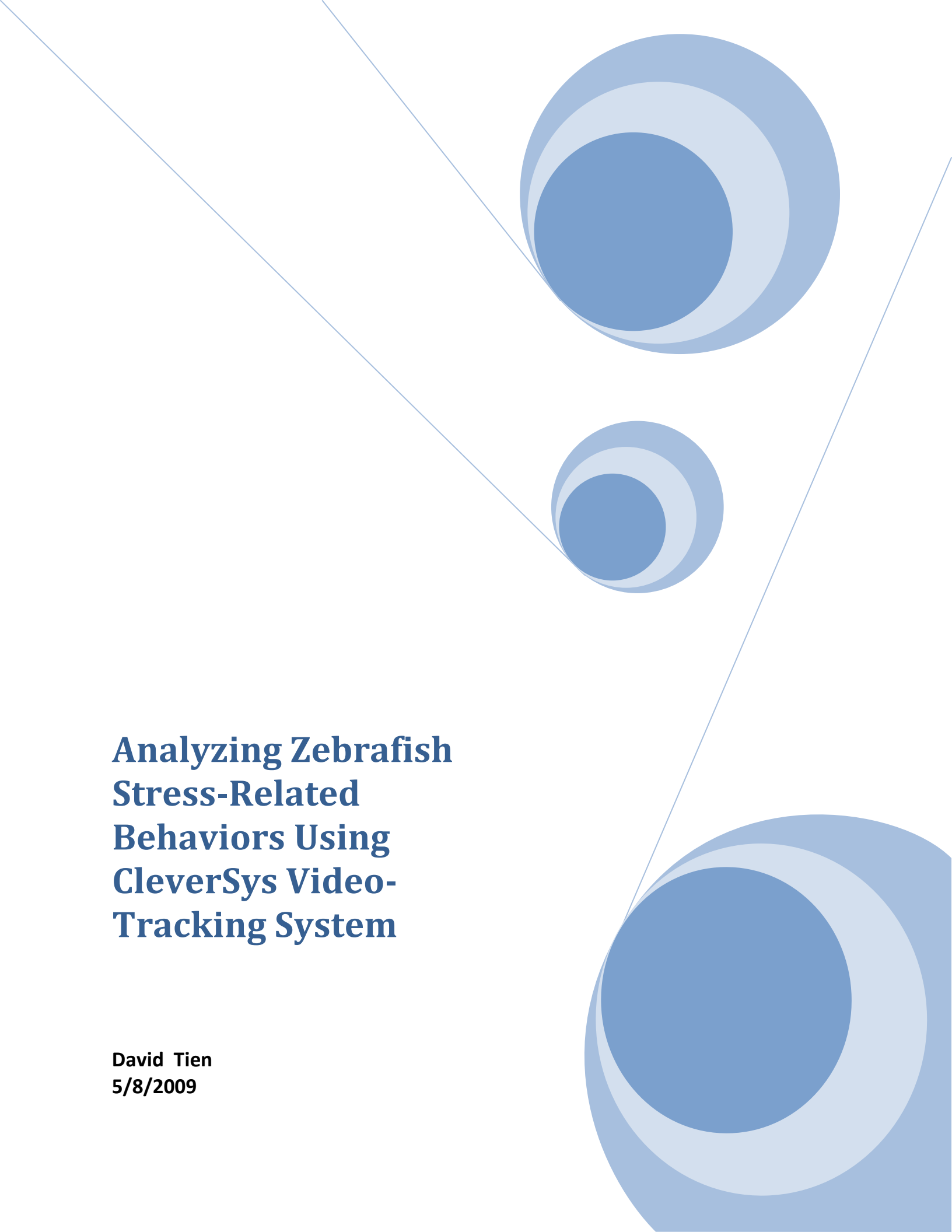

Analyzing Zebrafish Stress-Related Behaviors Using CleverSys Video-Tracking System

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Introduction

Zebrafish are becoming a commonly used experimental organism in biomedical research because its physiology and morphology are homologous to humans. These similarities allow researchers to explore the pathways and mechanisms of the Zebrafish, and see how it can be related to humans' mechanisms and treatments (Shin and Fishman, 2002). Moreover, all of the "classical" vertebrate neurotransmitters can be found in the zebrafish (Mueller et al., 2004). Because of their physiological similarities, researchers have been using zebrafish to study a wide range of research domains, including investigation of social behavior (Pyron 2003; Engeszer, Ryan et al. 2004; Bass and Gerlai 2008), olfactory-related behaviors (Mann, Turnell et al. 2003; Vitebsky, Reyes et al. 2005), anxiety (Barcellos 2007), addiction (Darland and Dowling 2001; Ninkovic and Bally-Cuif 2006; Kily, Cowe et al. 2008), sleep (Cirelli and Tononi 2000), and learning and memory (Williams, White et al. 2002; Colwill, Raymond et al. 2005). Zebrafish corticosteroid stress axis also exhibits strong physiological responses to stress (Alsop and Vijayan, 2008). In this experiment, we will explore the effects of chronic ethanol exposure, withdrawal ethanol exposure, and chronic selective serotonin reuptake inhibitor (SSRI) fluoxetine on the stress and anxiety level of zebrafish, assessed using a new CleverSys video-tracking system.

