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Abstract

The purpose of this research was to investigate the effects of forced exercise on anxiogenic behavior in *Danio rerio* living in different environmental conditions. To study this relationship, zebrafish were introduced to the following housing environments for several weeks. Fish in Group 1 were exposed to brown gravel and green, plastic plants (enriched environment), and fish in Group 2 were exposed to no brown gravel and no green, plastic plants (impoverished, structurally-barren environment). Fish from both housing environments were randomly assigned to be exposed to exercise or no exercise (control). Fish in the exercise group were forced to swim against a current generated by a submersible water pump daily. Fish that were in the non-exercised group were treated in an identical manner each day, but the water pump was off (controlling for the effects of stress related to the procedure). The light/dark and novel tank tests were used to measure anxiety-like behaviors. It was hypothesized that subjects exposed to daily forced exercise would be protected from the adverse effects of living in a deprived environment. Results indicated a significant main effect of exercise only for the number of entries into the top portion of the novel tank, which suggests a decrease in anxiety. All other behavioral measures of anxiety were not significant.

Keywords: anxiety, enrichment, exercise

The Effects of Forced Exercise on Anxiety in *Danio rerio* Living in Impoverished Environments

As a dynamic structure, the brain continuously experiences cellular and molecular changes to adapt to the environment, which can in turn alter and shape behavior. Environmental enrichment is a form of environmental enhancement that causes anxiolysis (anxiety reducing) and promotes neurogenesis (formation of new neurons) in both mammals and non-mammalian species. Environmental enrichment generally involves enhancing the complexity of the social or structural environment of animals. The environment of many animals, including zebrafish, can be enriched with the addition of objects or altering socialization. For zebrafish, enrichment often includes the accompaniment of other zebrafish or the addition of artificial plants. The present research aims to investigate the potentially enriching effects of forced exercise in zebrafish.

Environmental enrichment enhances the psychologic and physiologic health of laboratory animals and is often used for laboratory-raised animals (Collymore, Tolwani, & Rasmussen, 2015). One mammalian study investigated the effects of environmental enrichment in adult Wistar rats on activity levels in an elevated zero-maze (Sampedro-Piquero, Zancada-Menendez, Begega, Rubio, & Arias, 2013). The researchers also assessed ability in a radial-arm water maze and looked at changes in the metabolic activity of brain regions associated with anxiety responses and memory using cytochrome c oxidase histochemistry (COx). The researchers found an anxiolytic effect in the elevated zero-maze. Additionally, in terms of the rats' neuronal metabolic activity, a reduction was observed in the COx activity in brain regions linked to anxiety responses, including areas in the thalamic and hypothalamic nucleus, amygdala, and ventral hippocampus. Moreover, researchers conducted a study on the positive effects of environmental enrichment on cognitive functioning, which is usually impaired throughout the aging process of rats (Sampedro-Piquero & Begega, 2017). The researchers found that the use of environmental enrichment effectively simulates an active lifestyle in this rat population and reduces the cognitive effects of aging. Therefore, environmental enrichment has been linked to many benefits in mammals.

One study conducted by Collymore, Tolwani, and Rasmussen (2015) aimed to determine if introducing environmentally-enriching materials, such as an artificial plant, would lead to anxiolytic behaviors and place-preferences in adult zebrafish. Researchers housed fish either in groups of five or alone for three weeks in various housing environments. These housing environments included: single-housed enriched, single-housed barren, group-housed enriched, and group-housed barren. Each group consisted of thirty subjects. During the fourth week, fish were randomly selected from each housing environment, and then they were tested using the novel-tank, light-dark, and place-preference tests. Researchers found that housing fish alone in a barren, desolate environment resulted in higher anxiety levels. The place-preference test, where zebrafish were offered a choice between complex and barren environments, indicated that zebrafish housed in barren tanks, both alone and in groups, spent less time focusing on the artificial plant device than interacting with conspecifics. The researchers' results indicated that by introducing an artificial plant for enrichment, single-housed zebrafish may benefit when group housing is not possible.

