Predator-Induced Stress and Anxiety in Zebrafish

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I. Introduction

Zebrafish have been widely used as a model for genetic, behavioral, and physiological research. Zebrafish mutant phenotypes in genetic screens correlate to pathophysiology in hematopoietic disorders, cardiovascular disorders and kidney disorders (Dooley and Zon 2000 and Shin and Fisherman 2002). Brain structures found in zebrafish have been associated to homologous parts of the cholinergic basal forebrain in humans (Mueller et al. 2004). Additionally, zebrafish have all of the essential neurotransmitter systems found in vertebrates (Mueller et al. 2004). Its ease of maintenance and relatively small size have also contributed to why it is so often chosen as a behavioral model in the laboratory (Gerlai et al. 2009). Zebrafish are becoming popular models for stress research as they produce a strong anxiety response when exposed to a novelty which makes stress levels easier to quantify (Blaser and Gerlai 2006).

Stress and anxiety have been extensively evaluated in zebrafish. Research has been conducted in which drugs such as ethanol, nicotine and cocaine were used to provoke anxious behaviors (Blaiser 2006; Levin and Bencan 2007; Lopez-Patino 2008). Zebrafish have been assessed for addiction properties (Ninkovic and Bally-Cuif 2006) and drug toxicity because their toxicity profile is comparable to that of mammals (McGrath and Li 2008). Anxiety related disorders continue to be one of the most prevalent neuropsychiatric conditions in humans. There is a lack in medical treatment for such conditions because their mechanisms are still poorly understood (Gerlai et al. 2009). While predator-induced defenses in animals that produce natural responses have been considered to be useful models of human emotional disorders (Apfelbach et al. 2005), zebrafish may provide important insight into understanding the neural basis of predator avoidance (Miklosi and Andrew 2006).

After visual contact with a predator fish, there was an increase in the whole body cortisol level in zebrafish, indicating that visual sensory cues could evoke a stress response in the prey fish (Barcellos et al. 2007). Predator exposure has also resulted in increased serotergic activity in another fish species (Winberg et al. 1993). At least two notions have been proposed to explain the source of predator avoidance behavior. First, prey may acquire anti-predatory responses through learning after it has been exposed to a harmful predator. Alternatively, there could be a genetic component that would instinctively influence the prey's behavior to avoid the predator. There is some literature regarding both areas of interest. Many experiments have evaluated the differences between responses from natural versus foreign predators in fish (Gerlai 1993; Berejikian et al. 2003, Bleakley et al. 2005). Mounting evidence supports the importance of the role that learning plays in the development of anti-predatory responses. Kelley and Magurran illustrated that while visual predator recognition skills came were grounded in unlearned predispositions, anti-predatory behavior using olfactory skills can be modified with experience particularly during the initial stages of the predator-prey interaction (Kelley and Magurran 2003). Olfactory cues also enabled zebrafish to recognize the predator after a single exposure to the predator fish followed by subsequent samplings of the smell alone (Korpi and Wisenden 2000). However, a recent experiment by Bass and Gerlai demonstrated that Zebrafish exhibited significantly more stressful behaviors to its natural predator, the Leaf Fish, than to the allopatric predator. Importantly, these zebrafish were never exposed to the predator fish, suggesting that the responses originated from a genetic level rather than through experience learning (Bass and Gerlai 2008). An animated image of the natural predator using a computer software application also resulted in similar behavioral responses that consisted of decreased mean velocity and increased mean turn angle and increased variability of turn angle (Gerlai et al. 2009).

The present paper describes the behavioral response of the zebrafish when exposed to a sympatric and allopatric predator given visual and olfactory cues. One experiment using the Indian Leaf Fish was performed in Dr. Kalueff's lab in Georgetown University, and another experiment using the Oscar Fish was performed in his lab in Tulane University.

II. Materials and Methods

2.1 Experimental subjects: Zebrafish

Adult (3-4 months old), half male and half female zebrafish were tested in this study (Figure 1.A). The fish were obtained from local pet stores(Petco, Rockville, MD and 50 Fathoms, Metairie, LA). The fish were fed daily and their water was changed weekly.

