# THE EFFECT OF CANNABIDIOL (CBD) ON THE PHYSIOLOGY AND IMMUNOLOGY OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*) *IN-VITRO* AND *IN-VIVO*

by

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**A Thesis**

*Submitted to the Faculty of Purdue University*

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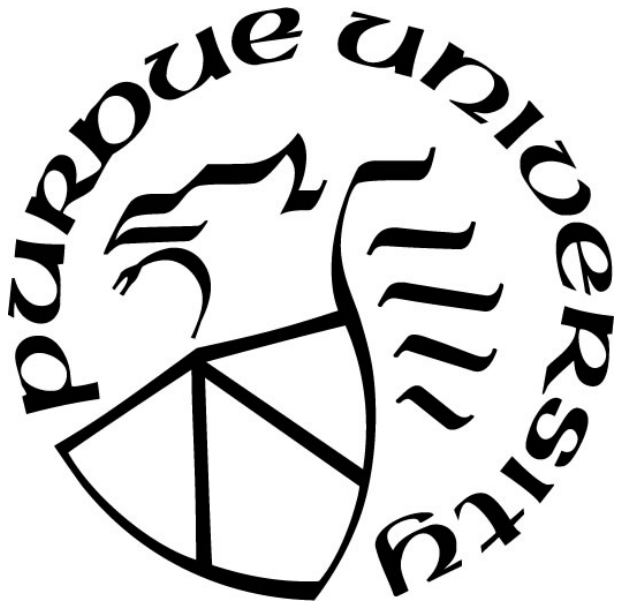
**Master of Science**

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# THE PURDUE UNIVERSITY GRADUATE SCHOOL
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*I would like to dedicate this thesis to my brother Adib Mortuza.*
*Your years in life were few, but I will carry you in my memory forever.*

*My youngest brother, Akib Mortuza will have to wait until the next thesis to get his name in.*

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# ABSTRACT

As the human population increases and the demand for aquaculture increases, aquaculturists are coming up with new ways to mitigate stress in fish to increase their production. Cannabidiol (CBD) is an up and coming nutraceutical that may have potential to reduce stress in not only humans but also other vertebrates such as fish. In this project the effect of CBD on the stress physiology and immunology of Nile tilapia was evaluated both *in-vitro* and *in-vivo*. In the *in-vitro* study, spleen cell proliferation was conducted to observe the effect of CBD on fish T and B cells and were compared to mouse T and B cell proliferation. In the *in-vivo* study, the fish were reared in a recirculating aquaculture system. The effect of CBD on the stress physiology of the fish in short term and long term were evaluated. Based on the short-term acute study, a longer chronic study was designed where tilapia were fed with and without CBD (0.001% of feed weight) and with and without hydrocortisone stress hormone (0.01% of body weight) every day for four weeks. This experiment compared the various growth and feed utilization parameters as well as physiological and immunological parameters such as, plasma cortisol, blood glucose and protein levels, liver and spleen somatic indices (HSI and SSI, respectively), and lysozyme activity of the fish. From our current research, CBD shows potential in stress modulation and in immune modulation. It may have different effects based on the species, whether they need to enhance their immune response or reduce inflammation to be healthy. It also seems to have had different effect on different parts of the immune system. Hematological parameters were not significantly affected by acute stress. CBD did not make any substantial difference in growth. However, in the presence of stress, CBD was able to lower lysozyme activity down to the normal control levels. By administering the proper dosage of CBD on a case by case basis, health benefits can be achieved. Further investigation into the matter may not just be useful in stress mediation in aquatic organisms but may also have implications in human medicine as well.

# CHAPTER 1. GENERAL INTRODUCTION

The human population is increasing and with it, the demand for food is increasing as well. Our food supply is straining to keep up with the demand. Aquaculture may be the solution to keep up with the increasing demand of food. However, in aquaculture, farmers grow fish in high densities to increase profit. This and other environmental factors lead to stress in fish which, in turn, leads to increase in fish's susceptibility to diseases and ultimately to reduction in productivity. To mitigate diseases, farmers use various drugs that lead to production of poor-quality protein, death of non-target species, and environmental pollution. Alternative to these artificial drugs would be to use nutraceuticals to reduce stress. Nutraceuticals are functional food that provide medicinal benefit.

In this 4-part thesis, the stress mitigating and immune modulating potential of Cannabidiols (CBD) is explored. CBD is a cannabinoid found in the extract of marijuana plant. Clinical research on CBD includes studies on stress, anxiety, cognition, movement disorders and pain. However, there is a lack of sufficient high-quality evidence to support the recent claims of CBD in reducing stress, especially in fish. Our experiments aimed to investigate the effects of CBD on the modulation of acute and chronic stress which affect growth and immune response in Nile tilapia both *in-vitro* and *in-vivo*. To test the immune modulating potential, mouse and fish spleen cells were exposed to CBD in various concentrations (*in-vitro*) to see their proliferative effect. For the *in-vivo* acute study, tilapia were fed with commercial feed supplemented with 0%, 0.001%, 0.002% and 0.003% CBD (based on feed weight) with or without stress hormone, cortisol, to see the stress mitigating potential of CBD. For the acute stress study, the fish were sampled at 72 hours to measure their hematological factors. For the chronic stress study, the CBD concentration from the acute study that showed the most stress mitigating potential was used to supplement the commercial feed in the presence or absence of stress hormone in the feed. The fish were sampled at the end of 4 weeks to measure their physiological and immunological status and their growth.

The results of this research are important in understanding the effects of CBD in stress modulation and immune stimulation in not only fish but also other vertebrate organisms. Sound scientific evidence can lead to further medical applications of CBD in humans. With increasing legalization of CBD, increase in production and decrease in price, it may be worth exploring the

potential of using CBD in fish aquaculture to reduce stress. This will reduce the usage of artificial drugs and antibiotics leading to eco-friendly, healthy, and economic farming of fish.

## 1.1 Aim of the experiments

**Overall objective**

- To study the effects of CBD on stress physiology, growth, and immunology of Nile tilapia both *in-vitro* and *in-vivo* (long term and short term).

***In-vitro*: T cell and B cell proliferation assay**

**Chapter 1.** Effects of cannabidiol (CBD) on the immunomodulation of mouse and fish (*Oreochromis niloticus*) spleen immune cells (T and B cells) *in vitro*

- To see if CBD has immune modulatory properties for fish in comparison to mouse.

***In-vivo*: Stress physiology, Immune response, and growth**

- **Acute stress**

**Chapter 2.** Effects of cannabidiol (CBD) on the hematological parameters of Nile tilapia (*Oreochromis niloticus*, Linnaeus) under acute stress *in-vivo*

- Does CBD mitigate acute stress in Nile tilapia?
- Which concentration mitigates stress the best?

- **Chronic stress**

**Chapter 3.** Determination of feed utilization and growth performance of stressed and non-stressed Nile tilapia, *Oreochromis niloticus*, fed cannabidiol (CBD) -supplemented diets

- The most promising concentration of CBD from the acute study was tested long term (4 weeks) to study the effects of CBD on the growth of Nile tilapia.

**Chapter 4.** Effects of cannabidiol (CBD) on the physiology of Nile tilapia (*Oreochromis niloticus*) as a stress mitigating agent *in-vivo*

- The most promising concentration of CBD from the acute study was tested long term (4 weeks) to study the effects of CBD on the stress physiology and immune response of Nile tilapia.

# CHAPTER 2. EFFECTS OF CANNABIDIOL (CBD) ON THE IMMUNOMODULATION OF MOUSE AND FISH (*OREOCHROMIS NILOTICUS*) SPLEEN IMMUNE CELLS (T AND B CELLS) IN-VITRO

Formatted and submitted to the Journal of Immunoassay and Immunochemistry.

## 2.1 Abstract

This experiment tests how mouse and fish spleen cell proliferation are affected by different concentrations of (final concentrations per well: 11.49 µg/mL, 2.30 µg/mL, 0.46 µg/mL, 0.092 µg/mL) of 99% pure CBD oil. Spleen cell proliferation assay was conducted in the presence of concanavalin A (Con A) and lipopolysaccharide (LPS) mitogen for both mouse and fish spleen cells. Results indicate that for mouse spleen T cells, 11.49 µg/mL of CBD was effective in increasing the T cell proliferation when the cells were simulated by Con A. For mouse B cells, CBD seems to have had an inhibitory effect on the cells by reducing the proliferation caused by the mitogen. Similar results are seen in the case of fish spleen cells stimulated with LPS, where the inhibition of spleen cells is seen at the final CBD concentration of 0.46 µg/mL. From our current research, CBD may have different effects based on the species, whether they need to enhance their immune response or reduce inflammation to be healthy. It also seems to have different effect on different parts of the immune system. By administering the proper dosage of CBD on a case by case basis, health benefits can be achieved.

Keywords: Cannabidiol, Spleen cells, T cells, B cells, Mouse, Nile Tilapia, *in-vitro*.

## 2.2 Introduction

Cannabidiol (CBD) is a fairly new nutraceutical. It is a derivative of the marijuana plant (*Cannabis sativa, Cannabis indica, Cannabis ruderalis*). It is a non-psychoactive compound unlike Tetrahydrocannabinol (THC). Clinical research on CBD includes studies on stress, anxiety, cognition, movement disorders, and pain (Morales et al., 2017). CBD can have therapeutic potential such as, neuroprotection (Fasinu et al., 2016), anxiety and psychosis (Morales et al., 2017), epilepsy (Anavi-Goffer et al., 2012), depression (Breuer et al., 2016), and pain (Burstein, 2015). It also has immunological potential such as reducing inflammation (Ben-Shabat et al., 2006),

cancer (Juknat et al., 2016; Burch et al., 2021), and bacterial infection (Ryberg et al., 2007). Despite the widespread therapeutic usage of CBD, not a lot of research has been conducted on the effects of CBD on the immune response of vertebrate organisms such as fish. Some of the existing research seems to be inconclusive. Some studies used commercial CBD, which tends to contain only 1-3% pure CBD with other essential oils with unknown properties. Therefore, it is very important to evaluate the effect of pure CBD on the immune systems of mouse and fish. Most of the immunological research conducted on CBD is via mouse models, and therefore, has a well-established protocol and is a good model to compare against.