Several reliable paradigms are available for testing anxiety in zebrafish, as used in the aforementioned study (Collymore, Tolwani, & Rasmussen, 2015). An effective apparatus to measure anxiety in fish, known as the novel tank test, involves measuring the amount of time subjects spend swimming near the bottom or top half of an aquarium tank. When fish are first exposed to the novel environment, they have a tendency to spend more time at the sides and bottom of the tank. This type of behavior is similar to the thigmotaxis paradigm in rodents. For the novel tank test, a longer latency to migrate to the upper half of the tank, less time spent in the upper half of the tank, and a higher number of recorded erratic movements and freezing behaviors have been linked to anxiety-related behaviors in zebrafish (Collymore, Tolwani, & Rasmussen, 2015).

A study conducted by Blaser and Rosemberg (2012) assessed these measures of anxiety. Researchers assigned three groups of fish to a black, a white, or transparent tanks, then they studied fish depth preference using the novel tank test. Also, two groups of fish were placed in a deep or a shallow tank and later tested for preference of a black or white color. Researchers hypothesized that zebrafish would prefer the deeper portion of a tank over the shallow portion, and the black half of the black-white tank. Researchers found that the wall color of the tank affected depth preference significantly. The fish in transparent tank displayed the most depth preference. However, the depth of the tanks did not significantly affect color preference. They also found that the two tests are complementary but not necessarily transposable. This study is relates to the proposed research because both tests are measures for anxiety to be used in the research.

Another paradigm measuring anxiety is the light/dark test. This test involves acclimating subjects to a water-filled tank, which is dark on one side and light on the other, then video-recording and measuring the amount of time animals spend on each of the two sides. Anxious fish will spend more time in the darker area (displaying anxiogenesis), whereas less anxious fish explore the lighter region more. This scototaxis (light/dark partiality) procedure is a behavioral model for fish that has been confirmed to measure the antianxiety effects of many pharmacological agents (Maximino et al., 2010). Researchers, for example, investigated the effects of poisonous substances that result in anxiety-related behavior. Fish were placed in the main compartment of a half-white, half-black chamber and allowed to acclimatize to the environment, and then they were allowed to swim around the chamber for fifteen minutes. The number and length of passes in each side of the chamber (white or black) were logged by the observer for the entire trial. Tilapias, guppies, goldfish, and zebrafish, all important model species to the behavioral neurosciences, validated preferences in anxious animals for the dark section. An increase in dark section activity would indicate anxious behavior, yet an increase in white section activity would reflect anti-anxiety behavior.

Furthermore, anxiety causes the release of certain stress hormones, such as cortisol. A study conducted by Alsop and Vijayan (2008) investigated the corticoid stress axis in zebrafish because the onset of activation of cortisol synthesis is unknown. Researchers tested the hypothesis that the molecular events that underlie the appearance of corticosteroid signaling are associated with steroid production, and they determined cortisol levels and the time of expression of genes involved in corticosteroid synthesis, signaling, and metabolism. They introduced zebrafish embryos and larvae to a stressor and noted the time of activation of the corticosteroid stress axis. Researchers found that the expression of cortisol genes showed that the zebrafish corticoid stress axis is developed following egg hatching. These data showed that the corticoid-signaling pathways found in zebrafish were similar to those of mammals, implying that there is strong support for zebrafish as a model organism for corticosteroid axis stress research.

Another study, conducted by Egan et. al (2009), investigated zebrafish stress models by manipulating both environment and pharmacology, and researchers noted how behavior and physiology changed as a result. They exposed animals to alarm pheromones, fluoxetine, caffeine, or ethanol and discovered that alarm pheromones and caffeine yield anxiogenic (anxiety-producing) effects. Ethanol and fluoxetine, however, yielded anxiolytic effects. Researchers used different genetic strains of zebrafish (wild-type, leopard, and albino) to look at behavioral differences and measured cortisol levels to relate the drugs to levels of anxiety. They found that zebrafish can act as models of stress and affective disorders.