Like other species, exposure to a novelty environment will trigger robust anxiety responses in the zebrafish (Blaser and Gerlai, 2006). When the zebrafish is placed in a novel tank, it will seek protection by diving to the bottom of the tank and stay there until it feels comfortable and safe enough to explore (Levin et al., 2007). By using the novel tank diving test, we will be able to collect and compare different behavioral parameters from the zebrafish, such as latency to enter the upper-half, transitions into the upper-half of the tank, erratic movement, and freezing bouts to measure anxiety level.

Methods

Animals and Housing

Adult Zebrafish (>90 days; 50/50 male, female) were retrieved from a local commercial distributer (50 Fathoms, Metairie, LA). All the fish were housed in approximately 40-L tanks in groups of 40-50 fish per tank. All of the fish were naïve, and were given at least ten days to adapt to the laboratory environment. The tanks were filled with de-ionized water treated with Prime Freshwater and Saltwater Concentrated Conditioner as recommended by the manufacturer (Seachem Laboratories, Inc., Madison, GA). The room and water temperature were maintained at 25-27°C with illumination provided by fluorescent light tubes from the ceiling. The lights were turned on from 9AM to 7PM every day. All fish were fed with a mixture of ground flake food (Tetramin Tropical Flakes; Tetra USA, Blacksburg, VA).

Video-tracking Protocol

To prevent human errors and variability in recording of the zebrafish movements, we used CleverSys Inc. (Reston, VA) *TopScan* (*TopView Animal Behavior Analyzing System*) to record the zebrafish movements. The zebrafish were placed individually in a 1.5-L trapezoidal tank (approximately 15.2cm H x 27.9 TW) maximally filled with aquarium treated tap water. The tank was divided into two equal horizontal halves, marked by a sharpie on the outside of the tank (Fig. 1.A). The tank was placed on a stable, level surface, and all environmental distractions were kept to a minimum. A Gigaware 2.0-Megapixel Webcam with Auto-Focus was placed 22.86 cm (9 inches) in front of the novel tank to ensure that the novel tank is within the webcam vision range. A standard letter size yellow (21.59 cm x 27.94 cm; 8½ in x 11 in) sheet of paper is placed 5.08 cm (2 inches) behind the novel tank to ensure a uniform background for the video analysis. Two 75-watts light bulbs were placed behind the yellow screen to boost the

contrast of the background and the zebrafish. One 65-watts light bulb was placed behind the webcam, shining on the novel tank; and one 65-watts overhead light was placed over the novel tank (Fig. 1.B). A laptop was hooked up to the webcam, and the videos were recorded via the program *ArcSoft WebCam Companion 3*.

Zebrafish were transported from their home tank to the novel tank with careful handling to reduce the net-stress. Once placed in the novel tank, the recording was started, and the zebrafish movement was recorded over a period of 6 min. After six minutes, the zebrafish was removed from the novel tank and dissected into two parts: the brain and the whole-body.

The zebrafish was given a number and the recorded video file name was changed to correlate to that zebrafish number. The recorded videos were then imported into *TopScan* to be analyzed. In general, *TopScan* is able to provide several endpoints that human observation cannot. For example, total distance traveled, velocity, distance travel in the top/bottom portion, and a traceable map of the path that the zebrafish took (Fig. 1.D). The protocol first required the opening the video for analysis. The protocol next instructed a background frame to be set, which can be achieved by going under "Background" and click on "Set this frame as background". The protocol then required the setting of the arenas/parameters: go to "Design" to set to top and bottom arenas by using the "polygon" tool under the "Arena Design Tools". After setting the arenas, the protocol demands the activation of the arenas. To activate, the investigator has to go to the "Area" section of the "Event" and click 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" box tells the program to interpret every movement that the zebrafish made. After 6 minutes, the analysis was done, and the data were exported to Microsoft Excel. In Microsoft Excel, the data provided the information about the amount of time the zebrafish enter the top, the duration it spent in the top/bottom, duration percentage in

top/bottom, distance (in mm) traveled in top/bottom, latency to reach the top (in sec), and the velocity (mm/sec). Further analyzing of the data gave the ratio of duration spent on top verse the duration spent on the bottom portion and the total distance traveled. The program can also provide information about erratic movement and freezing bouts. Erratic movements were defined as sharp or sudden changes in direction of movement or repeated darting behavior. Freezing bouts were defined as absolutely no movement, except for the movement of the eyes and gills. A significant decrease in exploration was marked by longer latency to reach the top, longer freezing bouts, fewer top entries, or elevated erratic movement, which represent stress responses indicative of high-anxiety phenotypes.

Drugs

For chronic ethanol exposure experiments, the zebrafish home tank water was treated with 0.3% ethanol for one week. Tank water was replaced every two days and 0.3% ethanol was added to the tank before the zebrafish were introduced back into the home tank. For the withdrawal experiment, the zebrafish were place through the same treatment as the chronic zebrafish with the exception that the withdrawal zebrafish were exposed to the ethanol one week prior to the discontinuation of treatment. To induce the withdrawal symptoms, the zebrafish were transported to an identical tank without ethanol treatment for 12 hours before the novel tank diving test was performed.