Therefore, in this research, we tested the effects of CBD on immune cells of mouse and fish. T and B lymphocytes are major white blood cells of the adaptive immune response that are produced in the bone marrow. The cellular response is mediated by T lymphocytes that mature in the thymus and the humoral response is regulated by B lymphocytes that mature in bone marrow (LaRosa and Orange, 2007). In the thymus, T cells learn to differentiate between self and non-self-antigens and then they migrate to the spleen. T cells can combat against antigens and fight against diseases through clonal expansion (Dwyer and Johnson, 1981). T cells have the ability to recruit or activate other immune cells. In addition, they can also directly eliminate virally infected or cancerous cells (Kumar et al., 2018). On the other hand, B cells are able to produce antibodies to neutralize antigens or enhance the destructive effects of immune cells on the antigens (Hoffman et al., 2016). B cells are also able to aid in the activation of T cell responses. T cells have the ability to recognize processed antigens presented by Antigen Presenting Cells (APC) while B cells can recognize the three-dimensional native form of an antigen. Antibodies produced by B cells are called immunoglobulin. There are five classes of immunoglobulin (Ig G, Ig A, Ig M, Ig E, Ig D) and depending on the type of antigen, B cells are able to go through class switch for appropriate responses (Elena and Damaris, 2013). These immunoglobulins can also be named as B cell receptor and they can bind to antigens and initiate multiple immune responses such as phagocytosis, neutralization of receptors, complement activation (Elena and Damaris, 2013). On the other hand, there are two major types of T cells, helper T cells and T cytotoxic cells. T helper cells aid other cells against antigen in the immune system and T cytotoxic cells have the ability to kill virally infected cells and tumors. Unlike antibodies produced by B cells, T cells do not have the ability to bind directly to antigen (LaRosa and Orange, 2007).

Although our immune cells help us fight against antigens, excessive proliferation of these cells can harm our own cells (Rosenblum et al., 2015). Sometimes, overreaction of these immune cells can lead to autoimmune disorders, a condition where the body's immune system attacks its own healthy cells (Rosenblum et al., 2015). There are various types of autoimmune diseases that can range from organ specific (T cells or antibodies react to self-antigens within localized tissues) to systemic (reactivity against a specific antigen or antigens throughout various tissues in the body) (Smith and Germolec, 1999). There has been much research on the topic of autoimmune diseases. However, many questions regarding etiology and effective suppressive agents against autoimmune diseases are still being investigated and discovered. CBD has been shown to act as an immunosuppressant and reduce inflammation (Ben-Shabat et al., 2006). According to Kozela et al. (2011), CBD was administered (5 mg/kg i.p.) at the onset of a disease. They mentioned that CBD was found to reduce T cell proliferation *in-vitro*. In another experiment, 0.01–20 μg/mL or 0.03–64 μM of CBD was shown to inhibit phytohemagglutinin (PHA)-stimulated IFN-γ production in T cells (Watzl et al., 1991). McKallip et al. (2006) also stated in their experiment that CBD-induced apoptosis was observed in Jurkat and MOLT4 human T cells. Unlike T cells proliferation experiments with CBD, few research exists on the effect of CBD on B cells. Zimmerman et al. (1977) found in their experiment that when CBD was administered at 25mg/kg through intraperitoneal (i.p.) injection to sRBC-induced plaque-forming cells, antibody production was reduced. Another research conducted by Kaplan et al. where CBD was administered orally, it was observed that CBD reduced the production of antibodies (Kaplan et al., 2008). Additionally, Wu et al. (2008) demonstrated that CBD induced apoptosis in B cells. Overall, these findings suggest that CBD seems to not just reduce inflammation but also inhibit major lymphocytes and antibody production.

For this study, we worked with mouse and fish model systems. For the mouse system, we chose BALB/c mice as it is widely used in immunological research and is a great model organism for the human immune system. We chose Nile tilapia as our fish model, as it is one of the most cultivated fish around the world due to their hardiness (Gupta and Acosta, 2004). Nile tilapia originated in Africa and were widely cultivated in Egypt (Barcellos et al., 2001). Nile tilapia is widely used as a fish model in research and would be a good standard to compare against mouse immune system. Benefits of using a fish model is that they have a similar stress physiology and immunological response compared to other vertebrate animals including humans. Fish innate

immune system consists of almost all the same elements as that of mammals (Magnadóttir, 2006). Therefore, findings may have implications for human medicine. Most importantly, stress is a big issue in aquaculture where farmers try to maximize their productivity by culturing fish in high densities. This stresses the fish by polluting the water and thus lowering dissolved oxygen levels which leads to lower productivity (Hough et al., 2016). To mitigate this, farmers often use drugs that may pollute and affect non-target species. Nutraceutical use is suggested in reducing stress as an alternative. CBD could potentially be used to modulate stress and immunity to increase the productivity of fish in traditional aquaculture systems.

The aim of the experiment was to determine the effect of CBD on the immune response of BALB/c mouse and Nile tilapia *in-vitro.* This was done across various dilutions of CBD, such as 1:5, 1:25, 1:125 and 1:625 or final concentrations of 11.49 µg/mL, 2.30 µg/mL, 0.46 µg/mL and 0.092 µg/mL in each well. These dilutions were chosen to see the effects of CBD on the cellular and humoral immune response of a terrestrial and aquatic organism model over a large range of concentrations. We did so by testing the effects of CBD on mature T cell (from spleen) proliferation to test the cellular immune response. T cells were stimulated using Concanavalin A (Con A) which is a highly antigenic substance (Raetz and Whitfield, 2002) originally extracted from jack beans. We tested for the humoral immune response of mouse and Nile tilapia by testing on B cell proliferation as it is an important part of the humoral immune response. B cells were stimulated by using lipopolysaccharide (LPS), a highly antigenic substance, widely used for the purpose (Malek and Castro, 2010).

## 2.3 Methods

### 2.3.1 Animal maintenance

Nile tilapia (mean length 23.5 cm and mean weight 240 g) were collected from Troyer Farms, Indiana. The fish were kept in a recirculating aquaculture system for the duration of the experiment (pH: 6.0-7.0, ammonia: 1.0-3.0 mg/dL, temperature: 25-28 °C, dissolved oxygen (DO) 5.00-7.00 mg/L). The fish were fed with commercial feed Purina® AquaMax® Fingerling Starter 300 (Purina Mills, MO, USA) twice a day at 1.5% of their body weight (3% daily). The mice were obtained from Jackson Laboratories (Bar Harbor, ME). Both mouse and fish were taken care of

following Purdue University Animal Care and Usage Committee (PACUC) approved protocols.

### 2.3.2 Sampling

The fish were euthanized with Tricaine Methane sulfonate (MS-222) (Sigma-Aldrich; St. Louis, MO) at 400 mg/L within 2 minutes of catching the fish to reduce fish stress from handling. The spleen was collected from the fish using aseptic techniques. 3-month-old mice were sacrificed via cervical dislocation and dissected using aseptic techniques to obtain its spleen and the spleen was immediately put in RPMI 1640+10% FBS media.

### 2.3.3 Experimental design

The spleen cell proliferation experiments were conducted following the experimental methodology outlined in Ottenweller et al. (2004). The spleen T and B cell proliferation assay experiments were conducted with both mouse (BALB/c) and fish (Nile tilapia) spleen in the presence of mitogen. Separate 96 well plates were used for mouse and fish spleen proliferation as they needed to be incubated at different temperatures. Each of the factors were plated in triplicates. In the case of T cells, Con A (Concanavalin A from *Canavalia ensiformis*, Type IV, lyophilized powder from Sigma) was used as a mitogen. LPS (Lipopolysaccharides from *Escherichia coli* O26:B6, ≥10,000 EU/mg, purified by phenol extraction from Sigma) was used in the case of spleen B cells. 7.5 µL of 100 µg/mL Con A (final concentration of 3.45 µg/mL per well) was used in the case of mouse T cells (Biswas et al., 2020) and 50 µg/mL Con A (final concentration of 1.72 µg/mL per well) was used in the case of fish T cells (Edholm et al., 2007) following previous experimental protocols stimulating T cells. 7.5 µL of 80 µg/mL LPS (final concentration of 2.76 µg/mL per well) was used in the case of mouse B cells and 40 µg/mL LPS (final concentration of 1.38 µg/mL per well) was used in the case of fish B cells (Chen et al., 2020) as per previously established experiment stimulating B cells. The experiment was conducted across various final concentrations of CBD (11.49 µg/mL, 2.30 µg/mL, 0.46 µg/mL and 0.092 µg/mL per well), in each well of the 96 well plates in the presence of mitogen. A negative and a positive control was also established per species and for mitogens Con A and LPS. Negative controls only contained spleen cells (mouse/fish) in media and positive controls only contained spleen cells (mouse/ fish)

in media along with the appropriate mitogen (Con A/ LPS). The volumes, concentrations as well as the final concentrations of the factors added to each of the wells are also provided.