Another way to decrease anxiety (i.e., anxiolysis) in many animals is with exercise. For example, a study conducted by Taylor Hendershott, Marie Cronin, Stephanie Langella, Patrick McGuinness, and Alo Basu (2016) investigated the influence of exercise as a form of enrichment on cognition and emotion in mice. Researchers evaluated anxiety, sensory gating, social interaction, and spatial learning in mice. They found positive effects in both the male and female mice on anxiety behavior and sociability. This was due to an apparent increase of exploratory activity.

Not only does exercise cause anxiolysis in mammals, non-mammals have been shown to have physiological benefits from exercise as well. One surprising reptile species that has been shown to benefit from exercise is the American alligator (*Alligator mississippiensis*) (Eme, Owerkowicz, Gwalthney, Blank, Rourke, & Hicks, 2009). Like mammals, the alligators' response to exercise training involved an increase in convective oxygen transport mechanisms, such as heart size and haematocrit (blood oxygen carrying-capacity). Other unorthodox exercise models include the roundworm (*Caenorhabditis elegans*) (Laranjeiro, Harinath, Burke, Braeckman, & Driscoll, 2017). The researchers' results indicated that a single swim exercise session promotes positive physiological changes. Their data showed that acute swimming-induced changes for roundworms are similar to acute exercise responses seen in humans. *Danio rerio*, commonly known as zebrafish, are frequently-used model organisms that have also exhibited physiological advantages from exercise (Gilbert, Zerulla, & Tierney, 2014).

A study conducted by Palstra et. al (2010) investigated swimming economy and the effects of exercise on growth in zebrafish. Both individual zebrafish and groups were tested. Researchers found that exercised zebrafish were able to increase total body length by 5.6%, and they increased body weight by 41.1% when compared to non-exercised zebrafish. This study is the first to show significant exercise-related growth in adult zebrafish. The results of the study indicated that zebrafish are good exercise models for growth. Another study conducted by Palstra, Schaaf, and Planas (2012) looked at cardiac hypertrophy and cardiomyocyte regeneration in zebrafish. There are known positive effects of exercise for the mammalian immune function; however, zebrafish can also be used as models to look at stimulation of immune function following exercise. Researchers looked at the beneficial effects of exercise on muscle growth, immune functioning, and the cortisol stress response.

Palstra, Rovira, Rizo, Torrella, Spaink, and Planas (2014) investigated the cellular and molecular mechanisms involved in adult zebrafish swimming. By training fish at a low swim speed or an ideal swimming speed, increased vascularization was found in the exercised animals. The results of the study showed that forced exercise in zebrafish promotes increased muscle mass and increased vascularization. Researchers attribute these phenotypic adaptations to transcriptional changes as a result of the forced exercise. These results offer additional support for the validity of using zebrafish as an exercise model.

Additionally, exercise has been linked to neurogenesis, or the formation of new neurons (Raichlen, & Gordon, 2011). This suggests that behaviors, like a decrease in anxiety induced by enrichment, might also be modified by exercise-induced synaptic plasticity. Aerobic exercise consistently and robustly enhances the synaptic connectivity of neurons in the hippocampus, and these changes are strongly correlated with enhanced learning. Controls that are not exercised, in contrast, do not display similar neurogenesis and their hippocampal-dependent learning is not enhanced. While robust synaptic plasticity has been proven to occur in the zebrafish brain throughout life, including the evolutionary precursor of the mammalian hippocampus, very little is known about whether the neural changes that occur importantly underlie specific behaviors.

Von Krogh, Sørensen, Nilsson, and Øverli (2010), investigated neurogenesis and neuronal growth in zebrafish exposed to various environments. Researchers housed adult male zebrafish alone in enriched or barren environments for one week. The technique of immunohistochemistry was used to look at telencephalic cell proliferation. Higher cell growth was found in zebrafish that lived in enriched environments. Cortisol levels were also measured, and they were found to be elevated in zebrafish housed in enriched environments. This study found that environmental changes can significantly influence cellular dynamics in the brain of zebrafish.