A selective serotonin reuptake inhibitor (SSRI) fluoxetine (5 µg/L) was administered daily for two to four weeks to investigate the anxiolytic effects of chronic antidepressant (Brooks, Smith et al. 2003). In each experiment, the zebrafish were individually tested in the novel tank diving test, euthanized in 5% Tricane, and immediate dissected and frozen at -80°C for further physiological assessments later.

Results

As detected by the CleverSys *TopScan*, chronically exposure ethanol zebrafish spent strikingly more time in the upper portion of the novel tank per entry compared to the controls (Fig. 2). They also spent more time in the upper half of the novel tank throughout the trails. On the other hand, the ethanol withdrawn zebrafish demonstrated significantly fewer transitions to the upper half, increased frequency of freezing bouts, longer time spent freezing, and less time in the upper half (Fig. 3). Overall, it is consistent with the anxiolysis elicited by the chronic fluoxetine treatment.

On average, the chronic ethanol zebrafish enter the top 15 times, and spent 111.38 seconds in the upper half portion of the novel tank, have a latency of 51.76 seconds, and traveled a total distance of 13402.13 mm (Fig. 2.F) with a velocity of 27.88 mm/sec (Fig. 2.D).

For the ethanol withdrawn zebrafish, they rarely entered the upper half portion (Fig. 3.C), did not exhibit much erratic movements (an average of 0.37) (Fig. 3.I, Fig. 3.J), and freeze significantly more compare to the control (Fig. 3.K, Fig. 3.L). The ethanol withdrawn fish also freeze for a long period of time (Fig. 3.M), on an average of 36.78 seconds each trail (Fig. 3.N). The longest one froze for 55.17 seconds, while the shortest one froze for 6.17 seconds within a six minutes trail.

The chronic administration of the SSRI fluoxetine presents numerous anxiolytic effects on the zebrafish behavior (Fig. 4). Compared to the control, the experimental zebrafish spent considerably shorter latency to reach the top portion of the novel tank (Fig. 4.A). Once in the top, they spent a significantly longer amount of time up in the top portion overall and per minutes compared to the control (Fig. 4). Furthermore, the experimental zebrafish transition to the upper portion more frequently overall, especially during minute one and three (Fig. 4). At this time, the zebrafish have become familiar to the environment and started to explore the

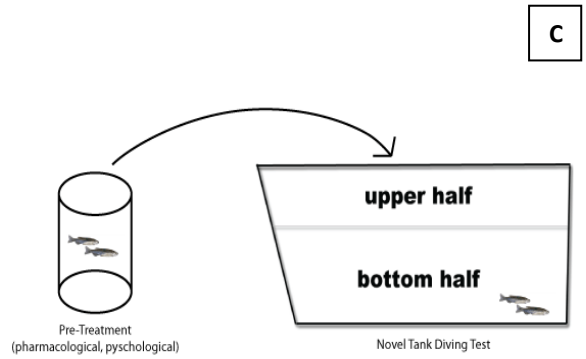
upper portion of the novel tank. The experimental zebrafish physiologically exhibited a significantly lower level of whole-body cortisol compared to the control group. This indicate that the SSRI treatment effectively reduced the stress-level of the zebrafish; thus, lowering the stress hormone cortisol.