### 2.3.4 Cell standardization

A single cell suspension of cells from mouse and fish spleen were made separately by placing the organs on top of a heat sterilized sieve in a petri dish. Cells were then suspended in 1 ml of RPMI 1640 (Mediatech, Inc, Herndon, VA) after macerating with a sterile plunger and collecting the cells in a non-adhesive syringe. Isotonic solution (pH 7, 295 µL) was added along with Trypan Blue (100 µL, to dye the cells) and lyse buffer (100 µL, to lyse red blood cells) to an aliquot amount of the tissues in media (5 µL). A hemocytometer was used to count the number of cells under a microscope. Then the suspensions were diluted appropriately to achieve a final cell count of $1x10^7$ cells/ mL for both mouse and fish spleens separately.

### 2.3.5 Lymphocyte proliferation assay

Once the cells were standardized, two 96 well plates were obtained, one for mouse and the other for fish spleen cells. 100 µL of the standardized spleen cells were then plated in each of the wells of the mouse and fish spleen cell plates in 100 µL of RPMI 1640+ 10% FBS solution. This made for a final cell count of $1x10^6$ cells per well of each of the plates. All the treatment groups were plated in triplicates. For mouse T cell proliferation, 7.5 µL of mitogen Con A (stock 100 µg/mL) was put in each of the wells (except (-) control), making for a final concentration of 3.45 µg/mL per well. For mouse B cells, 7.5 µL of mitogen LPS (stock 80 µg/mL) was put in each of the wells, making for a final concentration of 2.76 µg/mL per well. All the wells received 7.5 µL of mitogen except for the (-) control wells, which only contained cells in media and 7.5 µL of sterile deionized water in the place of a mitogen. The same procedure was followed for the fish spleen cell plate. In the case of fish spleen cells however, the mitogen used for T and B cells were less concentrated. For fish T cell proliferation, 7.5 µL of mitogen Con A (stock 50 µg/mL) was put in each of the wells, making for a final concentration of 1.72 µg/mL per well. For fish B cell proliferation, 7.5 µL of mitogen LPS (stock 40 µg/mL) was put in each of the wells, making for a final concentration of 1.38 µg/mL per well.

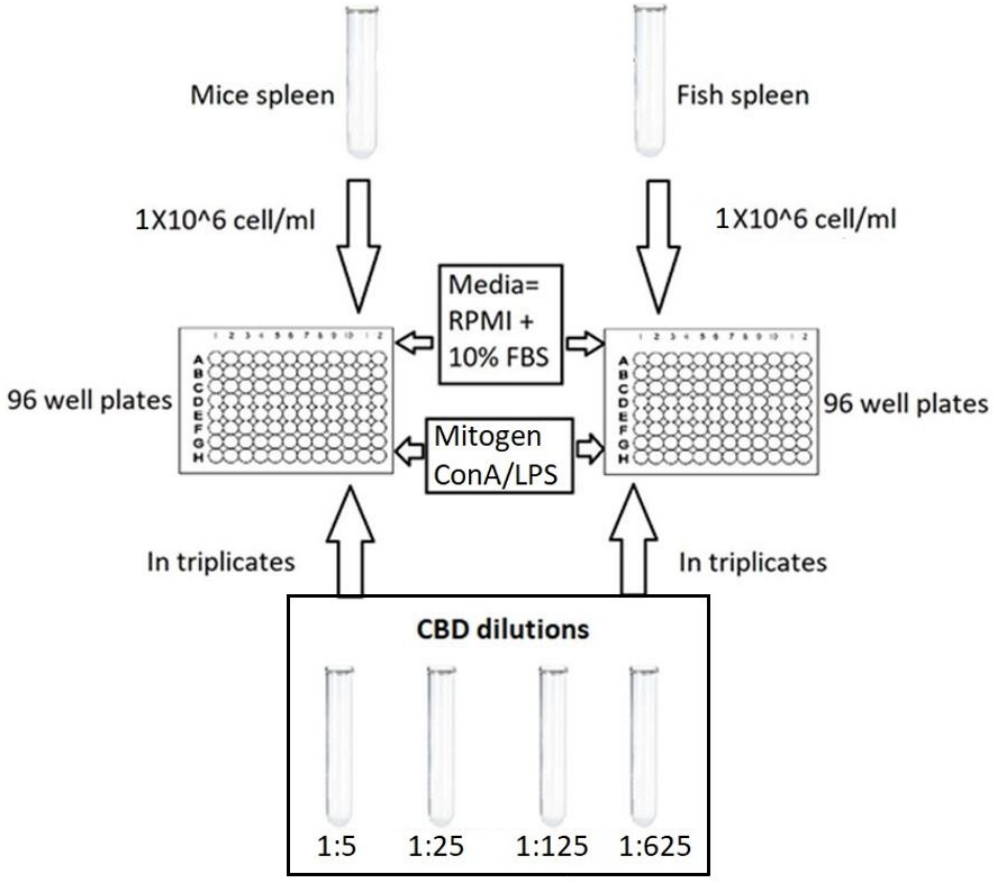


Figure 2.1. Diagrammatic representation of the Spleen T and B cell proliferation assay. Mouse and fish spleen are standardized in media ($1x10^6$ cell/mL) and plated separately in each well of the two 96 well plates. Media is added to all the wells and mitogen (Con A or LPS) is added to the appropriate wells of both the plates. Various dilutions of CBD are plated in the appropriate wells in triplicates in each plate. Then the mouse plate is incubated at 37ºC and the fish plate is incubated at 28ºC.

The CBD (99% pure isolate from Sigma Aldrich®, MO, USA) used in this experiment was diluted to 1:5, 1:25, 1:125 and 1:625 or stock concentrations of 250 µg/mL, 50 µg/mL, 10 µg/mL and 2 µg/mL. Each of these dilutions were then plated in triplicates of 10 µL per well making for a final concentration of 11.49 µg/mL, 2.30 µg/mL, 0.46 µg/mL and 0.092 µg/mL in each well. Only (-) and (+) control wells did not receive any of the CBD dilutions and in the place of CBD, 10 µL of sterile deionized water was added to fill the blank. This procedure was repeated for mouse T and B cells as well as fish T and B cells. Figure 2.1 diagrammatically represents the process outlined.

Mouse cells were then cultured at 37 °C, in 5% $CO_2$ and 95% humidity for 48 hours and fish cells were cultured at 28 °C in the same conditions. The lower incubation temperature for fish is due to the lower basal body temperature of fish compared to mammals. This is because, unlike mammals, fish are poikilotherms (Nivelle et al., 2019) and are not exposed to the high body temperatures mammals have due to high metabolism (Scholander et al., 1950). Nile tilapia have been found living in nature between the temperatures of 8-42 ºC. For this research the fish were maintained between 25-28 ºC. The fish spleen incubation temperature was determined based on previously conducted experiments (Dominguez t al., 2004; Khan, 2016). 28 ºC is a few degrees higher than the average temperature at which Nile tilapia are found and therefore should make for a good cell incubation temperature for poikilotherms such as Nile tilapia. 48 hours later, the cells were observed under an inverted microscope for proliferation and potential contamination. Then 10 µL (working concentration: 0.0375 µCi (Curie)/ µL) of radioactive 3Thymidine (3H) (Moravek Biochemicals, CA, USA. Stock Concentration: 1mCi/mL) was added to each well of the plates so that each well contained 0.375 µci. The cells incorporated the radiolabeled 3H as a component of their replicating DNA while performing mitotic division. The quantity of radioactive thymidine that gets taken up is proportional to cell proliferation. This was done for both mouse and fish spleen plates.

A cell harvester (Brandel Cell Harvester: model# M-24) was used to harvest the DNA from the cells. Filter paper (Whatman™) strips were used to harvest the components. 10% TCA (trichloroacetic acid) was used to precipitate the DNA from the cells and then ethanol was used to wash any free thymidine off the filter paper and fix the DNA. The filter papers were washed 5 times with 10% TCA. The filters were then dried for 3 hours and put into scintillation vials with 3 ml of scintillation cocktail (Ecolume™ scintillation cocktail, MP Biomedicals) to submerge in. The vials were then put into a scintillation counter (Beckman Coulter™ LS 6500 Multi-Purpose Scintillation Counter) to determine the radioactivity count in counts per minute (CPM) using a beta counter filter (Geiger counter). The experiment was replicated, and the data shown in this paper is a compilation of the runs. The CPM values obtained were made into percentages (% of control) for ease of understanding and were compared to the (-) control (100%).

### 2.3.6 Statistical analysis

The data obtained from these experiments were analyzed by one-way analysis of variance (ANOVA) using SigmaStat® 14.0, Systat Software Inc to see if there were significant difference between any of the treatment groups. For all data that was statistically significant ($P < 0.05$), a Tukey's HSD test (post ANOVA comparison of multiple means) was performed in order to determine any differences between treatments. The data are presented as means $\pm$ and standard errors of means (SEM).

## 2.4 Results and discussion

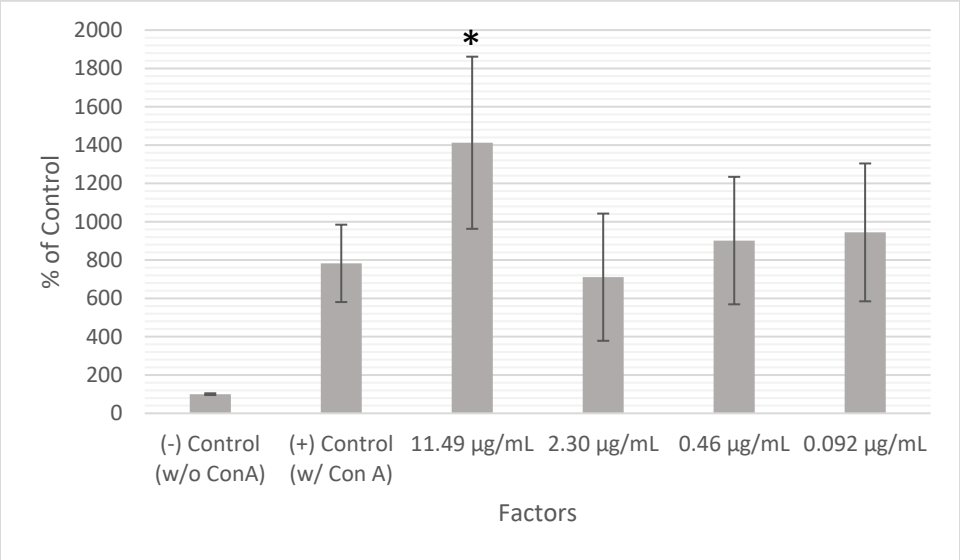


Figure 2.2. % of Control of mouse T cells applied with various factors. (-) Control= media+ cells; (+) Control= media+ cells+ mitogen. The concentrations of CBD provided are the final concentrations in each of the wells with Con A. Results are presented as means± SEM. *= significantly different from (-) Control ($P \leq 0.05$).