Though many beneficial physiological effects of exercise have been found in zebrafish, virtually nothing is known about the behavioral effects of exercise in zebrafish. A study conducted by Luchiari and Chacon (2013) investigated the influence of physical activity on learning for an associative conditioning task in zebrafish. Researchers operationally defined learning as the approach of a zebrafish to a designated feeding area following classical conditioning. The zebrafish were randomly selected from a stock aquarium to be exercised for 20 consecutive days. Groups of four fish were placed in contact with a stringent water current. Each group was forced to swim until they were exhausted. The research indicated that physical activity accelerates the accomplishment of completing the associative learning task in zebrafish. This study is critical for the proposed research because it is the only behavioral study published on the behavioral effects of exercise in fish, and this exercise model used was used in the proposed research.

The proposed study is novel and is focused on assessing the effects of forced exercise on anxiety in zebrafish living in different environmental conditions. It was hypothesized that subjects exposed to daily forced exercise would be protected from the adverse effects of living in a deprived environment. It was also hypothesized that the exercise and enrichment group would receive the greatest anxiolytic benefit. The experiment was designed to determine if living in a physically-barren environment (absence of artificial plants and gravel) would result in anxiogenesis in zebrafish. Finally, the research determined if the detrimental effects of living in an impoverished, structurally-barren environment, which causes anxiogenesis in fish, can be lessened or prevented by forced physical activity.

Method

Subjects

The subjects required for this research were 60 adult male and female zebrafish (*Danio rerio*). Fish were transported from a local pet store (Pet & Aquatic Warehouse) and housed in the behavioral neuroscience lab at Lynchburg College.

Materials

The materials needed for this research include: approximately 60 zebrafish, previously cycled ten-gallon tanks (with dimensions 50.80 cm x 27.94 cm x 33.02 cm), American Aquariums non-reflective frosted material light/dark testing chamber (with dimensions 45 cm x 10 cm x 15 cm), Pentair Aquatic Eco-Systems novel tank testing chamber (trapezoidal shaped, 1.5L), Jecod/Jebao DCT Marine Controllable Water Pump (DCT-6000), Reef Octopus Varios 2 submersible DC water pump (3000L/hour), glass pipe (with dimensions 45.7cm long, 10.2cm diameter, 5.1 cm diameter at both ends), KONA Lab flow meters, Agrimaster Poly Stock Tank (265L), gravel, green Aquatop Cabomba Aquarium Plants (9" high with weighted base), Logitech C920 webcams, Dr. Meter LX1330B Digital Illuminance/Light Meter, 0-200,000 Lux Luxmeter, two computers, CleverSys AquaScanfish software, nets, stop watches, luxmeter, thermometers, commercial fish food (TetraMin tropical flakes), and water treatment materials (FritzZyme nitrifying bacteria, tap water conditioner).

Procedure

The experiment investigated whether the anxiogenic effects of living in an impoverished, structurally-barren environment can be lessened or prevented by daily forced physical activity. After arrival in the lab, five fish were randomly selected for housing in each 10 gallon tank.

Fifteen naïve zebrafish (in three separate tanks) were housed in each of the following aquarium environments: Group 1- approximately 2.5 cm of brown gravel and three green, plastic plants placed in the gravel (enriched environment) and Group 2- no brown gravel and no green, plastic plant (impoverished, structurally-barren environment). Initially, all groups were fed TetraMin tropical flakes three times weekly, but after the exercise condition was introduced, all groups were fed daily. Water changes were administered every two weeks for all groups, and the fish were exposed to the same 14-hour light cycle (14-hours-lights on/10-hours-lights off).

After being exposed to the enrichment conditions for 34 days, zebrafish received daily forced exercise or no exercise (control) for 14 consecutive days. Daily aerobic activity was manipulated in four groups of zebrafish as follows: *Group 1*-received no exercise and no environmental enrichment, *Group 2*-received no exercise and environmental enrichment, *Group 3*-received exercise and no environmental enrichment, and *Group 4*-received exercise and environmental enrichment. Fish in the exercise groups were forced to swim against a current each day that was generated initially by a Jecod/Jebao DCT Marine Controllable Water Pump (DCT-6000) connected to a transparent glass pipe. This pump had a rapid fill rate and was later replaced with a Reef Octopus Varios 2 submersible DC water pump (3000L/hour) after five days of exercise. Groups of five zebrafish swam together against a water current each day. Fish that were in the non-exercised groups were treated in an identical manner each day, but the water pump was off (controlling for the effects of stress related to the procedure). Fish were returned to their home tank using nets and transport containers.