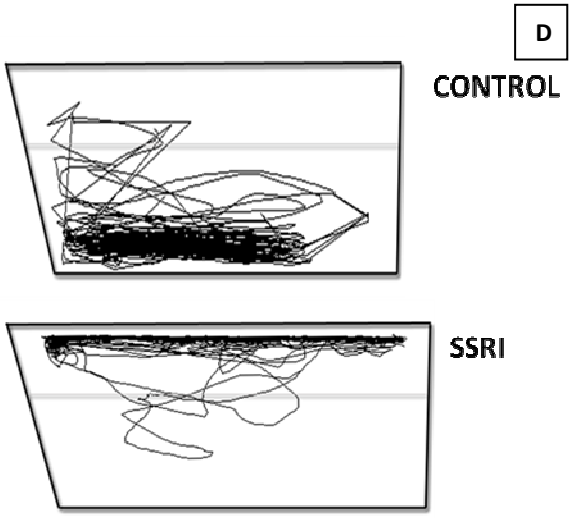
Discussion

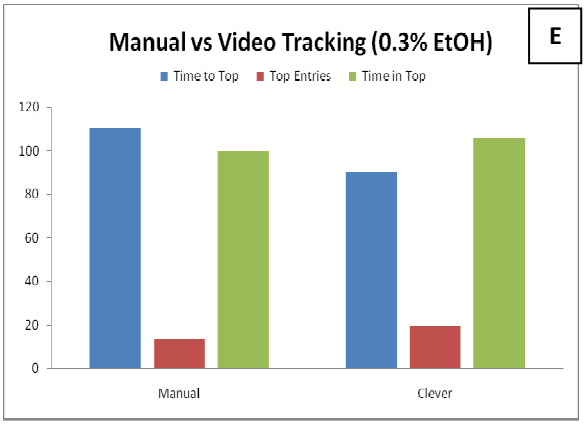
Our data confirms that when a zebrafish is presented to an unfamiliar environment it will evoke moderately simple, but robust anxiety-like behavioral responses, which can be measured by using the novel tank diving test. By using psychoactive drugs, the behavioral and physiological endpoints of the zebrafish can be manipulated because of its high sensitivity. Due to this high sensitivity and manipulation, the novel tank paradigm processes a great potential to be use in the screening of novel compounds of possible therapeutic value. In addition, the classic tests in rodents use to assess anxiety-like behavior (e.g. light/dark and place preference) have recently been adapted to zebrafish (Ninkovic and Bally-Cuif 2006). These classic tests can serve to enhance the novel tank diving test in measuring anxiety-like responses.

In conclusion, the video-tracking system has proven to be a useful and effective way of measuring the zebrafish responses in the novel tank paradigm. The preliminary results obtained using this method shown that the video-tracking system can successfully distinguish between the control and experimental cohorts of zebrafish. Moreover, the data produced by the video-tracking system exhibit a high correlation with the data produced by manual observation and recording. This confirms the comparability of the two methods. By successfully reducing the inter-rater variability, the video-tracking system method produced more precise and consistent results, and ensures higher data validity. The video-tracking system also offers data that cannot otherwise be obtain via human observation, such as velocity in top verse bottom regions of the

novel tank, distance travelled in top/bottom, and mapping of the pathways the zebrafish took throughout the trail. Furthermore, the reduced time required for the interpretation of the video data provides this method with a significantly increase in the throughput of the experimental processes.







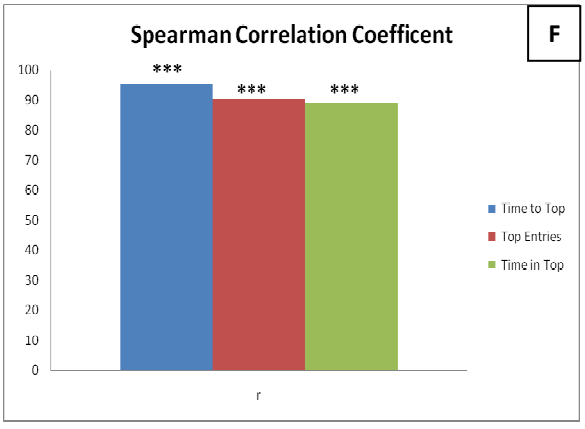
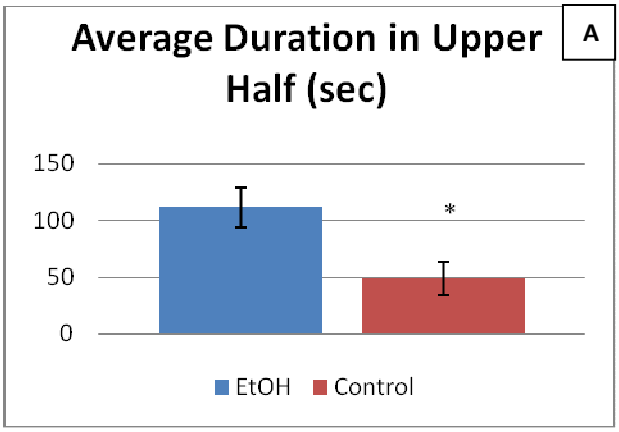
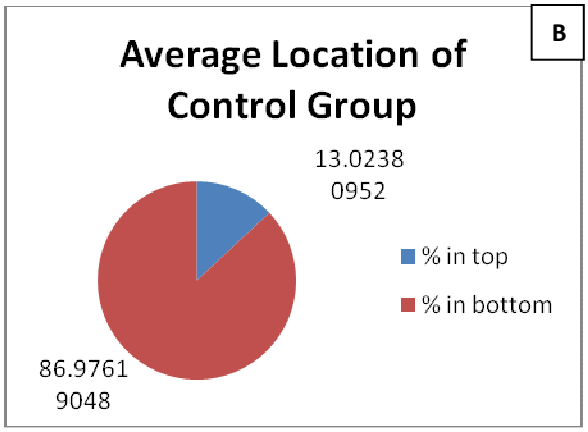
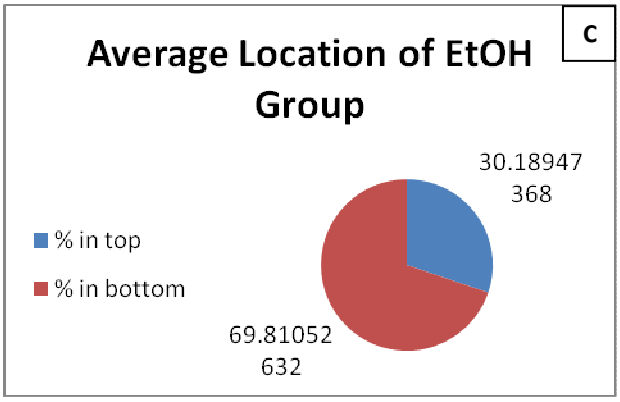
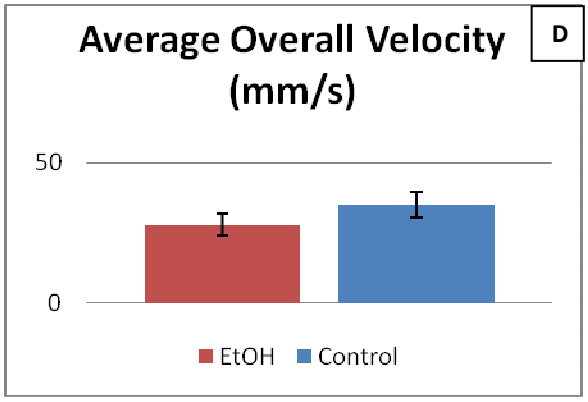


