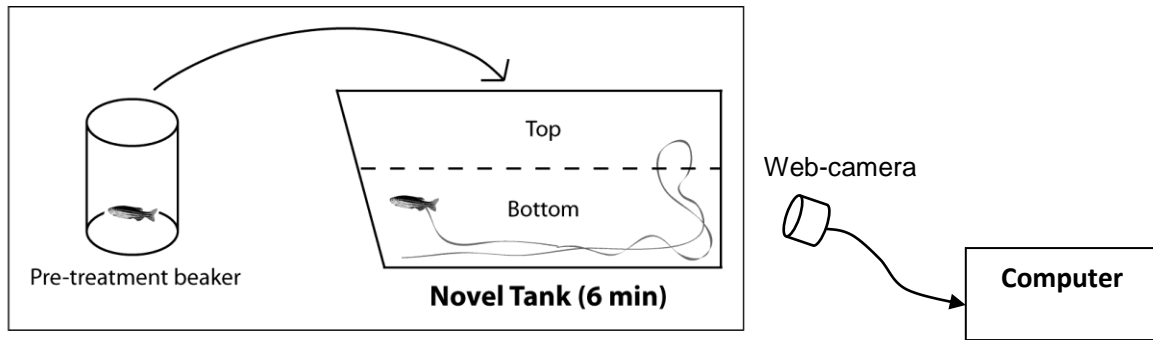
### **Conclusion**

Overall, video-tracking of zebrafish yields objective analysis of behavioral endpoints and therefore provides researchers with an important tool for the investigation of anxiety and stress behavior in this animal model (Fig. 2-4). Representing a significant improvement over more subjective manual recordings (that are prone to human error), the video-tracking approach introduces the capability of observing additional behavioral endpoints not captured in manual recordings (Table 1). This research strategy allows an accurate and standardized measurement of anxiety related behavior in zebrafish for sound data collection and analysis. Furthermore, such standardization promotes reproducibility in experimental design, strengthening the investigator's ability to draw valid conclusions from zebrafish study data and results.