Figure 2.2 shows the % of control of mouse spleen T cells when various dilutions of CBD were applied. (+) control group (~800%) exhibited higher % of control than the (-) control group (8 times higher). Although this was not significantly different, the trend shows the known ability of Con A behaving as an antigenic substance in stimulating the T cell response (Palacios, 1982; Larsson and Coutinho, 1979). At 11.49 µg/mL (~1400%), CBD increased the % of control of the spleen cells significantly in comparison to the (-) control (100%). In lower concentrations of CBD,

there was no significant proliferation of the spleen cells compared to the (-) control. The proliferation seen at 2.30, 0.46 and 0.092 µg/mL are mostly due to the mitogen added as the same trend is also seen in the absence of any CBD and in the presence of mitogen in (+) control. Therefore, at 11.49 µg/mL final concentration, CBD was able to significantly increase spleen T cell proliferation in the presence of a mitogen. In lower concentrations however, it made no difference in comparison to cells that were exposed to only the mitogen. A similar trend was observed in other studies with T cell proliferation of C57 mouse spleen cells exposed to Con A and CBD. CBD at the highest concentration significantly increased the T cell proliferation compared to the control and lower concentrations made no difference (Ben-Shabat et al., 2006; Burch et al., 2021). Perhaps, in the presence of diseases, when the immune T cells are stimulated, CBD at aforementioned concentration would be able to increase the spleen T cell proliferation and enhance the immune system.

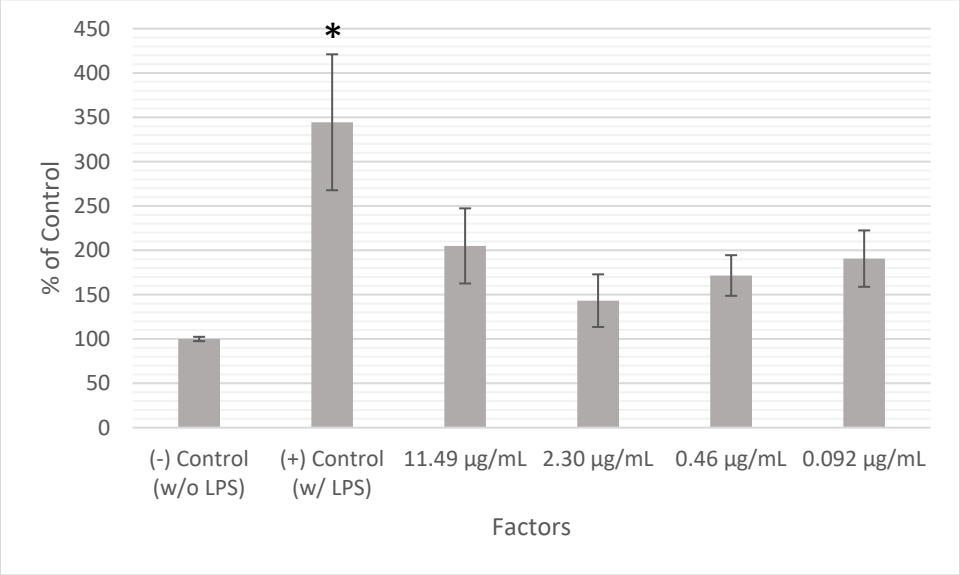


Figure 2.3. % of Control of mouse B cells applied with various factors. (-) Control= media+ cells; (+) Control= media+ cells+ mitogen. The concentrations of CBD provided are the final concentrations in each of the wells with Con A. Results are presented as means± SEM. *= significantly different from (-) Control (P≤0.05).

On the other hand, the mouse spleen B cell response (Figure 2.3) was slightly different from that of the mouse T cell response (Figure 2.2). (+) control (~340%) showed significantly higher % of control than the (-) control (100%). This demonstrates the property of LPS in acting

as a mitogen in stimulating the spleen B cell response (Tough et al., 1997). However, when applied with various dilutions of CBD and mitogen, the % of control seems to fall (from ~340% to ~200-140%) lower than that of the (+) control. In this case, the CBD is acting as an inhibitor of spleen B cell proliferation and undoing the effect the mitogen was having in stimulating the spleen B cell response and reducing the % of control of the samples. Previous studies have demonstrated the ability of CBD in reducing splenocyte counts and acting as an immunosuppressant.

Kaplan et al. (2008) demonstrated that CBD inhibited the production of interleukin-2 (IL-2) in phorbol ester and calcium ionophore (PMA/Io)-activated murine splenocytes. They also illustrated that CBD suppressed mitogen stimulated T and B cells as the concentration of CBD increased. The difference in our results could be originating from differences in methodology. For our experiment, we utilized Con A to activate T cells and LPS to activate B cells. Unlike our experiment, the authors utilized anti-CD3 antibodies to activate the splenocytes. Mice anti-CD3 antibodies have the ability to induce T cell proliferation in the presence of Fc receptor (FcR)-bearing accessory cells (Austyn et al., 1987). Also, instead of the 99% pure CBD extract we utilized in this experiment, the authors used 20-30% CBD extract. Their experiment had a final CBD concentration range of 3-15 ug/mL while our concentration of CBD ranged from 0.092 µg/mL - 11.49 µg/mL. Khuja et al. (2019) compared the effects of D9 tetrahydrocannabinol (THC) and cannabidiol (CBD) on the activation of lymphocytes *in-vitro* and *in-vivo* utilizing bone marrow transplantation (BMT) murine models (C57BL/6 and BALB/c). Overall *in-vitro*, the researchers found that treatment of CBD decreased the proliferation of activated lymphocytes and affected the secretion of cytokines. For *in-vivo*, the experiment summarized that both CBD and THC treatments inhibited lymphocyte recovery in a syngeneic transplantation model. Khuja et al. (2019) concluded that CBD was better at inhibiting lymphocytes proliferation compared to THC. Another research by Wu et al. (2008), investigated the potential mechanisms of T cells affected by CBD treatments. They found that apoptosis of splenocytes increased as the concentration of CBD and amount of time increased. The article mentioned that exposure of CBD to splenocytes resulted in production of reactive oxygen species (ROS) after 1 hour of the treatment. Additionally, caspase-8, the protein relating to apoptosis mechanism, increased after CBD treatments. After reviewing previous research in comparison to our own, it looks like CBD would make for a good immunosuppressant for mouse. However, there was a lack of research in this area on fish.

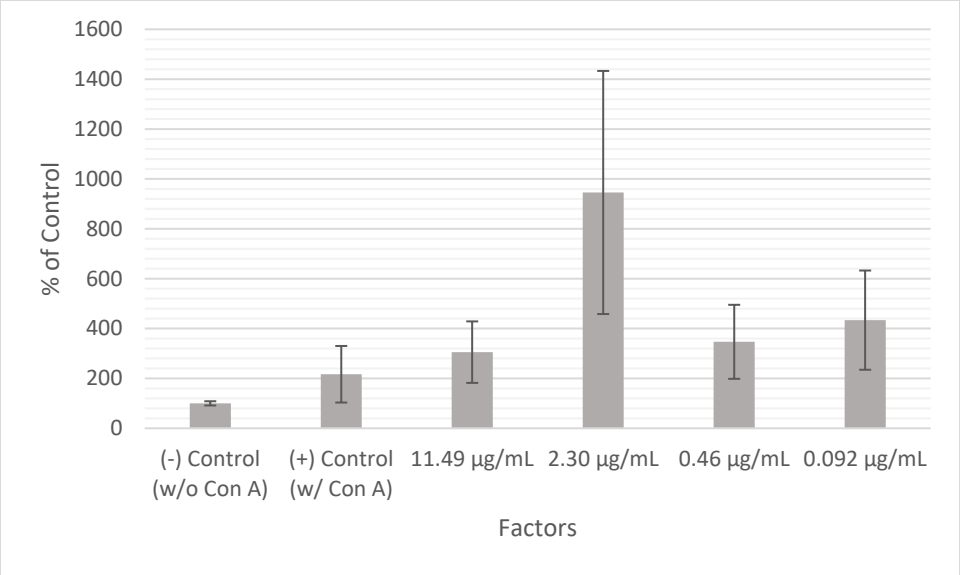


Figure 2.4. % of Control of fish T cells applied with various factors. (-) Control= media+ cells; (+) Control= media+ cells+ mitogen. The concentrations of CBD provided are the final concentrations in each of the wells with Con A. Results are presented as means± SEM.