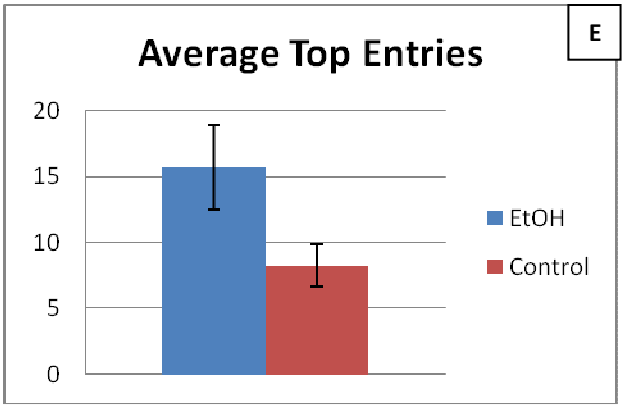
Figure 1: A) Novel Tank (1.5-L); B) Novel Tank Diving Test Setup; C) Upper and Bottom Halves Divisions); D) Mapped Traces for SSRI and Control Groups; E) Manual vs. CleverSys Video Tracking System; F) Correlation % between manual and video tracking recording on panel E.

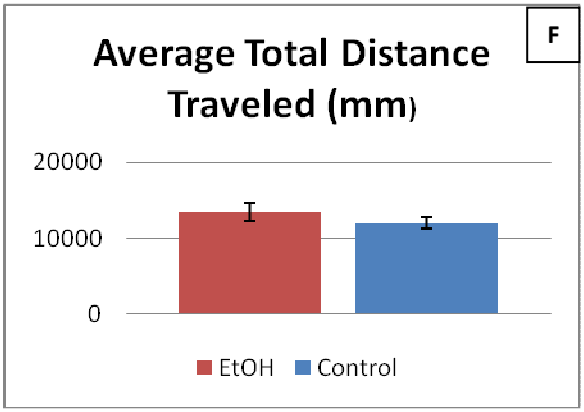












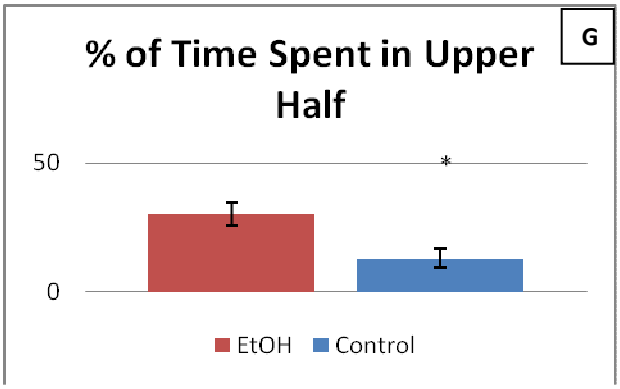
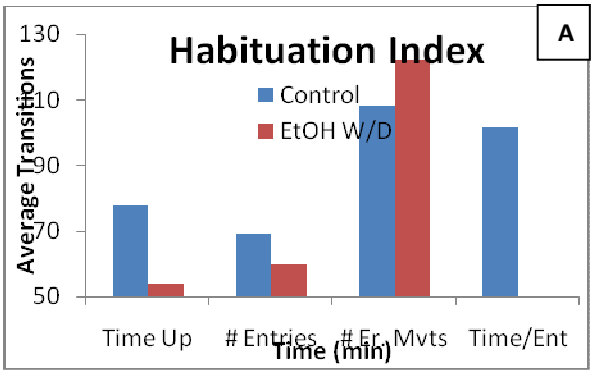
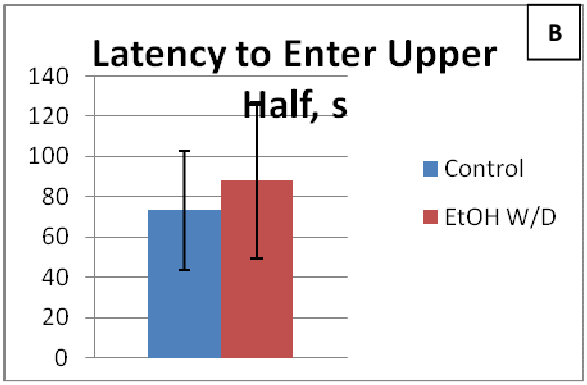
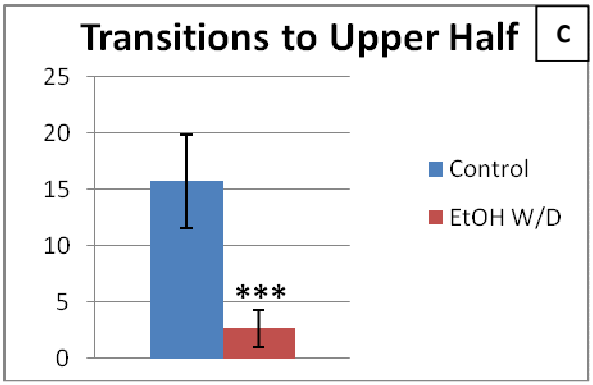
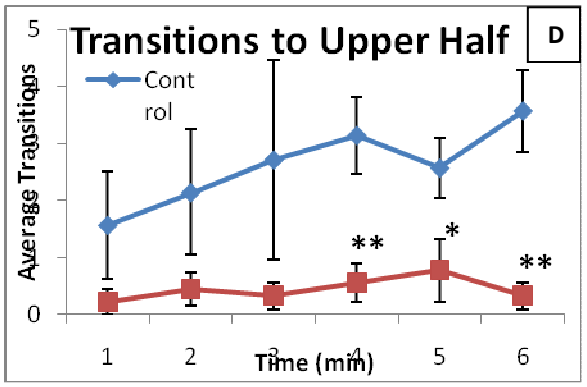