Prior to the onset of behavioral testing, the researchers' inter-rater reliability was assessed to ensure consistency between observations. A correlation of 0.93 was found across 25 observations. Following the 14 days of exercise, two behavioral tests, the light/dark test and novel tank test, were used to assess anxiety. Fish were coded by the Principal Investigator and were distributed to the blind researchers. The fish were tested in a counterbalanced manner, meaning that they were only exposed to one of the behavioral tests on the first day and were exposed to the second test on the following day to prevent exhaustion.

The light/dark test (measuring how much time fish spend in either the white or black section of the testing chamber) was used to assess anxiety. Prior to introducing each fish to the light/dark chamber, the water was changed in the chamber and the temperature was recorded. Then, fish were transferred from their home tanks to the light/dark chamber individually, using nets and a transport container. Fish were observed for five minutes. Once in the chamber, more than half of the fish's body had to be on one side of the chamber to be considered in that portion of the chamber (e.g. half of the fish's body located in the black or white portion of the chamber). The movement from each side of the chamber was monitored by the researchers via a Logitech C920 webcam situated above the testing chamber, and researchers used stopwatches to record time spent on each side. Following the light/dark test observations, zebrafish were returned to their habitat tank and the process was repeated for the next animal.

Anxiety was also tested using the novel tank test (fish swimming depth measured). Fish swimming at lower depths demonstrated more anxious behavior (anxious fish spend more time in the bottom third of the tank). Fish were transported to the novel tank chamber in an identical manner to the light/dark test. Fish were observed for six minutes. After being introduced to the chamber, greater than half of the fish's body had to be in one section to be deemed in that section of the chamber (e.g. half of the fish's body located in the top or bottom portion of the chamber).

The movement from each portion of the chamber was monitored by the researchers via a Logitech C920 webcam fixed above the testing chamber, and researchers used stopwatches to record time spent on each side. Following the behavioral testing, CleverSys AquaScanfish software was used to analyze the movement and thigmotaxis of the zebrafish through the testing chamber and a trace was generated (Kobla, 2018). All other conditions remained the same for both groups. After experimentation, all zebrafish were returned to a local pet store.

Results

It was hypothesized that zebrafish exposed to daily forced exercise would have lower anxiety than non-exercised fish, that zebrafish exposed to the enriched environment conditions would have lower anxiety, and that these effects would interact with one another. A 2 (Exercise) x 2 (Enrichment) factorial ANOVA was conducted to analyze the behavioral effects of the exercise and enrichment conditions. No interaction effects were observed for exercise or enrichment in both behavioral tests. This indicates that the effect of exercise on anxiety does not depend on the level of enrichment or vice versa. However, a significant main effect of exercise was observed for the number of entries into the top portion of the novel tank, $F(1, 48) = 4.85$, $p=0.033$; see Figure 3 and Figure 4. Higher number of entries to the top portion of the tank is considered an indication of lower anxiety. Fish that exercised daily for 14 days had lower anxiety ($M=49.84$, $SD=24.43$) than control fish that did not exercise ($M=36.78$, $SD=18.43$). See Table 1 for descriptive statistics. The factorial ANOVA did not reveal a significant main effect for total time spent in the top portion of the novel tank, $F(1, 48)=1.21$, $p= 0.276$. Additionally, the factorial ANOVA did not reveal a significant main effect for total time spent in the light portion of the light/dark chamber, $F(1,48)=0.37$, $p=0.547$ (Figure 1 and Figure 2).

