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Assessing Behavior Change Related to Acute Stress Exposure in the Zebrafish

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by

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Abstract

Stress affects how we function in all aspects of our lives. It is our physiological response to a threat. In fact, its origins were very adaptive. Stress can cause an animal to flee a predator and avoid being eaten. In today's society, stress can prompt us to work harder to achieve a good education or promotion at work so that we can afford food, shelter, and entertainment. But stress can also impair performance at work, on tests, and even can cause long-term bodily harm. In order to fully understand the deleterious effects of stress and thereby properly treat it, it is useful to better understand the accompanying changes in behavior. This experiment is designed to evaluate the effects of an acute stressor's ability to induce behavioral changes indicative of the stress response in zebrafish. This experiment will measure the baselines of three paradigms, and subsequently assess any behavioral changes that are the result of acute exposure to a novel stressor.

Zebrafish are an up and coming model organism, but there are many unknowns in the literature about their behavior. Since the 1980s, the use of the zebrafish as an animal model in neuroscience research has steadily increased.

The three paradigms reported here are open field, light/dark discrimination, and novel tank dive. Stable performance baselines will serve as control measurements and should replicate what has been reported in the literature. The treatment condition (for all three experiments) will consist of a 15-minute pretest exposure to an acute stressor.

The acute stressor, known as a "beaker stressor", places the fish in a 250 ml beaker containing 100 ml of water, away from its companions. The effects of confinement and isolation have been shown to increase the production and release of cortisol, a stress hormone (Speedie &

Gerlai, 2007, Champagne, Hoefnagels, Kloet & Richardson, 2010). Once the zebrafish have been exposed to the stressor for 15 minutes, they will be placed in one of three apparatuses to assess any associated behavior change.

Since zebrafish have a hypothalamic-pituitary-interrenal (HPI) axis similar to the HPA axis in humans, they are ideal experimental subjects for this area of research (Champagne, et al., 2010). Previous studies report that the activation of the HPI and HPA result in a biochemical cascade (Barton, 2002, Tsigos, 2002). Cortisol is one of the chemical messengers released during this cascade, a chemical associated with the physiological components of stress (Champagne, et al., 2010). This study should provide more information for human comparisons of reactions to stress as well as expand what we know about the zebrafish model.

1. Introduction

The HPI-axis is involved in stress regulation process of humans and animals. Often in conjunction with the sympathetic nervous system, which results in the release of glucocorticoids (GCs) and catecholamines, also known as stress hormones (Jaggi et al., 2011). These hormones are thought to act on the brain, contributing to cognitive and behavioral impairment (Sauro, Jorgensen & Pedlow, 2003, Egan, et al., 2009). These effects can have temporary consequences on functioning or can be long term.

Traditionally, rodents have been the behavioral neuropsychology's paradigm for research subjects. However, due to the zebrafish model's ability to be bred, fed and housed inexpensively, the ease with which can be mutagenized by chemical mutagens, and its HPI axis that is homogenous to humans', this new model is increasingly being utilized in neuroscience labs (Gerlai, 2003).

Anxiety in rodents has been typically measured in the presence of natural predators and aversive environments (bright and novel areas) (Blaser, Chadwick & McGinnis, 2010). A curious trend emerging in experiments utilizing zebrafish involves a lack of visceral reactions to the same stimuli that induced anxiety in the rodent experiments (Blaser, Chadwick & McGinnis, 2010). For example, not all natural predators elicit a fear response for this model in the laboratory (Bass & Gerlai, 2008). Due to the environment they were raised in, laboratory fish could be evolving without the natural survival instincts of avoiding certain predators. If this model is to be continually utilized, finding an empirically supported technique to induce the physiological stress reaction in zebrafish (both laboratory and naturally breed) must be a priority. This study aims to examine the behavioral underpinnings of this physiological response to confinement-induced stress.