In the case of fish spleen T cells (Figure 2.4), there was no significant difference between any of the groups. There is no evidence that the mitogen (Con A, final concentration of 1.72 µg/mL per well) used was able to stimulate the fish spleen T cells. Perhaps, a different mitogen needs to be used or a higher concentration is needed. Perhaps the incubation period needs to be longer see any spleen T cell proliferation in Nile tilapia. The only CBD dilution that stands out is at the final concentration of 2.30 µg/mL, which seemed to give rise to a higher % of control in the presence of mitogen than the control groups without any CBD. However, it is not significantly different from any of the other groups. No other studies were found on fish T cell proliferation assay using CBD to compare against. It is clear that not a lot of research has been done using CBD on fish splenocytes. Further studies need to be conducted to improve the protocol for fish spleen cell proliferation. Future studies with perhaps a higher concentration of mitogen and a longer period of incubation may lead to a clearer picture.

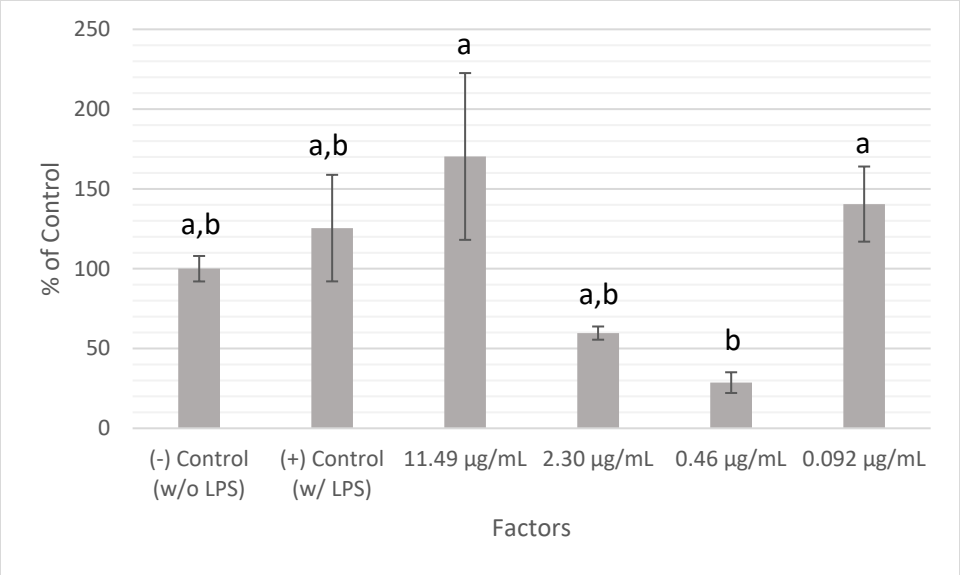


Figure 2.5. % of Control of fish B cells applied with various factors. (-) Control= media+ cells; (+) Control= media+ cells+ mitogen. The concentrations of CBD provided are the final concentrations in each of the wells with Con A. Results are presented as means± SEM. Different lettered graphs are significantly different from each other (P≤0.05).

Finally, in the case of fish spleen B cells (Figure 2.5), we see that there was no significant difference between (-) and (+) control. We do not know how effective the mitogen (LPS, final concentration 1.38 µg/mL per well) was in stimulating the spleen B cells. We do see a slight increase in % of control going from (-) to (+) control (100% to ~125%) and we see a further increase in % of control going from (+) control to the final concentration of 11.49 µg/mL CBD (~125% to ~170%). This gradual increase could be due to the mitogen having some effect and then the proliferation could be further increased by the presence of CBD. However, this is a sharp contrast to what begins to happen in the next 2 dilutions. Going from 11.49 µg/mL to 2.30 µg/mL and 0.46 µg/mL, we see a significant decrease in % of control or splenocytes (from ~170% to ~30% of control). It seems to be that CBD acts to inhibit the spleen cell proliferation when CBD is applied at the final concentration of 0.46 µg/mL. Just as in the case of fish T cell proliferation, we were not able to find other research that has looked at the effects of CBD on fish B cells or splenocytes. However, our findings here are consistent with our mouse B cell results (Figure 2.3) as well as other cited research conducted on mouse splenocytes where the ability of CBD in inhibiting spleen cells were exhibited. Perhaps at the right dosage, CBD can be used to selectively increase or decrease the immune response to either fight pathogens or reduce inflammation. It is also evident

that CBD impacts different species and within the same species different parts of the immune system differently.

Overall, the results indicate that mouse spleen T and B cells were stimulated by our current protocol and mitogen concentrations. However, the same cannot be said about the fish spleen T and B cells. Perhaps, a higher concentration of mitogen or different ones can be used along with longer incubation periods to properly evaluate the effects of CBD on the fish spleen cells. For mouse spleen T cells, 11.49 µg/mL final concentration of CBD was effective in increasing the T cell proliferation when the cells were stimulated by a mitogen. For mouse B cells, CBD seems to have had an inhibitory effect on the cells by reducing the proliferation caused by the mitogen. Similar results are seen in the case of fish spleen cells stimulated with LPS; the highest inhibition of spleen cells is seen at the final CBD concentration of 0.46 µg/mL.

## 2.5 Conclusion

Despite its widespread usage, whether CBD is as beneficial as it is claimed is not properly established. From our current research, CBD seems to not be a "one size fits all" and can have different effects based on the species, whether or not the organism is healthy and whether they need to enhance their immune response or reduce inflammation. By administering the proper dosage of CBD on a case by case basis, health benefits can be achieved. In order to determine the proper dose and usage, further studies need to be conducted on various species both *in-vitro* and *in-vivo*. Studies also need to be conducted on the effects of CBD on various immune signaling molecules. Such studies may not only have an impact on animal rearing but also have an impact on human medicine.

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# CHAPTER 3. EFFECTS OF CANNABIDIOL (CBD) ON THE HEMATOLOGICAL PARAMETERS OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*, LINNAEUS) UNDER ACUTE STRESS IN-VIVO

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## 3.1 Abstract

This study evaluates the effects of Cannabidiol (CBD) on the hematological parameters of acutely stressed and non-stressed Nile tilapia (*Oreochromis niloticus*, Linnaeus), reared in a recirculating aquaculture system. *O. niloticus* were fed with and without CBD (0, 0.001, 0.002 and 0.003% of feed weight) and with and without hydrocortisone stress hormone (0 or 0.01% of body weight) for 72 hours. This experiment compared blood glucose, hematocrit and protein levels of the plasma of the fish. Among the non-stressed groups, fish fed with the highest concentration of CBD showed one of the lowest stress in terms of the stress biomarkers, while one of the highest stress was detected in the stressed group supplemented with no CBD. No significant difference in stress was observed between the different concentrations of CBD supplementation of fish in 72-hour period.

Keywords: CBD; Acute; Nile tilapia; Stress physiology; *in-vivo*.

## 3.2 Introduction

In aquaculture, stress in fish has become a significant issue due to husbandry methods. Crowding, handling, and vaccinating are a few different husbandry methods used in aquaculture that may lead to stress and thus, lower growth rates and higher death rates from diseases (Hough, Glaze, Blumenthal, and Mustafa, 2016). To mitigate stress and its consequences farmers often use harmful substances, such as antibiotics and chemical drugs. These substances may lead to the creation of superbugs or affect non-target species. These harmful substances may also have the potential to affect human health if the chemicals remain in the fish's system and then consumed. In order to solve these issues, we must understand stress and how to modulate its effects.

Stress is a state of decreased fitness or a response to any external agent that challenges homeostasis and threatens its survival (Colombo, Pickering, Belvedere, and Schreck, 1990). It is a very deleterious and common phenomenon in farming, especially in aquaculture. A decrease in productivity due to disease and poor health can result from poor stress management, which can cause farmers to face significant loss in profit every year. Stress can be classified as either adaptive or maladaptive. The adaptive stress response occurs when the body learns how to overcome stress by maintaining the normal basal state of bodily activities. The maladaptive stress response is when prolonged exposure to a stressor leads to many different deleterious effects (Barton and Iwama, 1991). There are three hormones that control the stress response: adrenaline, noradrenaline, and cortisol. Together, these endogenous hormones target the sympathetic nervous system, which is responsible for initiating the fight or flight response. The fight or flight response is responsible for placing many vital mechanisms of the body on hold (digestion, immune response, growth, reproduction, etc.) and enhancing the blood circulation to the muscles, increasing heart rate, and preparing the body to either fight or flee. This fight or flight response can be useful for a short period of time (acute stress) but may become deleterious with prolonged exposure (chronic stress) (Barton and Iwama, 1991). Chronic stress can make an organism susceptible to various secondary diseases by weakening the immune and inflammatory response (Eddie and Norman, 2008). Depending on the intensity, frequency, and type of the acute stressor, acute stress may also have a similar effect on physiology as chronic stress. However, by mitigating acute stress, such negative effects could be averted (Barton and Iwama, 1991).

When an organism is exposed to stress, several physiological changes take place (Moyle and Schreck, 1990). These physiological changes caused by stress can be categorized as primary, secondary, and tertiary responses. After a stressor is detected by an organism, hormones (cortisol and adrenaline) are released into the blood by the central nervous system, initiating the primary stress response. Cortisol is made after corticotropin releasing hormone (CRH), released from the hypothalamus, signals the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH is carried by the blood to the interrenal cells in the head kidney from which the cortisol is released. Adrenaline (epinephrine) is released by chromaffin tissue that is found within the head kidney after the direct stimulation of the sympathetic nervous system, initiating the secondary stress response. The secondary response is characterized by changes in blood and tissue, which is the main focus of this research. According to Moyle and Schreck (1990), hematological

changes such as elevated blood glucose (hyperglycemia) and reduced blood clotting time may occur. Diuresis as well as electrolyte losses also occur, which leads to osmoregulatory dysfunction. Several tissue changes are also observed when an organism is stressed, such as: the depletion of liver glycogen and interrenal vitamin C, hemorrhage of the thymus, and hypertrophy of the interrenal body. The tertiary response is characterized by major physiological changes or whole-body responses. The tertiary response is usually triggered by the chronic stress response but depending on the intensity, frequency and type of stressor, it may also occur due to acute stress. Reduced resistance to infectious diseases, growth, reproductive success, and survival will occur. This causes delayed progression from one life stage to the next in fish, which can lead to decline in population (Moyle and Schreck, 1990). We can measure secondary stress biomarkers to determine the level of acute stress of an organism since it usually takes much longer to induce a tertiary stress response, as seen in chronic stress. In this paper we will focus on the secondary stress biomarkers (hematological biomarkers of stress) such as blood glucose, hematocrit and plasma protein of Nile tilapia (*Oreochromis niloticus*, Linnaeus).