Discussion

The purpose of this study was to investigate the effects of exercise and enrichment on anxiety in zebrafish. The researcher hypothesized that the no exercise-impoverished environment group (Group 1) would have the highest anxiety level. The researchers also hypothesized that the exercise-enriched environment group (Group 2) and the exercise-impoverished environment group (Group 3) would have moderate anxiety levels. Also, the exercise-enriched environment group (Group 4) would have the lowest anxiety. The results, as indicated by the factorial ANOVA, demonstrated that exercise significantly reduced anxiety levels (measured by number of entries to the top of the novel tank test). However, there were no interaction effects between exercise and enrichment, indicating potential procedural issues with this study. These results suggest that exercise may offer anxiolytic benefits to zebrafish, as shown by previous researchers for various mammalian species, but more research is needed to make definitive conclusions.

Limitations

There are several limitations associated with this study. One limitation of the present study was the relatively small sample size obtained. To potentially establish an effect, more fish need to be allotted in each group. Additionally, subject mortality was another limitation. By including more animals in the sample, the effects of this limitation could be reduced.

Another limitation of the present study were the relatively high levels of activity apparent in all of the subjects. Additionally, there were subjects of different sizes. To control for these issues, future researchers could request that the purchasing company select fish of similar activity levels and sizes.

One other limitation was properly controlling the rate flow of current in the exercise chamber. A stronger water current was used in the present study compared to a previous study conducted by Luchiari and Chacon (2013). Luchiari and Chacon (2013) used a current of 280L/h for days 1-10 and 520L/h for days 11-20, whereas the present study achieved a maximum current of approximately 1800L/h. This suggests that fewer days of exercise or shorter bouts of exercise may be required to establish an effect of exercise on anxiety. Also, the previous study (Luchiari & Chacon, 2013) did not specify whether or not controls were exposed to the same environment as the exercise fish, which could indicate a flaw in the methodology by which the present study was influenced.

Conclusion and Future Research

The present study offers new insight on the effects of exercise on anxiety in non-mammalian species. To better establish this phenomenon, future researchers could use a larger sample size and incorporate better sample selection methods. Additionally, researchers can further utilize AquaScan Software to allow for precise computer tracking and modeling of fish movement. Moreover, future researchers could investigate the effects of acute exercise on anxiety. This would involve introducing zebrafish to one bout of exercise and measuring anxiety. Overall, there is more to explore in this novel area of zebrafish behavioral neuroscience.

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Appendix A

Table 1. *Measure of Anxiety by Four Conditions*

Condition	Anxiety (time spent on top of tank in seconds)
No Exercise-Impoverished Environment	34.69 (17.35)
No Exercise-Enriched Environment	38.71 (19.82)
Exercise-Impoverished Environment	53.83 (31.12)
Exercise-Enriched Environment	46.15 (16.60)

Note: Higher numbers indicate low anxiety.