To fully understand whether this stressor is statistically valid, behavioral changes associated with stress must be operationally defined. In the rodent model, this was identified by extinction of exploratory behaviors, most commonly freezing behavior, as well as thigmotaxis (a term originally defined as movement away from a stimulus, that has come to be used in literature as a subjects adherence to the "safer" walls and avoidance of the less protected middle area of an apparatus) (Blaser, Chadwick & McGinnis, 2010, Stewart, et al., 2010). The zebrafish model likewise identifies the suppression of these investigative behaviors, as well as geotaxis (bottom dwelling and diving to the "safer" lower regions of an apparatus) and hyperactivity, as indicators of physiological reactions to stress (Blaser, Chadwick & McGinnis, 2010, Stewart, et al., 2010). Each of the three paradigms examined in this experiment have specific measures to examine mobility and behavior as a demonstration of the effectiveness of our novel beaker stressor.

The light dark paradigm is based on zebrafish's innate aversion to illuminated areas (Serra, Medalha & Mattioli, 1999, Blaser & Penalosa, 2011). In this part of the experiment, zebrafish are placed in a tank that is divided into a light area and a dark area; a control group is recorded without the stressor and an experimental group is recorded with the stressor. Time spent in each side of the tank is recorded. Research has shown elevated levels of cortisol can exacerbate a zebrafish's innate response to stay in the darker "safe zone" of a tank (Cachat et al., 2011). If the novel beaker stressor is an effective method of inducing stress in the subjects, they should spend significantly more time in the dark after they have been exposed to the stimulus.

The open field paradigm gained popularity by its wide use in the rat model. For the same reasons it was valuable in rodent research, many are finding it promising for the zebrafish model of biopsychology. In this paradigm, the subjects are allowed to freely explore the apparatus and time spent in the four quadrants, freezing, hyperactivity, and area traveled is measured. Zebrafish naturally stay close to the walls of an apparatus and as they habituate they gradually begin exploring the middle areas (Stewart et al., 2010). Increases in cortisol levels can lead to two opposite reactions: cessation or decrease of exploratory behaviors (less area traveled) or hyperactivity displayed as rapid movement of the organism (Blaser, Chadwick & McGinnis, 2010). If this novel stressor effectively raises cortisol levels, the zebrafish will spend significantly more time on the outer edges of the tank, explore less area, and/or have short bursts of hyperactivity and immobility.

Lastly, we utilized the novel tank dive to show alterations in behavior. It is based on the same concept as the open field for rodents, but is differential in that it examines vertical exploration. Zebrafish instinctually dive to avoid predation (Levin, Bencan, & Cerutti, 2007). This model has been shown to display that same diving behavior (geotaxis) in laboratory settings

with the subjects spending the majority of the recorded time at the bottom of a tank until they are comfortable to explore the upper regions (Levin, et al., 2007, Blaser, et al., 2010, & Egan, et al., 2009). If they are physiologically disturbed, by stress for example, they will not habituate and will instead remain at the bottom of the tank until they feel safe enough to exit the "safe zone" (Cachat, et al, 2011, Blaser, et al., 2010). After exposure to the beaker stressor, the zebrafish should spend a significantly longer amount of time on the bottom of the tank versus the top.

1.1 Value to academic discipline

The current study will examine how stress effects behavioral functioning in three different dimensions using the zebrafish model. Other studies utilizing the rat model have shown that stress resulting from restrained movement effects behavior. However, there have been no published studies examining zebrafish behavior during these tasks while using this novel stressor. Understanding the reaction to stress will further psychologist's understandings of the zebrafish as a model. This information will lay the foundation for experiments exploring zebrafish behavioral capacities while under stress.

Expanding what we know about the zebrafish model has a myriad of practical applications. For example, if pharmaceutical company wanted to create a drug that reduced stress, they would have to first run animals trials. The market for mutagenizing zebrafish has augmented in the past decade with studies already publishing the effects of ethanol, nicotine, cocaine, caffeine and fluoxetine (Echevarria, Hammack, Jouandot & Toms, 2010, Blaser, et al., 2010). To know how effective the drug is at treating stress, there must be empirical evidence that explains how stress manifests itself under specific conditions. This experiment should provide insight into the most useful ways to induce stress in this particular model.