2.2- Indian leaf fish

The Indian Leaf Fish (*Nandus nandus),* Figure 1.B, is a sympatric predator of zebrafish and it has been used to successfully induce a stressful reaction in zebrafish (Bass and Gerlai 2008).

The fish were housed in a separate tank from the zebrafish. To prevent predatory attacks during experimentation, proper care was taken. A juvenile 4-cm leaf fish, which cannot eat a full grown zebrafish, was used and the leaf fish was well fed by the time of the exposure. Also, experimentation took place in the daytime because leaf fish only feed at night. Interactions between the two fish were carefully monitored for particularly aggressive behavior to avoid any harm to the experimental animals.

2.2.1 Experimental Design

The effects of acute and chronic Indian leaf fish exposure on zebrafish were examined using the Novel Tank Test. This procedure was used to produce fear and anxiety related behavioral responses in the zebrafish. Zebrafish were individually placed in a 1.5 L tank containing an unrestrained leaf fish for a twenty minute interval. This enabled both fish to sense visual and olfactory cues from each other to provoke fear in the zebrafish. Control fish were also individually transferred into an exposure tank with the same conditions except the Leaf fish was not present in the tank. After the exposure duration, the zebrafish were transferred using a net to a new observation tank to undergo the novel tank diving test. Chronic leaf exposure was studied by placing the leaf fish in the home tank of the zebrafish, which contains approximately fifteen to twenty fish, for a twenty-four hour period before Novel Tank Testing.

Novel Tank Test

The novel tank (Fig. 2) test is an established experimental protocol by Bencan and Levin (2008) used to evaluate the level of anxiety in zebrafish. The zebrafish were individually placed in a 1.5-L trapezoidal tank (approx. 15.2 cm H x 27.9 cm TW) filled with aquarium treated tap water for a six minute time period. Two equal horizontal parts of the tank were marked by a rubber band surrounding the outside wall so that the observer could easily identify when the fish moved into either half. Disruptions during experimentation were reduced by keeping the tanks on a stable surface with minimal sound and constant light, and precaution was used while transferring the fish to avoid stress from the net. Behavioral observations of the swimming patterns were used to record latency to enter the upper-half, time spent in the bottom and top portions of the tank, number of entries into the top portion, erratic movements, freezing bouts, and freezing duration. Earlier literature describes anxious zebrafish spending a longer period of time in the bottom of the novel tank (Bencan and Levin 2008). Elevated anxiety levels were characterized by a longer latency to enter the upper half, more erratic movements (abrupt movements that show a change in direction and velocity) and greater time spent frozen (complete absence of movement in everything but eyes and gills).

2.3- Oscar fish (*Astronotus ocellatus)*

The Oscar fish is native to South America making it an allopatric predator of the zebrafish (Fig. 1C) . It is significantly larger in size compared to the zebrafish and it is generally a dark colored fish. One young adult Oscar fish (3 months old) was used during the experiment and it was obtained from 50 Fathoms Pet Store. It was housed separately from the zebrafish, fed daily with fish food pellets, and the water was changed weekly.

2.3.1- Experimental Design

The zebrafish were exposed to the stimulus predatory Oscar fish for ten minutes before performing the novel tank test, which provides the baseline data for the studies conducted in this lab. Since Oscar fish readily eat zebrafish if given the opportunity, an alternative design set up was used. An unrestrained Oscar fish was placed in a tank (30 cm x 15 cm) and the zebrafish were in a smaller plastic container with bars placed inside the corner of the tank. There was no physical contact with the predator, however, olfactory and visual cues were maintained. Control fish were placed in a similar plastic container inside of the tank without an Oscar fish.

The experimental zebrafish underwent the Novel Tank Test after exposure to the predator fish; the control zebrafish also underwent the test after exposure to the same conditions without the predator fish. For each of the behavioral endpoints, the stress and anxiety level of the fish was evaluated. More anxious behavior is typically manifested in a longer latency to the top half of the tank, fewer transitions to the upper half, more erratic movements and more freezing bouts as well as a longer time frozen.