Appendix B

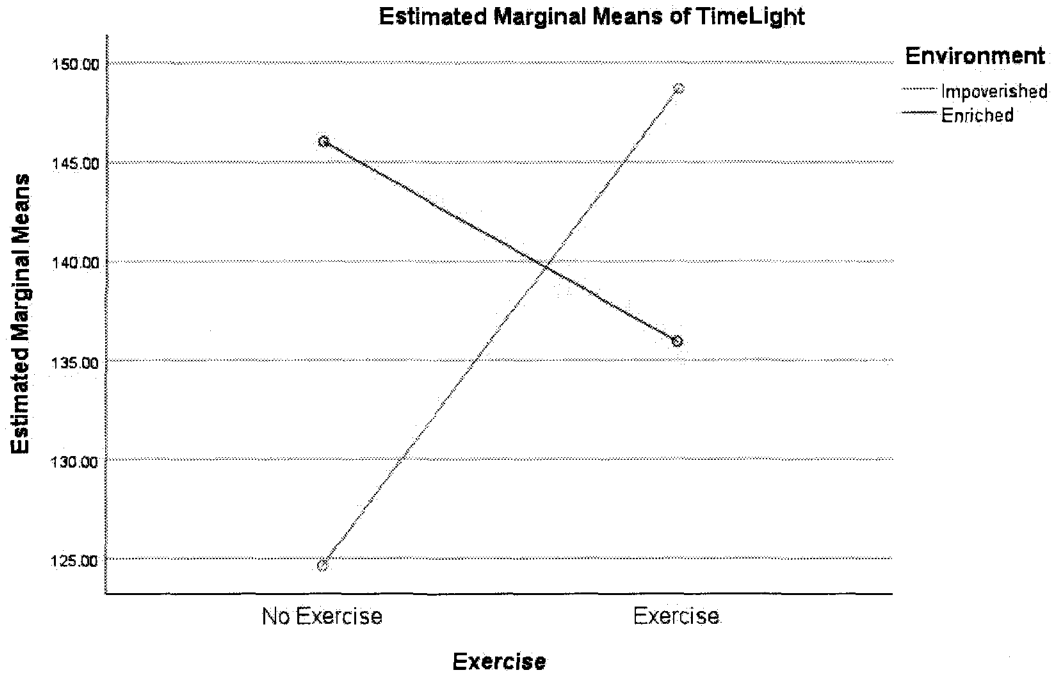

Figure 1. Means plot of exercise condition as a function of environmental condition for the total time spent in the light side of the light/dark chamber.

Appendix C

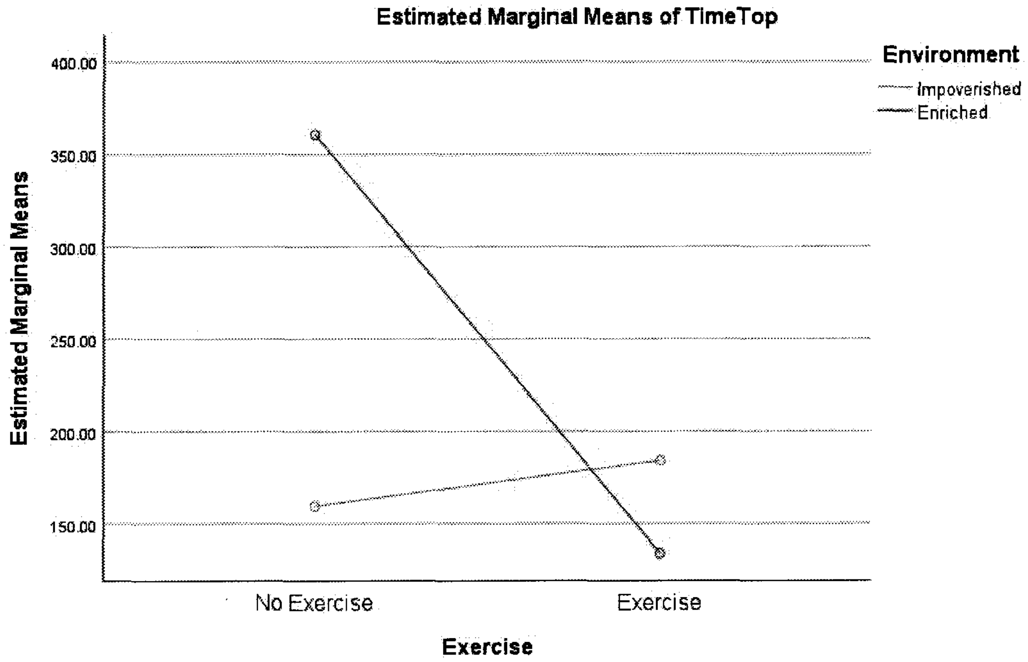

Figure 2. Means plot of exercise condition as a function of environmental condition for the total time spent in the top portion of the novel tank.