1.3 Overview and predictions

The goal of this study is to investigate whether elevated cortisol levels will affect zebrafish behavior. The primary prediction for this study is that the zebrafish's performance will be biased towards the "safe-zone" of each apparatus subsequent to the activation of the HPI-axis.

2. Materials and Methods

2.1 Subjects

Subjects for this study were adult zebrafish (*Danio rerio*) obtained from a local pet store. They measure 3—5 cm in length and were at least one year old. They were housed in an aquarium with a water temperature between 28°C and 30°C, a pH between 6.8 and 7.2 , and a light cycle of 14 hours on and 10 hours off. Prior to the conditioning procedures, fish were fed twice daily with flake fish food (TetraMin), frozen brine shrimp, and live brine shrimp.

Fifteen fish per condition were used, for a total of 90 fish. A zebrafish was randomly selected from the home tank and individually housed for 4 days before testing began in order to accustom the fish to being alone and provide a way to identify the zebrafish.

2.2 Apparatus

There are four behavioral apparatuses used in this experiment:

1) The light dark apparatus is a modified 10-gallon fish aquarium. The tank is divided exactly in half with one side covered in black shelf liner on all three sides and bottom and the other half is covered in white shelf liner on all three sides and the bottom (There are two circles cut into the white side that have black shelf liner). The tank is filled to 5 L for experimentation.

Figure 2.1 Light/Dark Discrimination

2) The open field apparatus is a modified 10 -gallon fish aquarium. Only half of the tank is utilized for our experiment. The two halves are sectioned off with opaque Plexiglas and rubber strips that prevent zebrafish from slipping past the barrier. The floor of the apparatus is lined in a grid of 1-inch squares. The four quadrants of the grid are also clearly sectioned off. The fish is placed in 5 L of water.

Figure 2.2 Open field tank

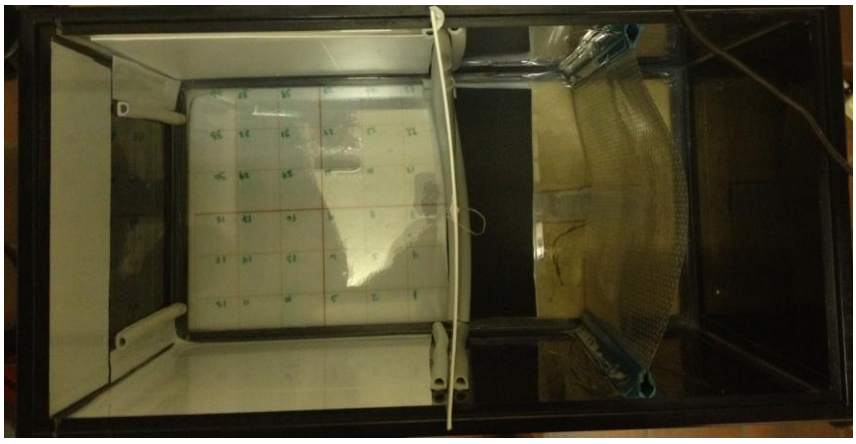

Figure 2.3 Open field tank

3) The novel tank dive apparatus is a modified 1.5 L holding tank (15.2 height x 27.9 top x 22.5 bottom x 7.1 width cm) The tank is sectioned off into two equal horizontal portions marked with a secure rubber band on the outside walls. The water is filled to maximum capacity.

Figure 2.4 Novel tank dive apparatus

4) The apparatus that was used to induce stress, known as the beaker stressor, was a 250 ml beaker filled with 100 ml of water. The experimental groups of fish were placed in the compact area for 15 minutes prior to testing.