Currently, the common practice, in aquaculture, is to use chemical treatments such as drugs and antibiotics to mitigate the effects of stress. However, as discussed earlier, the usage of antibiotics and chemicals in aquaculture can lead to environmental pollution and affect non-target species. Antibiotic usage can lead to the buildup of bacterial resistance and the emergence of superbugs. Therefore, scientists are on the search for alternative solutions such as nutraceuticals to mediate stress physiology of fish. Nutraceuticals are functional foods (often phytochemicals) that provide medicinal benefits (Lim and Webster, 2006). Nutraceuticals can potentially alleviate stress and boost immunity in various animals including fish (Mustafa, Randolph, and Dhawale, 2011; Ibrahim, Khan, Rinard, and Mustafa, 2015; Mustafa, Dhawale, Park, and Yoo, 2013).

Cannabidiol (CBD), a fairly new nutraceutical, is a derivative of the marijuana plant (*Cannabis sativa*, Linnaeus). CBD is a non-psychoactive compound unlike its counterpart Tetrahydrocannabinol (THC). Clinical research on CBD includes studies on stress, anxiety, cognition, movement disorders, and pain (Morales, Reggio, and Jagerovic, 2017). CBD has been shown to have therapeutic potential by functioning as a neuroprotectant (Fasinu, Phillips, ElSohly, and Walker, 2016), relieving anxiety, psychosis (Morales et al., 2017), epilepsy (Anavi-Goffer et al., 2012), depression (Breuer et al. 2016), and pain (Burstein, 2015). It has also shown to have immunological potential by reducing inflammation (Ben-Shabat, Hanuš, Katzavian, and Gallily,

2006), cancer (Burch et al., 2021; Juknat, Kozela, Kaushansky, and Mechoulam, 2016), and bacterial infections (Ryberg et al., 2007). Viudez-Martínez, García-Gutiérrez, and Manzanares (2018) aimed to assess the effect of CBD on different genes that target/control the hypothalamus-pituitary-adrenal axis under control and stress conditions in mice. They did this by administering 5 mg/kg, 15 mg/kg or 30 mg/kg (of mice body weight) of CBD intraperitoneally before exposing the mice to stress via restraining. Using real time PCR analysis, the relative gene expression of corticotropin-releasing factor was measured. Interestingly, CBD at 5 mg/kg and 15 mg/kg were able to block the gene expression caused by acute stress. Expression of stress related genes are certain to have a physiological impact on the whole organism's stress response which was yet to be tested with CBD.

Despite the widespread therapeutic usage of CBD, not a lot of research has been conducted on the effects of CBD on the stress physiology of vertebrates, whether it could be used to mitigate stress and its ability to compensate for growth in high stress conditions of aquaculture. Since there is a scarcity of research on the effect of CBD in mitigating stress in mice and the lack of research on the topic applied to fish stress model, we had to design our own experiment to determine the dosage of CBD needed to elicit therapeutic potential on fish in the short term. The results of this acute experiment helped design chronic studies we used to evaluate the effect of CBD on chronic stress models also applied to fish. We chose *O. niloticus* as our fish model as it is one of the most cultivated and researched fish around the world due to its hardiness (Gupta and Acosta, 2004). One benefit of using a fish model is that the fish are easily stressed in the presence of low-level stressors, this is because they live under water where the environment does not change as much as terrestrial environments (McCain, Quarrar, and Mustafa, 2015). Another benefit is that fish have a similar physiological and immunological response to stress as other higher-level vertebrates. Fish also have responses that are controlled by the nervous and endocrine system thus the findings may have human medical applications.

In our experiment, we aimed to test the effect of three different concentrations of CBD in mitigating acute stress to determine the most effective concentration. We do so by measuring its effects on blood glucose, hematocrit and plasma protein in both stressed and non-stressed *O. niloticus*.

## 3.3 Methods

### 3.3.1 Fish maintenance

Juvenile *O. niloticus* (average length 15±2 cm; average weight 65±5 g) were obtained from Troyer Aqua Farms in Geneva, Indiana. The fish were kept in a recirculating aquaculture system that was maintained and cared for following an approved animal care protocol. The water parameters were kept as follows: pH: 6.0-7.0, ammonia: 1.0-3.0 mg/dL; dissolved oxygen 5.00-7.00 mg/L; and temperature: 25±2 °C. The fish were fed with commercial feed Purina® AquaMax® Fingerling Starter 300 (Purina Mills, MO, USA) twice a day at 1.5% of their body weight (3% per day). All fish were taken care of following an approved protocol by Purdue University Animal Care and Usage Committee (PACUC) following the guidelines of the US National Research Council's "Guide for the Care and Use of Laboratory Animals".

### 3.3.2 Experimental design

In this study, the fish were divided into 8 feeding groups. Each group had two replicates with 3 fish per replicate (3 X 2 fish). Four of the 8 groups were stressed by feeding hydrocortisone (0.01% body weight) supplemented commercial feed (stressed group) as previously suggested (Barton, Schreck, and Barton, 1987) and the other four groups were fed with regular commercial feed (non-stressed group). Both the stressed and non-stressed groups were fed with four different concentrations of CBD (0%, 0.001%, 0.002% and 0.003% of feed weight) through feed supplementation to determine the best concentration to mitigate effects of stress on the hematological stress biomarkers.

The various fish groups were fed with 8 different mixture of feed twice a day at 1.5% of their body weight (that is, 3% per day). Group CC0 was fed with standard commercial feed with no supplement (CC0= Control feed, no CBD); group CCBD1 was fed with 0.001% (of feed weight) CBD (99% pure isolate from Sigma Aldrich®, St. Louis, MO) supplemented commercial feed (CCBD1 = Control feed with 0.001% CBD); group CCBD2 was fed with 0.002% CBD supplemented feed (CCBD2 = Control feed with 0.002% CBD); group CCBD3 was fed with 0.003% CBD supplemented feed (CCBD3 = Control feed with 0.003% CBD). The 4 stressed groups were as follows: group SC0 was fed with 98% hydrocortisone (at 0.01% body weight from Acros Organics, NJ, USA) supplemented feed (SC0 = Stress feed, no CBD); group SCBD1 was fed with

0.001% CBD and hydrocortisone (0.01% body weight) supplemented feed (SCBD1 = Stress feed with 0.001% CBD); group SCBD2 was fed with 0.002% CBD and hydrocortisone (0.01% body weight) supplemented feed (SCBD2 = Stress feed with 0.002% CBD); group SCBD3 was fed with 0.003% CBD and hydrocortisone (0.01% body weight) supplemented feed (SCBD3 = Stress feed with 0.003% CBD). Hydrocortisone is metabolized by the body to produce cortisol, which is an important stress hormone. The amount of hydrocortisone used was determined using a previously established protocol used for stressing fish (Barton et al., 1987). This allowed us to compare the effects of CBD on stressed and non-stressed *O. niloticus* with and without the presence of three different concentrations of CBD.

### 3.3.3 Feed preparation

The feed needed for each of the feeding groups were dependent upon the weight of the fish. The average weight of each of the group was used to determine how much food to prepare to feed at 1.5% of their body weight twice a day for three days. The feed were prepared by dividing the total commercial feed (Purina® AquaMax® Fingerling Starter 300) needed to feed the fish (for three days) in half (with and without hydrocortisone) and putting calculated dosages of hydrocortisone in the food used for hydrocortisone supplementation. Then the feed was thoroughly mixed to make for a final dosage of 0.01% hydrocortisone of fish body weight/day. The feed was then left out overnight to dry. The CBD supplemented fish feed were prepared by further dividing each of the feed (with and without hydrocortisone) into four parts and mixing 99% pure isolate Cannabidiol from Sigma Aldrich® at 0%, 0.001%, 0.002% and 0.003% CBD of feed weight. Then the feed was allowed to dry overnight and stored at 4°C. Ethanol was used throughout as a medium to dissolve and thoroughly mix the CBD and hydrocortisone into the feed. Proximate composition of non-stress feed and stress feed are presented in Table 3.1 and Table 3.2.