Appendix D

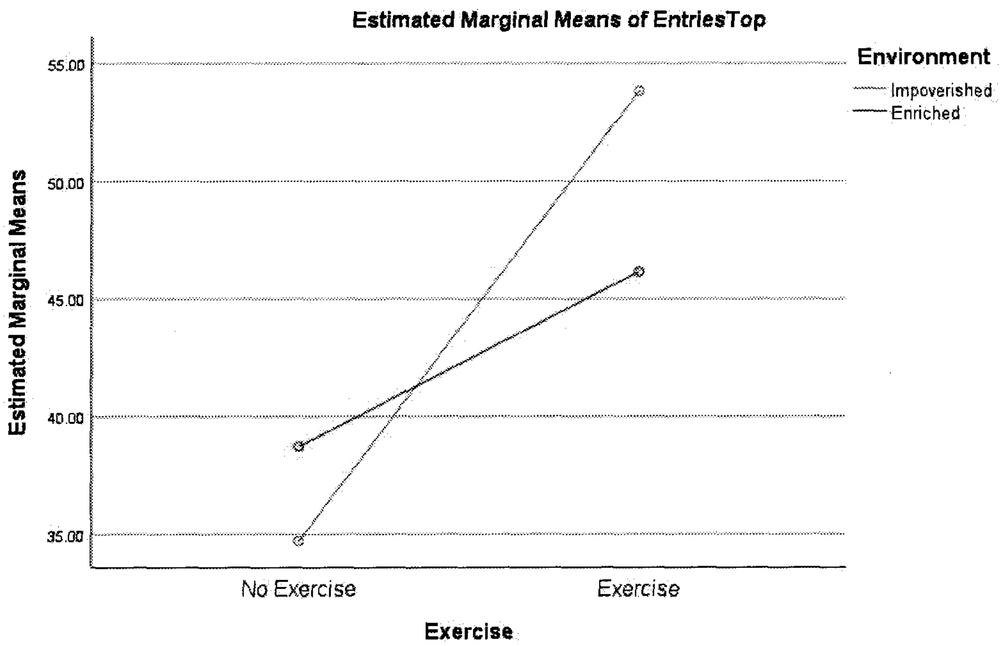

Figure 3. Means plot of exercise condition as a function of environmental condition for the number of entries to the top of the novel tank chamber.

Appendix E

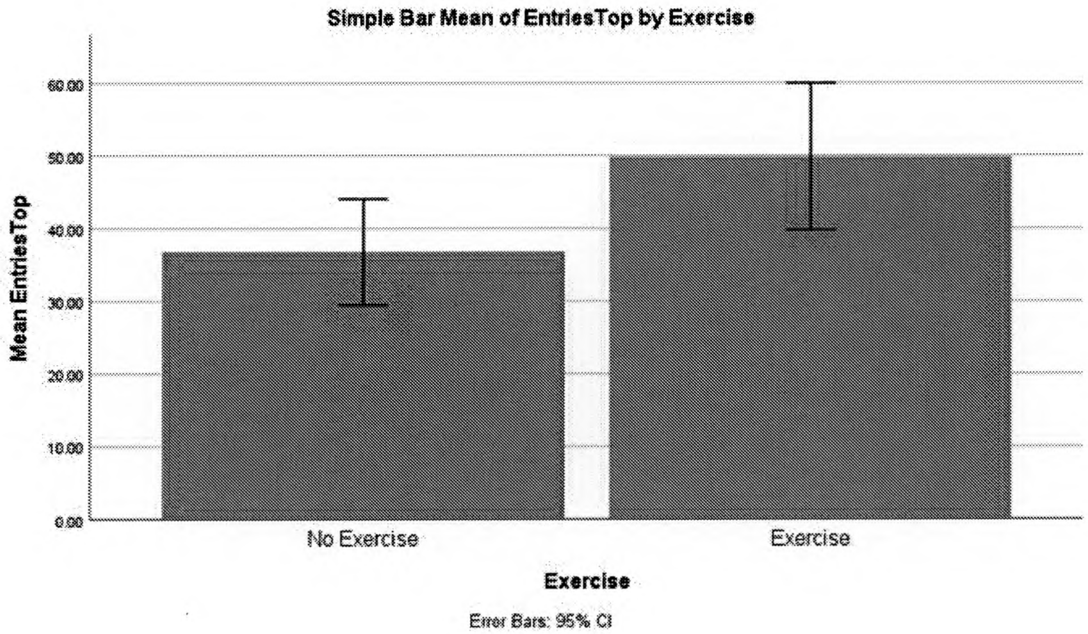

Figure 4. Group comparison of exercise condition as a function of the number of entries into the top portion of the novel tank.