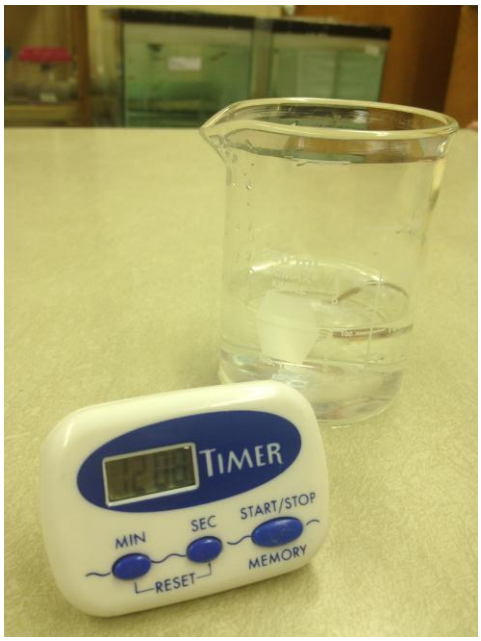

Figure 2.5 The Beaker Stressor

2.3 Procedures

There were six phases of the experiment: control and experimentation for light/dark, open field, and novel tank dive.

2.4 Control

For each task, 15 untrained zebrafish were used. Once zebrafish were used, they were not reused for another control or for additional experimentation.

1) In the Light/Dark Apparatus, the tank was filled to 3.5 L, then (without netting) the fish was poured into the tank and the 1.5 L of their holding tank combine to make 5 L of water for the zebrafish to swim in. The fish was then given 11 minutes to explore the apparatus with a camera recording all sessions. Coding begin at minute 1 and went to minute 11, totaling 10 minutes of data. The time spent in the light side of the tank was measured.
2) In the Open Field Apparatus, the tank was filled to 3.5 L, then (without netting) the fish was poured into the tank and the 1.5 L of their holding tank combine to

make 5 L of water for the zebrafish to swim in. The fish was recorded for 30 minutes while they explored the tank. We coded for one minute at the 5, 10, 15, 20, & 25-minute mark, totaling 5 minutes of data. The observer recorded time spent in all four quadrants during each minute, number of boxes the zebrafish swam through during each minute, and if there was any immobility or erratic swimming during the minute. Immobility was defined as not moving in any direction for five or more seconds. Erratic behavior was defined as crossing 12 inches or more in one second (revised methodology from Echevarria, Hammack, Jouandot & Toms, 2010).

3) In the Novel Tank Dive Apparatus, the tank was filled to maximum capacity (1.5 L). The fish was netted and placed in the apparatus then recorded for 6 minutes while they explored the tank. Coding began at minute 1 and went through minute 6, totaling 5 minutes of data. The observer recorded time spent on the bottom of the tank.

2.5 Experimentation

Testing involved inducing stress in the zebrafish using a "breaker stressor". Zebrafish's cortisol levels increase when they are isolated from their groups and confined within small areas (unpublished data). We induced this physiological change by placing them in the novel stressor for 15 minutes. This apparatus is a 250 ml beaker that has been filled to 100 ml (approximately 1.5" high with a diameter of 2.5"). Then the zebrafish was placed in the tank (using the same procedures as the control) of their respective paradigm and tested to see the effects of our stressor. Fifteen zebrafish were used for each control paradigm and then not reused for any subsequent paradigm.

3. Results

3.1 Light/Dark Discrimination

As previous research has indicated (Serra, Medalha & Mattioli, 1999, Blaser & Penalosa, 2011), our results also show that zebrafish have a significant preference for dark environments over lighted environments in the control group, with p=0.004 when a paired sample t-test was