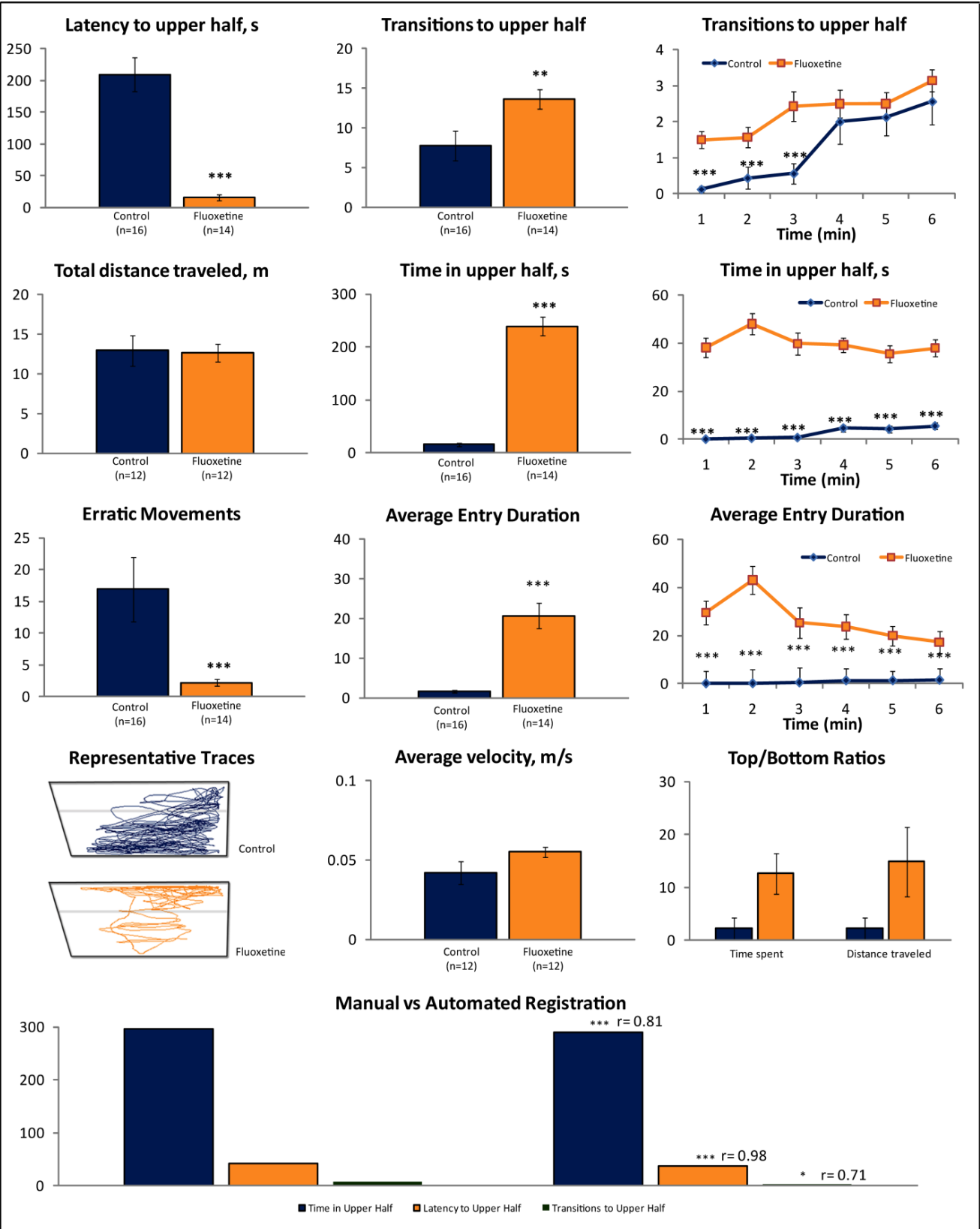
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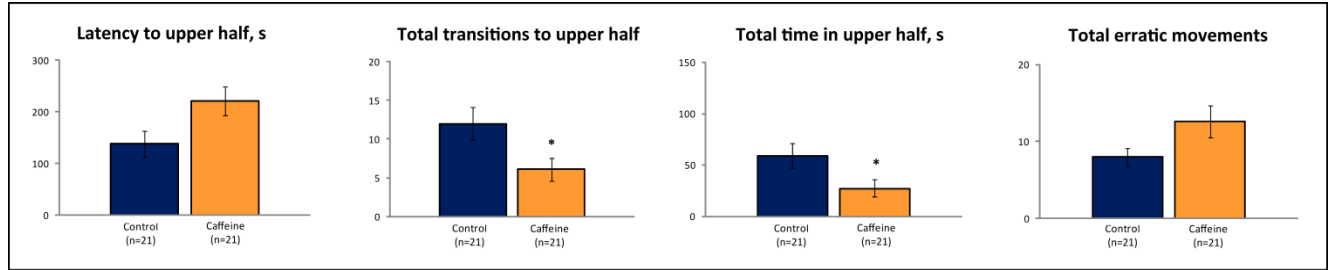
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**Figure 1. Novel tank diving test. Zebrafish are exposed to the experimental challenge in a pre-treatment beaker before being transferred into the novel tank for behavioral observation and phenotyping. Control groups undergo same procedures without challenge in pre-treatment beaker.**