III. Results

Acute Predator exposure, using the Indian Leaf Fish, revealed some unexpected behaviors among the endpoints (Figure 3). The Indian Leaf Fish spent most of the pre-exposure time on the bottom of the tank. As an anti-predatory response, the zebrafish found protection in the top of the tank, successfully avoiding the predator. Analysis of the data shows that this anti-predatory response behavior exhibited in the experimental tank must have transferred over into the novel tank. The experimental zebrafish had significantly shorter latencies to cross into the upper half of the tank (Figure 3.A). The immediacy of the fish leaving the bottom of the tank supports the notion of the fear response transferring over to the novel tank test. Consistent with the previous points, the experimental fish spent far more time in the top portion of the tank (Figure 3.D). Despite having fewer transitions to the upper half (Figure3.C) the average entry duration is significantly larger than that of the control (Figure 3.B). At each minute, the average entry duration is approximately 60 seconds, suggesting that the fish must have spent most of its time in the top half. Additionally, the overall greater number of erratic movements, with more at the beginning of the six minute novelty test, indicates that the experimental fish was obviously stressed (Figure 3.E). A minute distribution analysis indicates significance for this behavior during the first and second minutes, which suggests habituation (Figure 3.F).

The results of the Chronic Leaf Fish exposure are similar to the Acute Leaf Fish Exposure. The experimental zebrafish had a significantly shorter latency time to enter the upper half of the tank (Figure 4.A). Again, the immediacy of the transition into the upper half might illustrate a fear response trying to avoid the area previously inhabited by the Leaf Fish. There are a much larger number of erratic movements displayed in the behavior of the zebrafish exposed to the Leaf Fish (Figure 4.B). The average time spent in the upper half is also significantly larger than that of the control group (Figure 4.C). A minute analysis of the time spent in the upper half shows significance across all six minutes, demonstrating a fear-like response (Figure 4.D).

The results from the Oscar Fish exposure did not produce as significant findings in each behavioral endpoint. The control had a shorter latency to enter the upper half of the tank and on average, the control fish made more transitions to the upper half (Figure 5.A). The control fish also spent more time in the upper half (Figure 5.B). During pre-exposure, the Oscar Fish, like the Indian Leaf Fish, spent most of its time at the bottom of the tank. If the same logic is used in describing why the experimental fish spent more time in the top of the tank after exposed to the Indian Leaf fish, then the data from the Oscar experiment indicates that the zebrafish were less stressed after being exposed to the Oscar. Additionally, surprisingly, the control fish displayed more freezing bouts and longer durations of freezing than the experimental, even though a freezing bout is typically indicative of stress induced behavior (Figure 5.C). However, there is evidence that the Oscar Fish induced stress in the zebrafish because of the significantly larger number of erratic movements seen in the behavior of the experimental fish (Figure 5.D).

IV. Discussion

Our results demonstrated that the prey zebrafish were affected more by the sympatric predators than the allopatric predator because they exhibited significant stressful behaviors in more than one behavioral endpoint. For acute predator exposure using Leaf Fish, significance was found in the latency, average entry duration, transitions to upper half, time spent in the top region of the tank, and erratic movements. Similarly, for chronic exposure to the Leaf Fish, significance was found in latency to the upper half, erratic movements, and time spent in the top region. For exposure to the Oscar Fish, the allopatric predator, significance was only found in the number of erratic movements, indicating mild difference between the control and experimental groups.