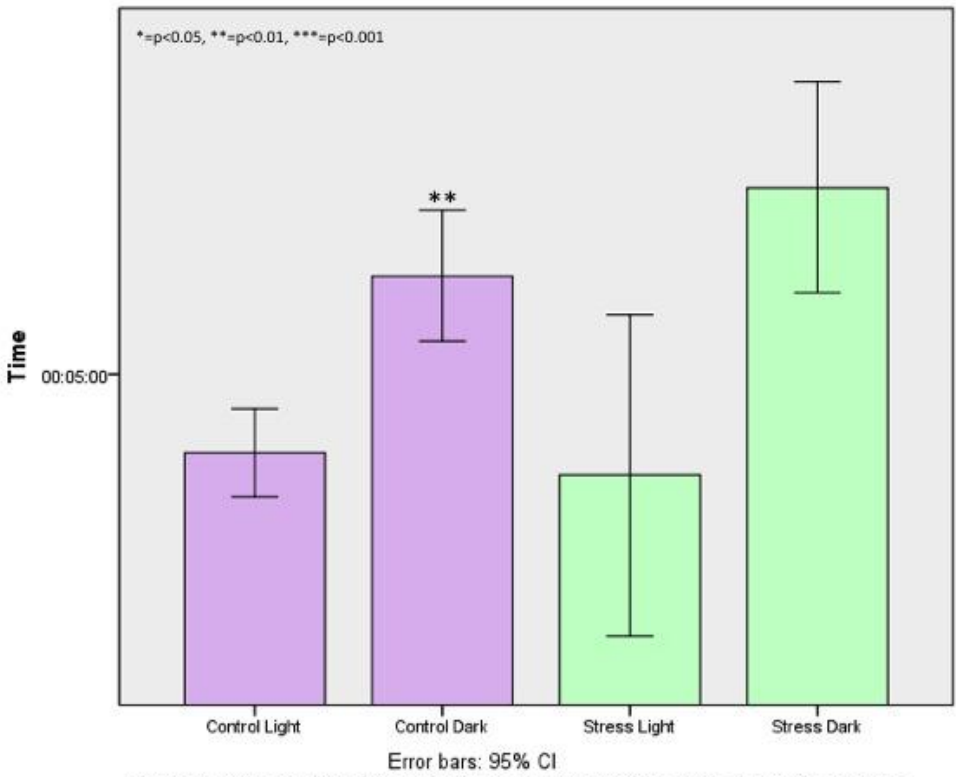

Figure 3.1 The results of the paired samples t-test. This graph shows the average time spent in light and dark for all thirty test subjects. Mean time for control groups spent in dark was significantly higher than time spent in light (p=0.004).

conducted. A paired samples t-test conducted under *stress* conditions revealed no significance.

3.2 Novel Tank Dive

As predicted, zebrafish spent more time on the bottom of the tank versus the top of the

tank in the experimental condition. A paired samples t-test revealed significance between the experimental measures for time spent on top and the experimental measures for time spent on bottom (p=0.017). There was no significant preference between control measures for time spent on top and control measures for time spent on bottom.