Figure 2 – Chronic Ethanol Exposure Zebrafish: A) the average duration time spent in the upper half of the novel tank (P = 0.021); B) the average location of the control zebrafish; C) the average location of the chronic ethanol zebrafish; D) the overall velocity (P = 0.13); E) the average transition to the upper half (P = 0.088); F) the average total distance travelled (P = 0.43); G) Percent of average time spent in the upper half (P = 0.0325).


A
Habituation Index
Control
EtOH W/D
Average Transitions
130
110
90
70
50
Time Up
Entries
Er. Mvts
Time/Ent
Time (min)


B
Latency to Enter Upper Half, s
140
120
100
80
60
40
20
0
Control
EtOH W/D


C
Transitions to Upper Half
25
20
15
10
5
0
***
Control
EtOH W/D


D
Transitions to Upper Half
Average Transitions
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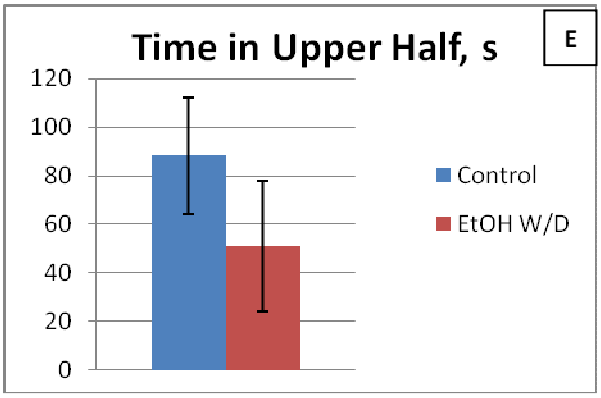
Cont
rol
**
*
**
1
2
3
4
5
6
Time (min)


E
Time in Upper Half, s
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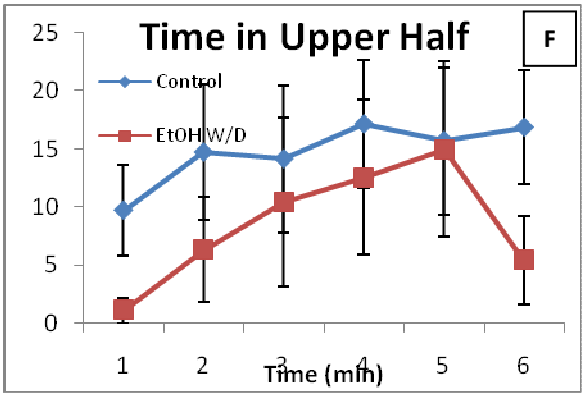
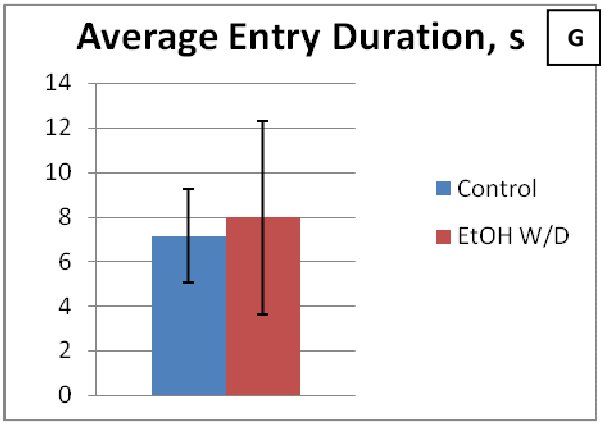
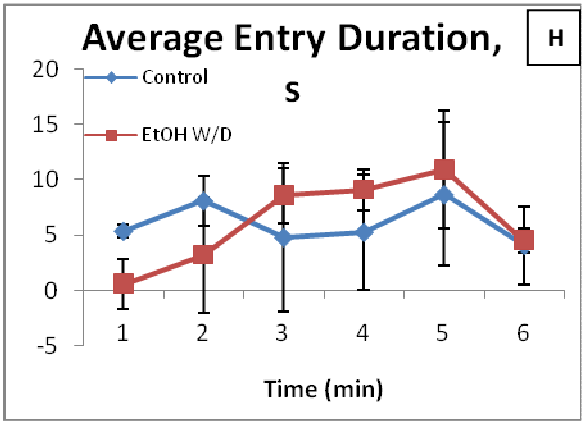
100
80
60
40
20
0
Control
EtOH W/D