The results of this experiment add to the increasing body of literature illustrating a similar trend in a greater response towards the natural predator in zebrafish (Gerlai 1993; Berejikian et al. 2003, Bleakley et al. 2005; Bass and Gerlai 2008). There are a number of directions that the results of this experiment could take future experimentation including determining the source of this type of behavior. In accordance with the results collected by Bass and Gerlai, the zebrafish were never exposed to either stimulus fish before experimentation suggesting that their responses were due to genetic predispositions rather than learning. Other experimental designs have been devised to further investigate this question. Korpi and Wisenden solely tested olfactory cues to assess if fish could learn to avoid novel predators. The zebrafish that were previously unresponsive to the pike scent learned to recognize it after being exposed to the fish and pike odor. The fish would remain in the area with the alarm cue for at least five minutes and this would enable them to become familiar with the nature of the predator. After retesting the experimental fish with the pike odor alone, they showed decreased activity and movement towards the bottom, indicative of anti-predatory behavior. This type of learning, in which learning occurs after a single presentation of the cue, is known as releaser-induced recognition. From an evolutionary standpoint, natural selection would favor the single trial learning over repeated encounters with predation (Korpi and Wisenden 2001).

The outcome of our experiments does support the use of zebrafish as a strong model for behavioral studies as the observed differences in their responses was easily quantified. Our lab is currently performing trials with a computer software program that can track the movements of the zebrafish. This technique would produce more precise data by reducing the variability between observers evaluating the fish swimming patterns. It would also enable us to analyze more behavioral endpoints such as velocity in the top vs. bottom regions of the novel tank and distance traveled in tank. Obviously, there are many factors that contribute to what extent genetics and environmental issues are involved in anti-predatory responses, but this seems to be a promising area of further research.

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http://www.focusonnature.be/files/images/zebra.preview.jpg
Figure 1.A- Zebrafish