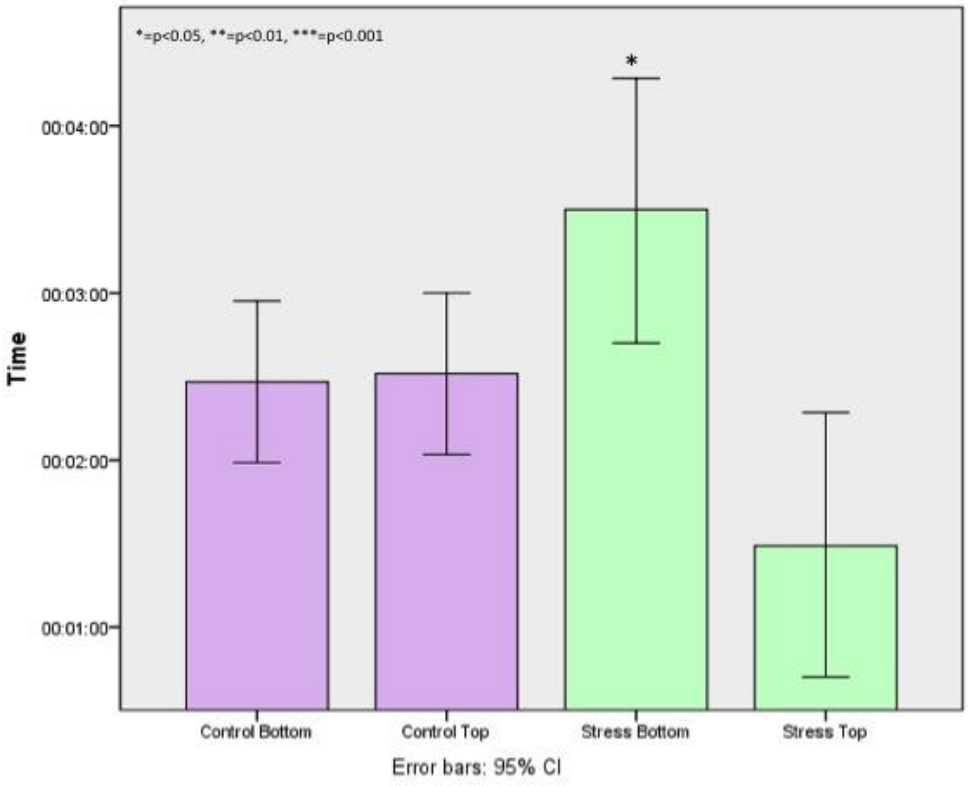

Figure 3.2 The results of the paired samples t-test. This graph shows the average time spent on top of the tank and time spent on bottom for all thirty test subjects. Mean time for experimental groups spent on bottom was significantly higher than time spent in top (p=0.017).

3.3 Open Field Paradigm

A paired samples t-test comparing the control condition to the experimental condition revealed a significant increase in immobility and erratic behaviors for the experimental group (figure 3.3). A repeated measures ANOVA for erratic behavior by time period was not

significant (figure 3.4). A repeated measures ANOVA analyzing immobile behaviors revealed increasing significance among the experimental group (figure 3.5, Table 3.1).

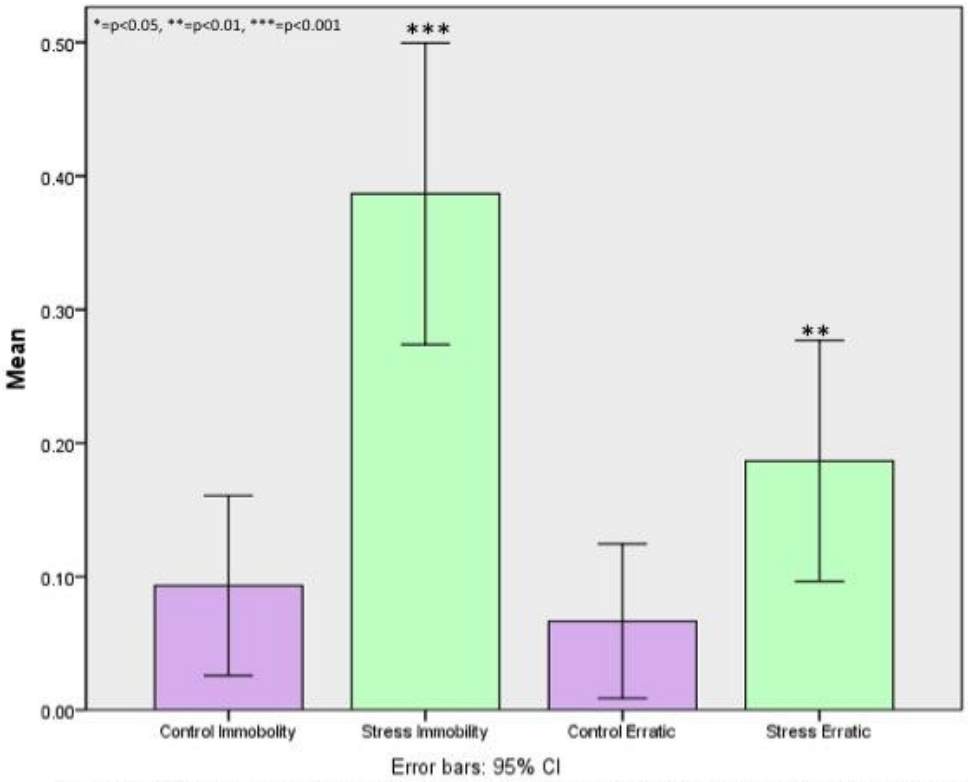

Figure 3.3 The results of the paired samples t-test for control group to experimental group for immobility and erratic behaviors. This graph shows all instances of erratic or immobile behavior for all thirty test subjects. Paired samples t-test resulted in $p < 0.0001$ significance for immobile behavior and $p=0.019$ significance for erratic behavior.