F
Time in Upper Half
25
20
15
10
5
0
Control
EtOH W/D
1
2
3
4
5
6
Time (min)


G
Average Entry Duration, s
14
12
10
8
6
4
2
0
Control
EtOH W/D


H
Average Entry Duration, s
20
15
10
5
0
-5
Control
EtOH W/D
1
2
3
4
5
6
Time (min)

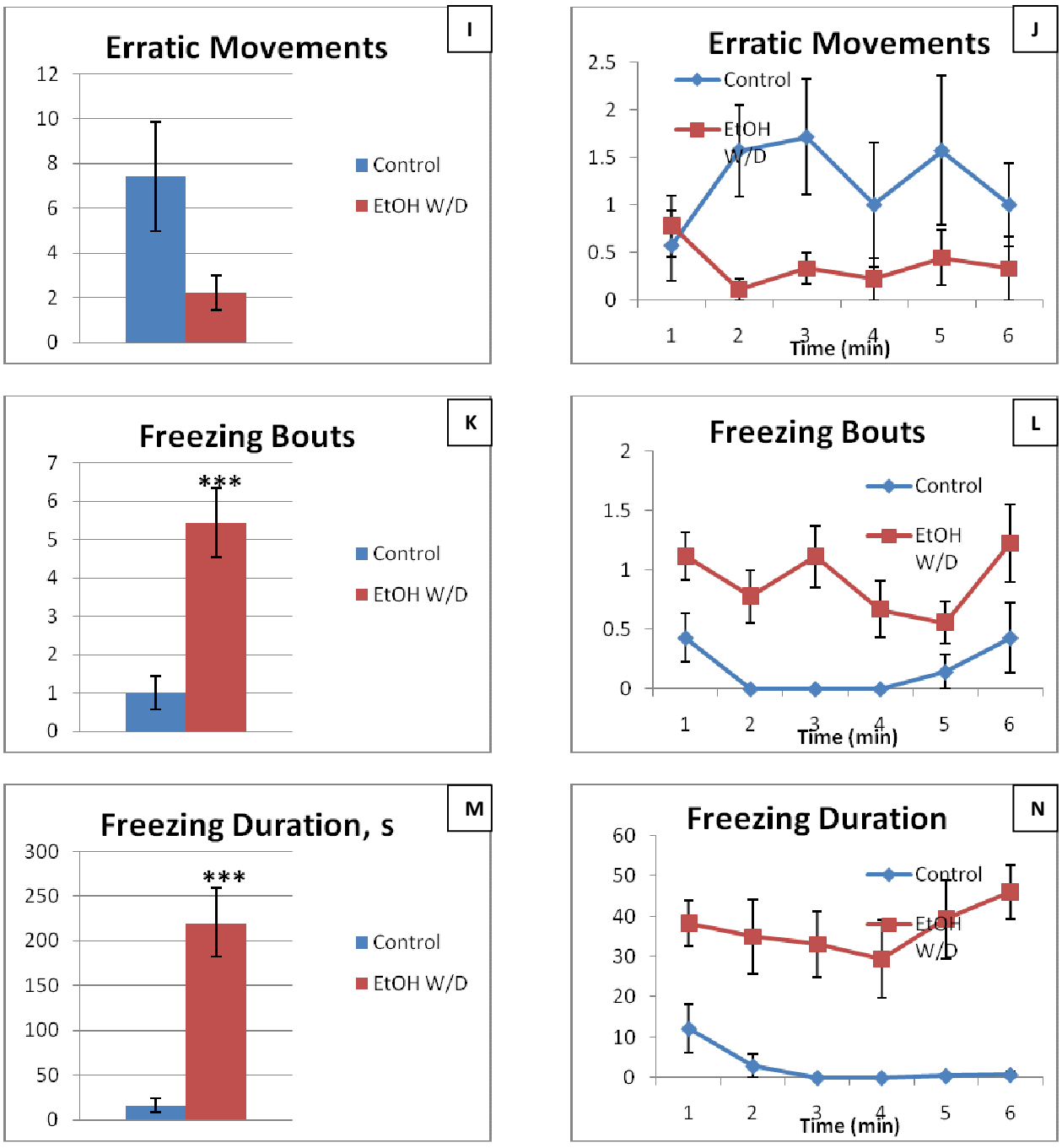


Figure 3 – Acute Ethanol Withdrawal (W/D) Zebrafish: A) average habituation index; B) average latency to enter the upper half; C) average transition to upper half; D) transition to upper half per minute; E) average time in upper half; F) time in upper half per minute; G) average entry duration; H) average entry duration per minute; I) average erratic movements; J) erratic movements per minute; K) average freezing bouts; L) freezing bouts per minutes; M) average freezing duration; N) freezing duration per minute. Mean ± S.E.M shown. P < .05 (*), .005 (**), .0005(***)