http://www.katsuayu.com/Nandusnandus011.jpg
Figure 1.B- Indian Leaf Fish

http://oscarfishcare.net/tigero.jpg

Figure 1.C- Oscar Fish

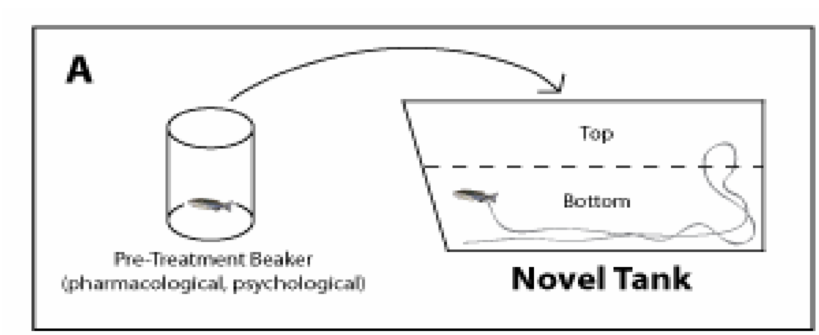

Figure 2. Novel Tank Test

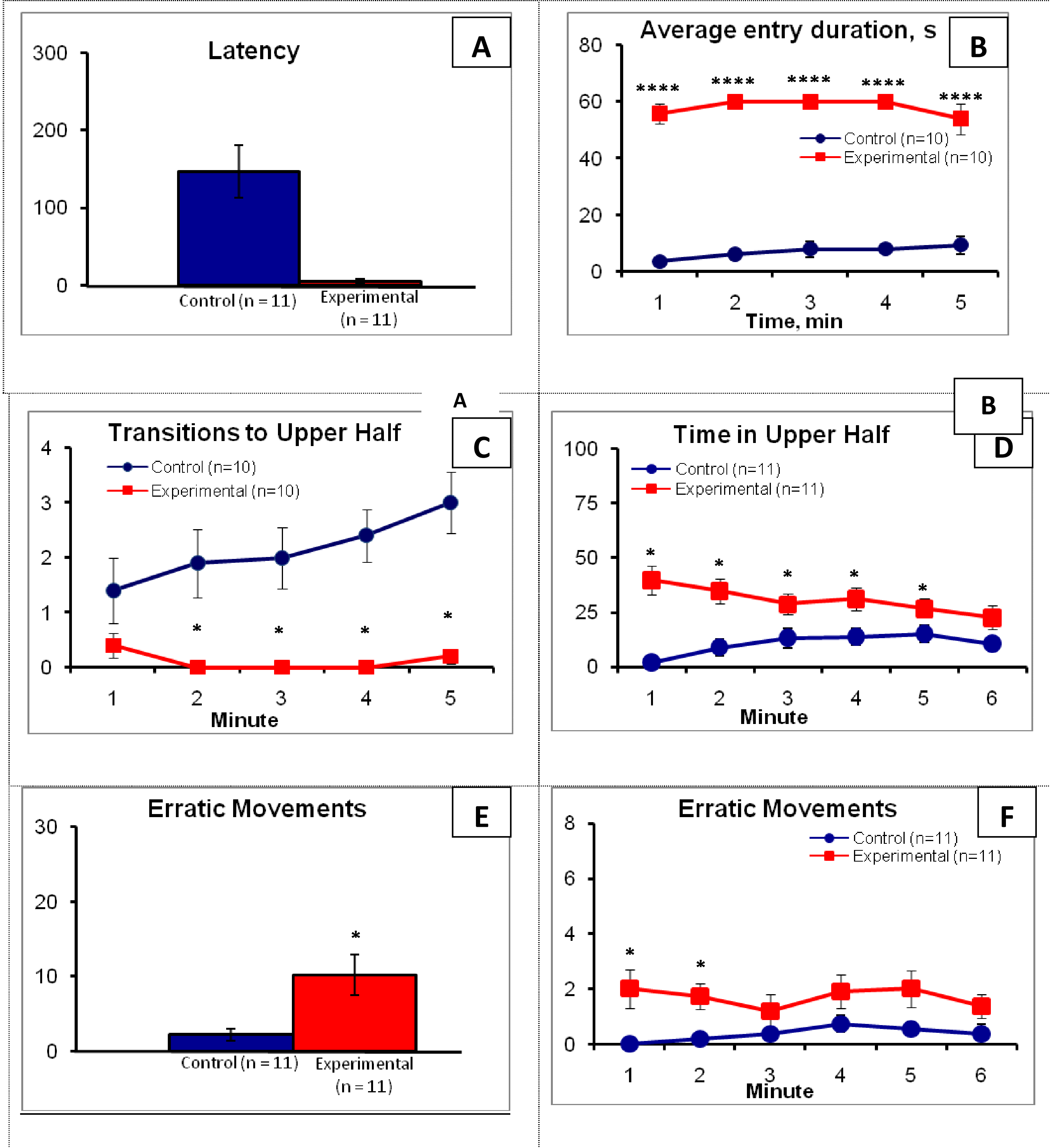
Latency
A
300
200
100
0
Control (n = 11)
Experimental (n = 11)
Average entry duration, s
B
80
60
40
20
0
****
Control (n=10)
Experimental (n=10)
1
2
3
4
5
Time, min
A
B
Transitions to Upper Half
C
4
3
2
1
0
Control (n=10)
Experimental (n=10)
*
Minute
Time in Upper Half
D
100
75
50
25
0
Control (n=11)
Experimental (n=11)
*
6
Minute
Erratic Movements
E
30
20
10
0
*
Control (n = 11)
Experimental (n = 11)
Erratic Movements
F
8
6
4
2
0
Control (n=11)
Experimental (n=11)
*
Minute

Figure 3 – Anxiogenic effects of acute predator exposure on zebrafish behavior: A) the average latency (s) to enter the upper half of the novel tank; B) average duration of upper half entries per minute; C) the average number of transistions to the upper half per minute; D) the average duration spent in the upper half per minute; E) averge number of erratic movements; F) average number of erratic movements per minute. Mean ± S.E.M shown. *p<0.05, **p<0.01, ***p<0.0005 (u-test).