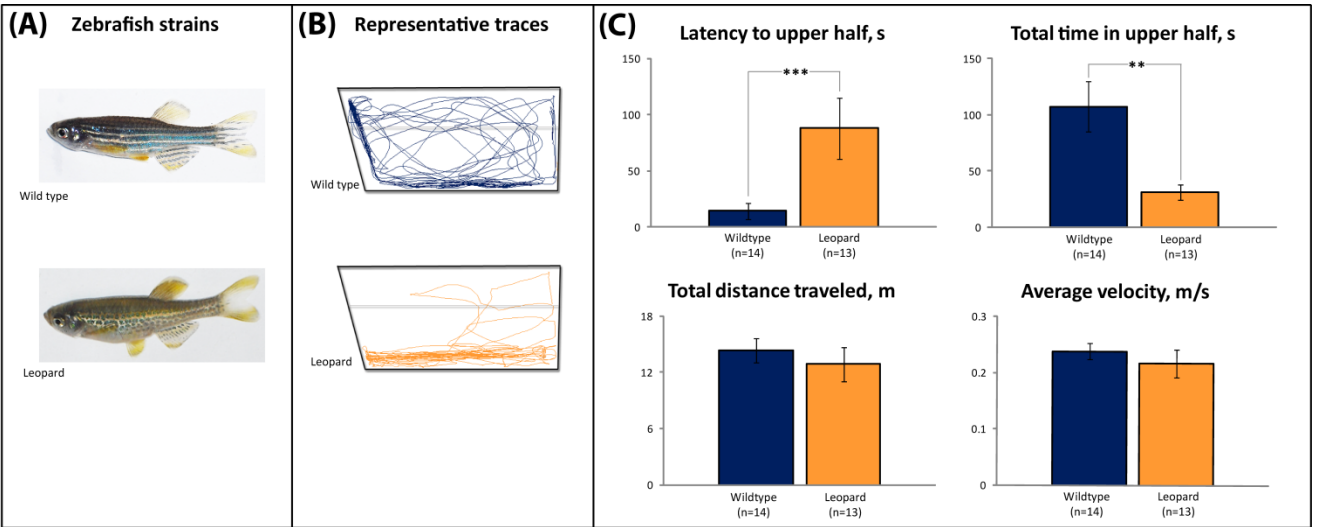


**Figure 2. Anxiolytic effects of chronic fluoxetine (100 µg/L, 2 weeks in the home tanks) on zebrafish behavior in the novel tank diving test, based on manual behavioral phenotyping and automated behavioral characterization with video-tracking software (CleverSys Inc). Data are presented as mean ± SEM, \*p<0.05, \*\*\*p<0.005 vs. control, U-test [5].**



**Figure 3. Anxiogenic effects of acute caffeine (100 mg/L, 15 min pre-exposure time) on zebrafish behavior in the novel tank diving test, based on automated behavioral characterization with video-tracking software (CleverSys Inc). Data are presented as mean  $\pm$  SEM, \* $P < 0.05$  vs. control, U-test [5].**





**Figure 4. – Strain differences in zebrafish novel tank diving test behavior. Two different strains of zebrafish used in this study (A) display specific patterns of their exploratory behavior, as illustrated by representative swimming traces (B) and measured behavioral endpoints (C) which were analyzed using video-tracking software (CleverSys Inc),  $**P < 0.01$ ,  $***P < 0.005$ , U-test [5].**

**Table 1: Summary of behavioral endpoints and their significance measured in the novel tank diving test (a - automatic observaion, m - manual observation, c –calculations based on manually or automatically recorded data)**

<b>Endpoint (units)</b>	<b>Registration</b>	<b>Definition</b>	<b>Interpretation</b>
Latency to enter the top (s)	m,a	The amount of time to first cross (by the center of mass of the body) from the defined bottom portion to the top of the novel tank	When introduced to a novel environment, zebrafish naturally dive to the bottom of the tank and gradually explore as it habituates to the test apparatus. The longer latency indicates higher anxiety levels
Time spent in top (s)	m,a	Total time spent in the top portion of the novel tank	A longer duration in the top of the tank indicates lower anxiety levels
Time spent top:bottom ratio	c	The ratio of the time spent on top over bottom	Lower ratio indicates higher anxiety level
Number of entries to the top	m, a	The number of crosses from the defined bottom portion to the top of the novel tank	More top entries indicate lower anxiety levels
Entries top:bottom ratio	c	The ratio of the number of entries to the top over bottom	Lower ratio indicates higher anxiety level
Average entry duration (s)	c	The amount of time spent at the top of the novel tank during each crossing	Calculated as time spent in the top divided by the number of entries to the top. Shorter average entry duration indicates higher anxiety level
Distance travelled in the top (m)	a	Total distance traveled in the defined top portion	Zebrafish with high anxiety would travel more distance in the bottom of the tank
Distance traveled top:bottom (m)	c	A ratio of the total distance traveled in the defined top portion versus the defined bottom	A lower top:bottom ratio indicates a higher stressed fish.
Total distance traveled (m)	a	Total distance the zebrafish traveled within the novel tank	Reflects general motor/neurological phenotypes. Zebrafish are generally quite sensitive to non-specific motor impairments and sedative drug effects (see troubleshooting section)
The number of erratic movements	m,a	Sharp or sudden changes in direction of movement or repeated darting behavior	Indicates increased fear/anxiety, and are generally higher in stressed zebrafish
Average velocity (m/s)	a	Magnitude and direction of zebrafish speed	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test

Freezing bouts (frequency)	m,a	Total immobility(>1s), except for the eyes and gills	Indicate increased anxiety and are generally higher in stressed zebrafish
Freezing duration (s)	m,a	Total duration of all freezing bouts	Indicates increased anxiety and is generally higher in stressed zebrafish
Meandering (°/m)	a	The degree of turning (vs. straight locomotion)	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Turning angle (°)	a	Total turning angle	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Angular velocity (°/s)	a	Magnitude and direction of zebrafish angular speed	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test