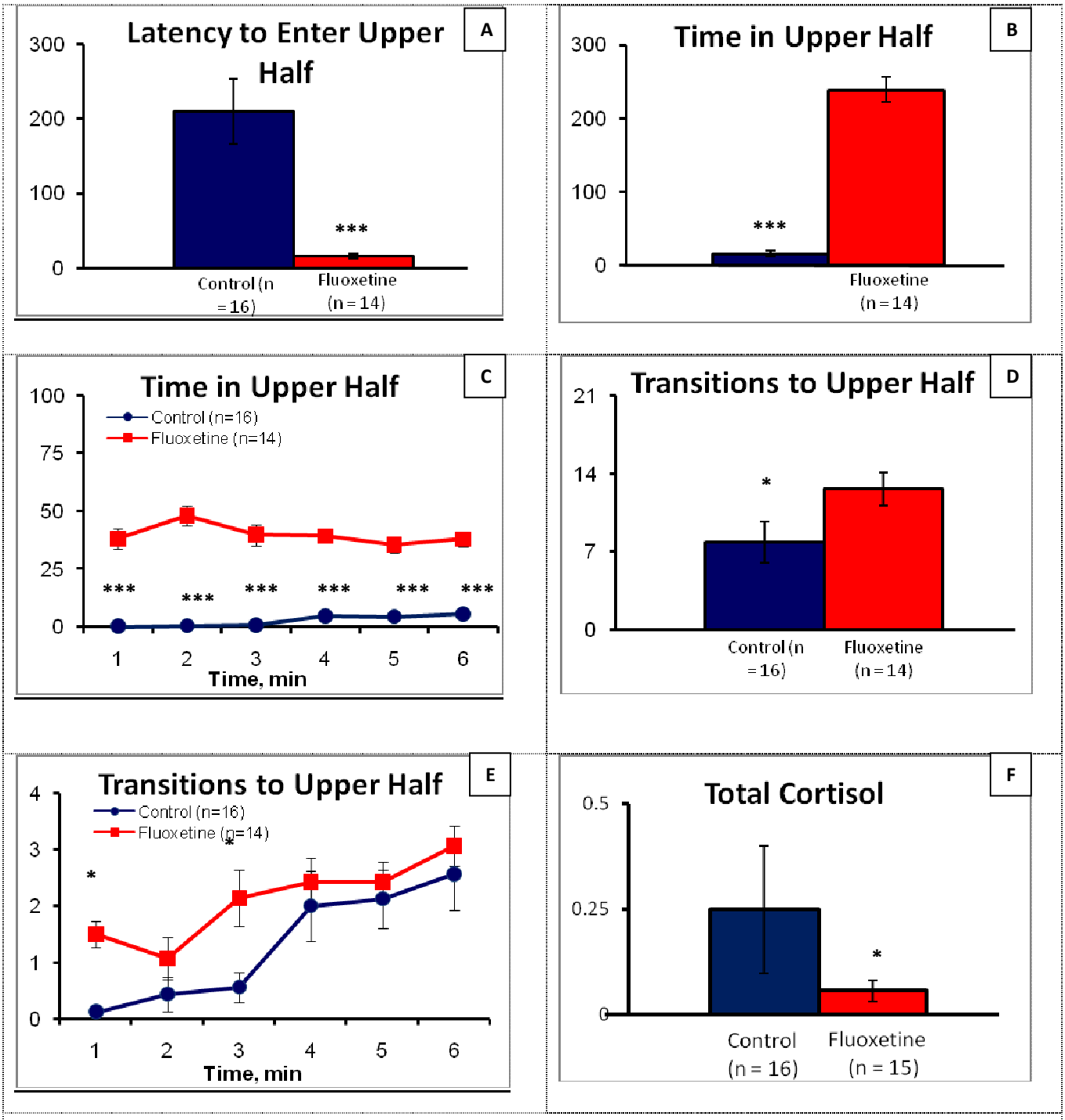


Figure 4 – Anxiolytic effects of Selective Serotonin Reuptake Inhibitor (fluoxetine) exposure on zebrafish behavior: A) average latency to initially enter upper half of tank; B) average overall time spent in upper half; C) average time spent in upper half per minute distribution; D) average overall transitions into upper half; E) average transisitions into upper half per minute distribution; F) average whole-body cortisol concentrations, per gram fish. Mean ± S.E.M shown. *p<0.05, **p<0.01, ***p<0.0005 (u-test).

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