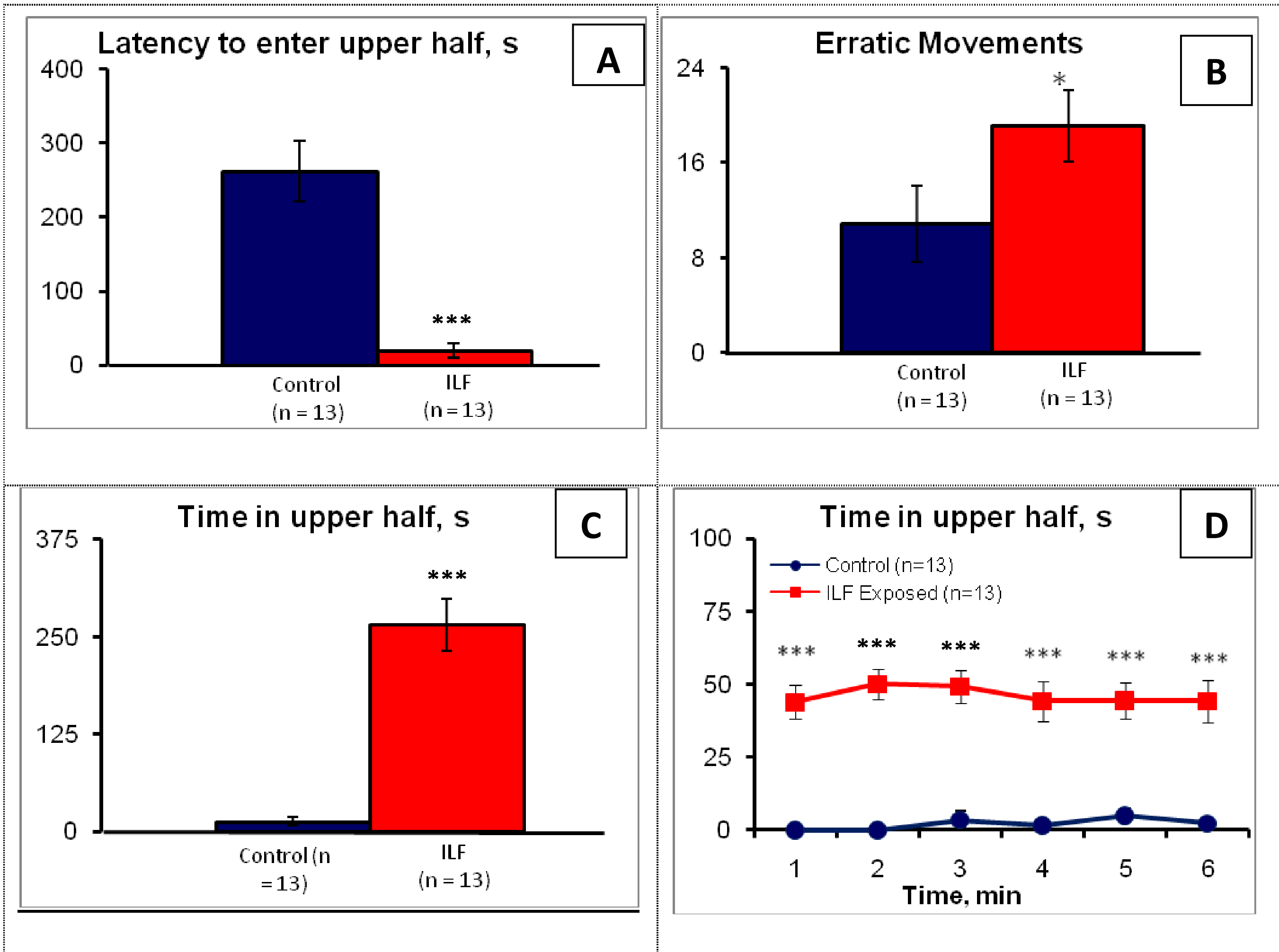

Figure 4 – Anxiogenic effects of chronic predator exposure on zebrafish behavior: A) illustrates latency to initially enter the upper half of the tank; B) average number of overall erratic movements; C) average overall time spent in upper half; D) average time spent in upper half of tank per minute distribution. Mean ± S.E.M shown. *p<0.05, **p<0.01, ***p<0.0005 (u-test).