Table 3.1. Proximate composition of feed ingredients used to prepare four non-stress treatment feeding groups represented by CC0 (Control feed, no CBD), CCBD1 (Control feed with 0.001% CBD), CCBD2 (Control feed with 0.002% CBD) and CCBD3 (Control feed with 0.003% CBD).

| Ingredients (% Feed Weight) | CC0 | CCBD1 | CCBD2 | CCBD3 |
|---|---|---|---|---|
| Crude protein | 50 | 50 | 50 | 50 |
| Crude fat | 16 | 16 | 16 | 16 |
| Crude fiber | 3 | 3 | 3 | 3 |
| Calcium (Ca) | 5.2 | 5.2 | 5.2 | 5.2 |
| Phosphorus (P) | 1.3 | 1.3 | 1.3 | 1.3 |
| Sodium (Na) | 0.6 | 0.6 | 0.6 | 0.6 |
| CBD | -- | 0.001 | 0.002 | 0.003 |

Table 3.2. Proximate composition of feed ingredients used to prepare four stress treatment feeding groups represented by SC0 (Stress feed, no CBD), SCBD1 (Stress feed with 0.001% CBD), SCBD2 (Stress feed with 0.002% CBD) and SCBD3 (Stress feed with 0.003% CBD).

| Ingredients (% Feed Weight) | SC0 | SCBD1 | SCBD2 | SCBD3 |
|---|---|---|---|---|
| Crude protein | 50 | 50 | 50 | 50 |
| Crude fat | 16 | 16 | 16 | 16 |
| Crude fiber | 3 | 3 | 3 | 3 |
| Calcium (Ca) | 5.2 | 5.2 | 5.2 | 5.2 |
| Phosphorus (P) | 1.3 | 1.3 | 1.3 | 1.3 |
| Sodium (Na) | 0.6 | 0.6 | 0.6 | 0.6 |
| CBD | -- | 0.001 | 0.002 | 0.003 |
| Hydrocortisone* | 0.01 | 0.01 | 0.01 | 0.01 |

*% Body Weight

### 3.3.4 Fish sampling

To calculate the hematological stress parameters, the fish (3 X 2 fish/ group, 8 groups) were sampled at the end of the 72 hour period by euthanizing them using 400 mg/L tricaine methane sulfonate (MS-222; Western Chemicals, WA) to reduce any stress caused by handling.

Blood was collected from the caudal vein using syringes that have been heparinized in order to prevent blood clot. The blood was immediately transferred to 1.5 mL Eppendorf tube and placed on ice. The following parameters were then measured using the collected blood.

***Blood glucose***

Blood glucose is one of the most important parameters used to measure stress. The concentration of glucose in the blood is very sensitive to stress allowing slight changes in blood glucose to be detected immediately, making it valuable for measuring acute stress. Changes in blood glucose can occur due to an increase in respiration, decrease in metabolic activity and a decrease in immunity, all of which are indicative of stress (Ulrich-Lai and Herman, 2009). A glucometer (Freestyle, Abbott Diabetes Care, Inc., Alameda, CA) was used to measure the glucose concentration in the blood according to the manufacturer's protocol (Hossain, Blumenthal, and Mustafa, 2013).

***Hematocrit***

Packed cell volume (PCV) or hematocrit gives us the blood cell to plasma ratio. It counts the percentage of blood cells (red blood cells, white blood cells, platelets) in the blood (Asmathulla, Bidhan, and Papa, 2001). A glass capillary tube was filled with blood (~75%) using capillary action and then sealed on one side using a Crito-cap. The capillary tubes were then centrifuged at 10,000 rpm for 10 minutes in a micro-hematocrit centrifuge. A micro-hematocrit capillary tube reader was used to read the % PCV from the capillary tubes.

***Total plasma protein***

The total amount of protein in the plasma is a good indicator of health and stress levels (Asmathulla et al. 2001). An increased synthesis of certain proteins is induced to act as molecular chaperones to reconstruct damaged cells (Himmelfarb and Ellen, 2001) as part of the acute stress response. Total plasma protein is measured using a refractometer which measures the refractive index of all the dissolved solid materials in the plasma. Before use, the refractometer was calibrated using phosphate buffered sodium (PBS). The refractive index scale used to measure the amount of

protein was 1.333 to 1.360 g/100 mL. To take a reading, a few drops of plasma was placed on the prism in a way that covers the whole prism and the value was read.

### 3.3.5 Statistical analysis

SigmaPlot® 14.0, Systat Software Inc. was used to analyze the data. One-way analysis of variance (ANOVA) was used to determine the significance ($P < 0.05$) of the means. Comparisons between the groups were then made for significance using a Tukey's test. The means ± standard errors of means (SEM) are used to represent the analyzed data throughout the paper.

## 3.4 Results and discussion

Since there is a lack of research analyzing the effects of CBD on the acute stress physiology of fish, research that tested the effects of CBD on other animal models were reviewed for this study whenever fish model studies were not found. Currently, the effects of CBD on the stress physiology of fish is an innovative area of research, making our research one of the first to look at the potential of CBD to be used as a stress mitigating agent in fish. Glucose, cortisol and a few other biomarkers of stress such as hematocrit (packed cell volume) and protein can be used as a good indicator of stress (Martinez-Porchas, Martinez-Cordova, and Ramos-Enriquez, 2009). Consequently, this paper discusses the effect CBD has on glucose, hematocrit and protein in the presence of acute stress.

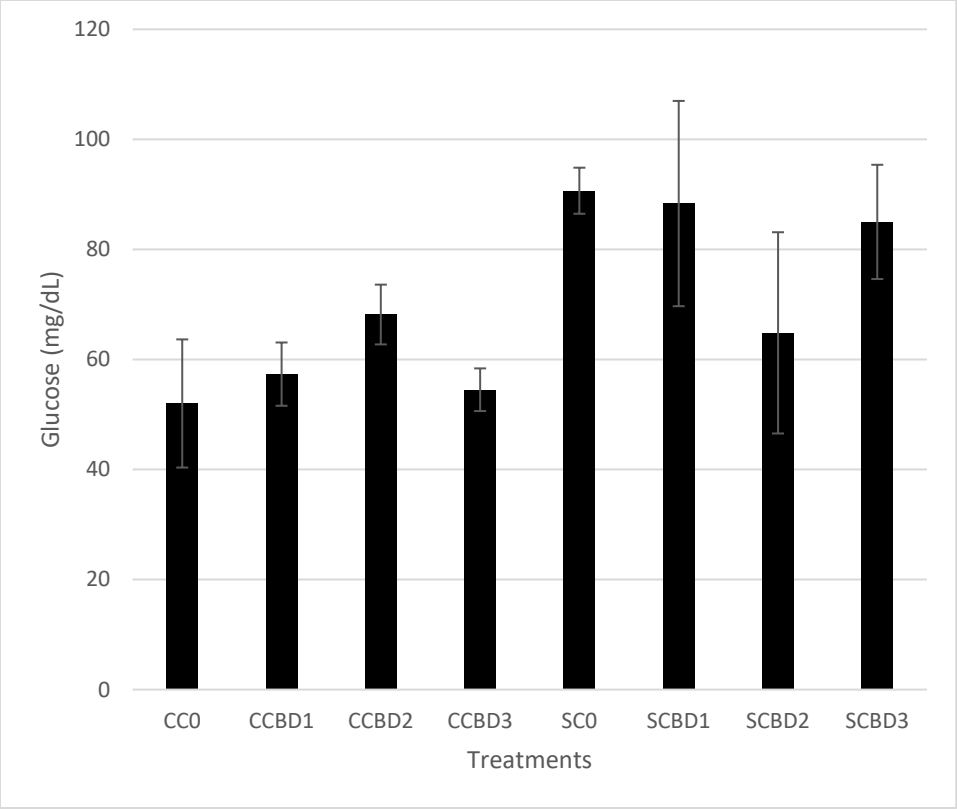


Figure 3.1. Blood glucose levels in mg/dL of Nile tilapia fed with various non-stress treatment feeding groups represented by CC0 (Control feed, no CBD), CCBD1 (Control feed with 0.001% CBD), CCBD2 (Control feed with 0.002% CBD), CCBD3 (Control feed with 0.003% CBD) as well as stress treatment feeding groups such as SC0 (Stress feed, no CBD), SCBD1 (Stress feed with 0.001% CBD), SCBD2 (Stress feed with 0.002% CBD) and SCBD3 (Stress feed with 0.003% CBD). Results are presented as means± SEM. There were no significant differences among the experimental groups (N = 6, P = 0.179, DF = 7).

As mentioned earlier, glucose is a good stress biomarker. Stress increases the energy demands of the organism, this energy is used to fuel the fight or flight response and to maintain homeostasis, causing an increase in blood glucose to keep up with the increase in energy demand. In order for the body to keep up with the demand of blood glucose, the pancreas releases glucagon which causes the liver to break down glycogen into glucose (Taborsky, 2010). This increase in blood glucose production is a sign of increased stress. Although no statistical significance was found, according to figure 3.1 (N = 6, P = 0.179, DF = 7), we see that stress groups (SC0, SCBD1, SCBD2, SCBD3) in general had higher glucose levels (~90-85 mg/dL) than the non-stress groups (CC0, CCBD1, CCBD2, CCBD3) (~55-50 mg/dL) with the exception of CCBD2 and SCBD2. This is to be expected as the exogenous cortisol in the plasma of the stress groups would lead to higher plasma glucose levels due to stress. Similar results have been reported on the blood glucose

levels of fish under stress in various studies outlined in Martinez-Porchas et al. in 2009. Looking at all the non-stressed groups, it looks like CBD was not able to reduce glucose levels. However, comparing within the stress groups, SCBD2 had the lowest glucose level (~65 mg/dL) from the highest blood glucose value of ~90 mg/dL under control stress condition (SC0), although it was not significant. It may be that CBD reduces stress only in the presence of significant acute stress and therefore no effects are seen in non-stress conditions. To our knowledge, the blood glucose reducing ability of CBD on fish have not been investigated. However, the effect of CBD on blood glucose have been tested by Romero-Zerbo et al. (2020) on mice models of prediabetic and non-alcoholic fatty liver disease. In the study, no significant difference was found in blood glucose between groups treated with CBD vs without CBD (Romero-Zerbo et al., 2020) which is on par with our findings here.