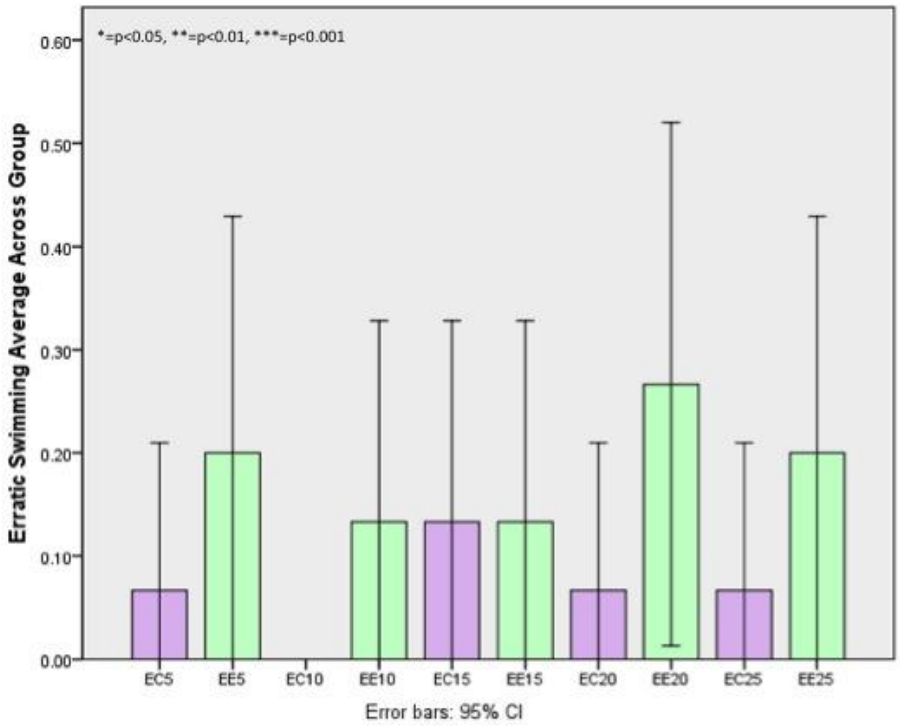

Figure 3.4 The results of the repeated measures ANOVA for control group to experimental group by time period. This graph shows all instances of erratic behavior for all thirty test subjects. There was no significant increases in erratic behavior by time period.