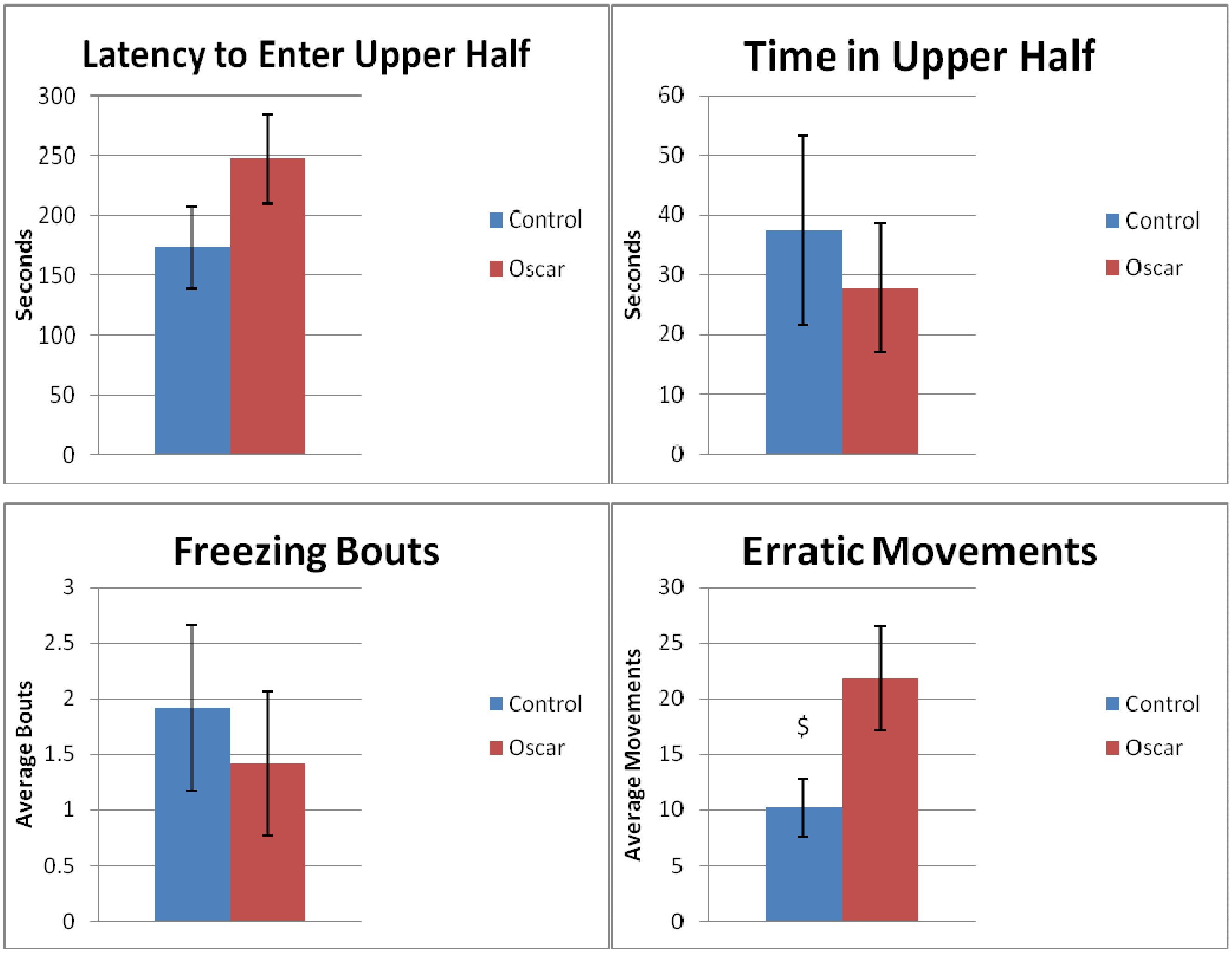

Figure 5- Effects of Oscar Fish exposure on Zebrafish behavior: A) Latency to Enter Upper Half illustrates latency to initally enter upper half of tank; B) Time in Upper Half is the average time spent in the upper half; C) Freezing Bouts are the number of time the fish did not move for an extended period of time; D) Erratic Movements are the number of jumpy, abrubt movements displayed by the zebrafish. Mean + S.E.M. shown. *p<0.05, **p<0.01, ***p<0.0005 (u-test).