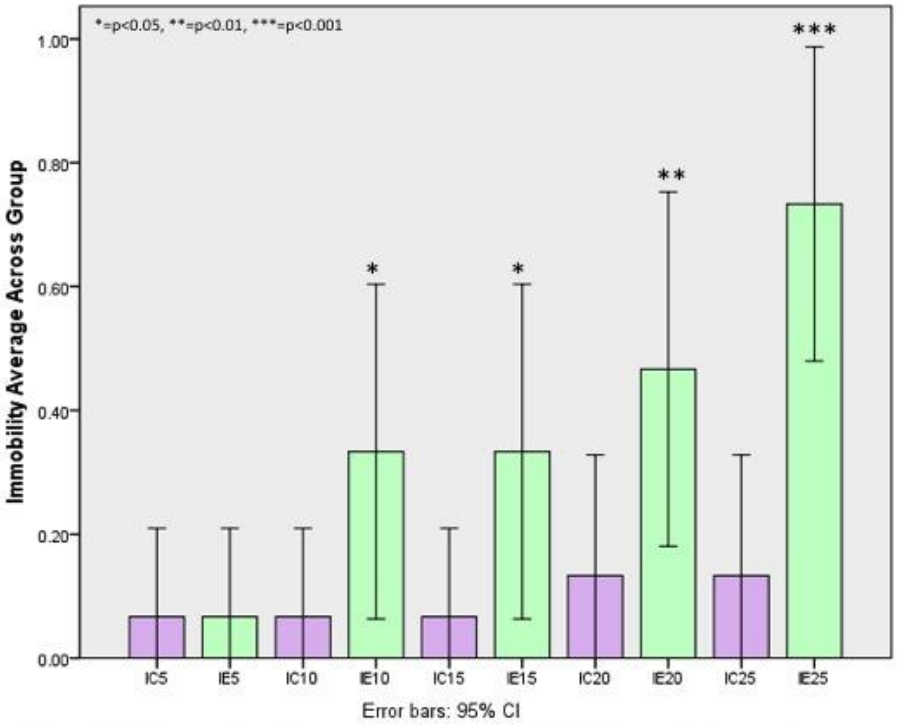

Figure 3.5 The results of the repeated measures ANOVA for control group to experimental group by time period. This graph shows all instances of immobile behavior for all thirty test subjects. Table 3.1 shows exact comparisons between time periods. There was no significance between time periods for control, but there was significance between time period for stress conditions.

Pairwise Comparisons

(I) factor1	(J) factor1	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Minute 5 Control	ExperimentMinute5	.000	.098	1.000	-.209	.209
	ControlMinute10	.000	.098	1.000	-.209	.209
	ExperimentMinute10	-.267*	.118	.041	-.520	-.013
	ControlMinute15	.000	.098	1.000	-.209	.209
	ExperimentMinute15	-.267	.153	.104	-.595	.062
	ControlMinute20	-.067	.067	.334	-.210	.076
	ExperimentMinute20	-.400*	.163	.028	-.750	-.050
	ControlMinute25	-.067	.118	.582	-.320	.187
	ExperimentMinute25	-.667*	.159	.001	-1.008	-.325
Minute 5 Experiment	ControlMinute5	.000	.098	1.000	-.209	.209
	ControlMinute10	.000	.098	1.000	-.209	.209
	ExperimentMinute10	-.267*	.118	.041	-.520	-.013
	ControlMinute15	.000	.098	1.000	-.209	.209
	ExperimentMinute15	-.267*	.118	.041	-.520	-.013
	ControlMinute20	-.067	.118	.582	-.320	.187
	ExperimentMinute20	-.400*	.131	.009	-.681	-.119
	ControlMinute25	-.067	.067	.334	-.210	.076
	ExperimentMinute25	-.667*	.126	.000	-.937	-.396

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

*. The mean difference is significant at the .05 level.

Table 3.1 The results of the repeated measures ANOVA for control group to experimental group by time period.

4. Discussion

The results of these studies give credence to the applicability of the novel beaker stressor. The light dark tank revealed that control groups would behave as literature has suggested, showing significant preference for darker areas. Once a stressful stimuli has been introduced, that behavior is abolished. Since typical behavior would be to prefer dark environments, this atypical behavior means the novel beaker stressor has some effect on zebrafish behavior.

The tank dive paradigm demonstrated that subjects were significantly more likely to spend their time on the bottom, safer area of the tank after exposure to stress-inducing stimuli. This task is designed to measure stress as displayed in bottom-dwelling behavior, and after exposure to our novel beaker stressor, the experimental group displayed increases in that behavior.

The open-field experiment revealed a significant number of erratic instances and an increasing significant number of immobile instances under stress conditions as time increased. This may be due to cortisol levels increasing throughout the experiment.

The data revealed in the open field paradigm strengthens and gives credence to the data from the light dark paradigm and tank dive paradigm. Subjects could be freezing in one area of the tank, contributing to an abnormal amount of time spent in that area, or subjects could be so erratic in their movements that they show no preference in their movement.

To check the reliability of the data coding between this experimenter and a research assistant, statistical analysis was conducted. Cronbachs alfa revealed an a=0.999 in regards to inter-rater reliability.

5. Conclusion

Our hypothesis was that the activation of the HPI and subsequent cortisol production would manifest behaviorally as performance changes on 3 well-known behavioral paradigms. To elicit these physiological changes we employed a novel stress paradigm developed by Dr. Echevarria. The "novel beaker stressor" had been previously linked to spikes in cortisol release. As predicted, behaviors were disrupted by exposure to our novel beaker stressor. These changes in behavior along with previous collected cortisol data collectively help to validate this novel stress paradigm.

One next logical step would be to investigate how a known anxiolytic (e.g. diazepam/Valium) might mediate the physiological stress response and thereby ameliorate stress induced performance deficits, like those reported here.

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