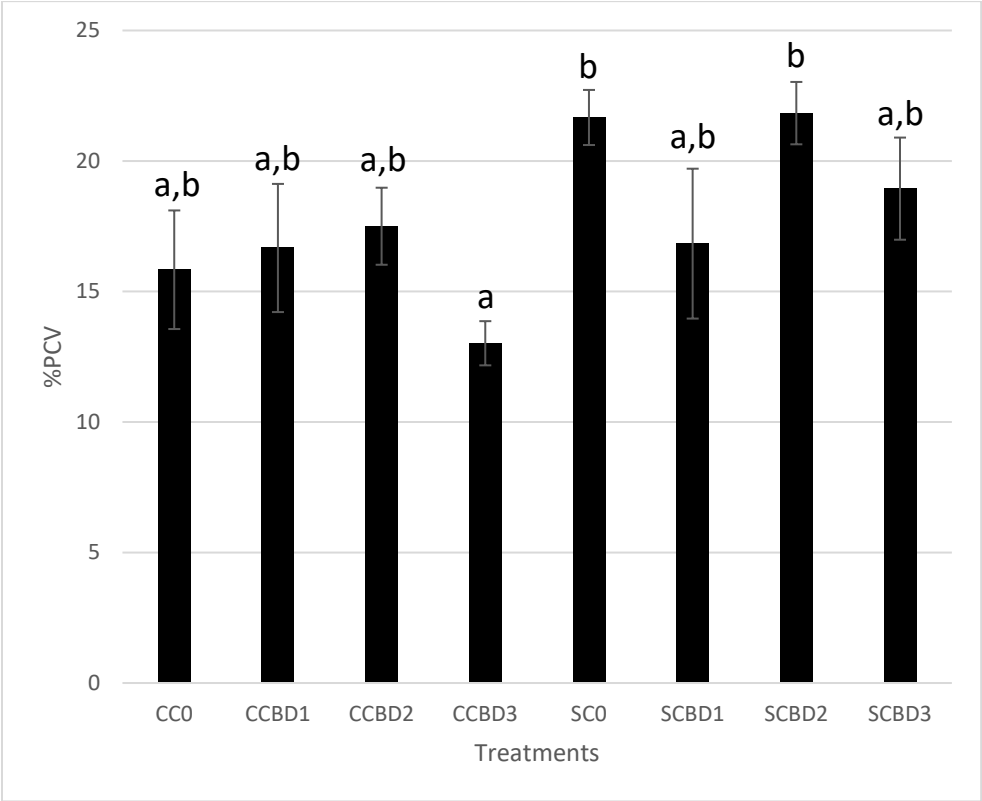


Figure 3.2. Hematocrit (PCV) in % of blood of Nile tilapia fed with various non-stress treatment feeding groups represented by CC0 (Control feed, no CBD), CCBD1 (Control feed with 0.001% CBD), CCBD2 (Control feed with 0.002% CBD), CCBD3 (Control feed with 0.003% CBD) as well as stress treatment feeding groups such as SC0 (Stress feed, no CBD), SCBD1 (Stress feed with 0.001% CBD), SCBD2 (Stress feed with 0.002% CBD) and SCBD3 (Stress feed with 0.003% CBD). Results are presented as means ± SEM. Bars with different letters are significantly different (N =6, $P<0.05$, F = 2.621, DF = 7).

Hematocrit or packed cell volume (PCV) is a good indicator of physiological and immunological function as blood cells perform major physiological functions in the body as well as immune modulation. Therefore, in stress conditions, there will be higher levels of blood cells to compensate for the increased demand of the body, causing a higher % PCV (Asmathulla et al. 2001). Such is seen in our results in figure 3.2 ($N = 6$, $P < 0.05$, $F = 2.621$, $DF = 7$) where SC0 and SCBD2 had the highest % PCV (~22 %). CCBD3 had the lowest % PCV (~13%) and there was a significant difference between SC0 and CCBD3 ($P = 0.035$), CCBD3 and SCBD2 ($P = 0.030$). The underlying implication of which is that under non-stressed condition supplied with 0.003% feed weight CBD (CCBD3) the fish had the lowest % PCV thus the lowest stress level. This is to be expected as in the case of CCBD3, there was no stress via exogenous cortisol and CBD was supplied at the highest concentration of 0.003% of feed weight. Under stress with no supplementation of CBD (SC0), the % PCV was the highest which is a sign of stress. Same was found in the case of stressed fish supplemented with CBD at 0.002% of feed weight (SCBD2) where CBD was not able to reduce any stress in comparison to the control stress condition (SC0).

Comparing between all the non-stressed groups there is no significant difference between any of the groups in % PCV. Meaning, CBD was not able to reduce hematocrit level in non-stressed condition. Although at the highest concentration of CBD (CCBD3), we see the lowest % PCV among all the groups. Between all the stress groups, SCBD1 and SCBD3 show slight reduction in % PCV and thus stress in comparison to SC0, although no significant difference was found. A drop from ~22% (SC0) to ~17-18% PCV (SCBD1 and SCB3) was observed. A study conducted by WHO (2007) looked at the effects of *Cannabis sativa* on the hematological parameters of guinea pigs. They found that guinea pigs fed with hemp powder had lower PCV levels than the other groups who were not (Karimi, Hayatghaibi, Yousefi, Saberivand, and Zavareh, 2007). Although, in their case the animals were not exposed to any stress and the supplement used was not pure CBD but rather the dried powder of the seeds. To our knowledge studies on the effect of CBD on the stress physiology focusing on hematology of fish is scarce and we were not able to find many that looked at its effects on the hematocrit level.

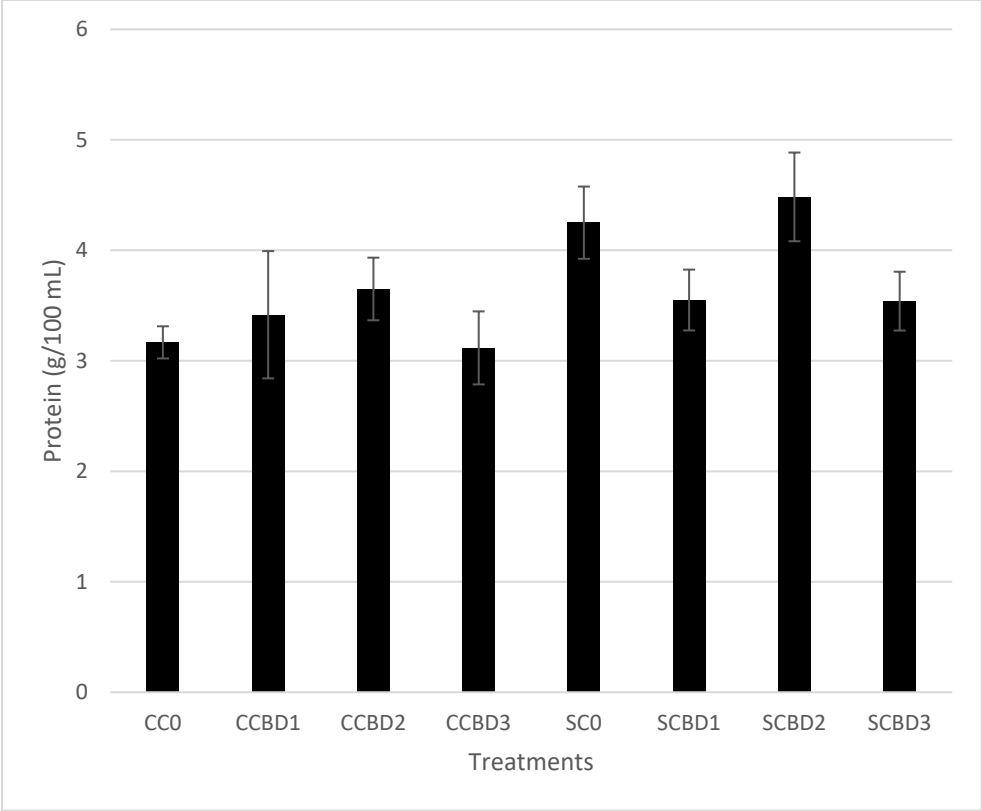


Figure 3.3. Plasma protein in g/100 ml of Nile tilapia fed with various non-stress treatment feeding groups represented by CC0 (Control feed, no CBD), CCBD1 (Control feed with 0.001% CBD), CCBD2 (Control feed with 0.002% CBD), CCBD3 (Control feed with 0.003% CBD) as well as stress treatment feeding groups such as SC0 (Stress feed, no CBD), SCBD1 (Stress feed with 0.001% CBD), SCBD2 (Stress feed with 0.002% CBD) and SCBD3 (Stress feed with 0.003% CBD). Results are presented as means ± SEM. There were no significant differences among the experimental groups (N = 6, P = 0.165, F = 1.597, DF = 7).

The last parameter is plasma protein which is another important secondary stress biomarker. As mentioned earlier, stressed organisms increase their plasma protein levels in order to supply proteins throughout the body. These proteins are used to repair any damage done to various tissues due to increased activity caused by stress (Himmelfarb and Ellen, 2001). Due to the increased need for protein, higher protein levels are expected in the blood when under stress. According to figure 3.3 (N = 6, P = 0.165, F = 1.597, DF = 7), SC0 and SCBD2 had the highest protein levels (~4.3 and ~4.5 g/100 mL) and one of the lowest protein levels was found in the CCBD3 group (~3.1 g/100 mL). This was the trend across all the hematological parameters measured with significant difference seen in % PCV. There is no significant difference between any of the stressed or non-stressed groups. 0.001% (CCBD1), 0.002% (CCBD2), and 0.003% CBD (CCBD3) had no

significant effect on stress modulation. Stressed fish fed with no CBD supplementation (SC0) had the highest protein level, as expected. CBD at 0.002% of feed weight (SCBD2) was not able to reduce protein and therefore failed to reduce stress under stress condition. Comparing all the stress fed groups, SCBD1 and SCBD3 show a slight reduction (from ~4.3 to ~3.5 g/100 mL) in protein levels in comparison to the control stress group, SC0, although the results here are not significantly different from each other. It has been said that stress does in fact increase blood plasma protein and that it could be used as a stress biomarker in a paper by George Iwama (1998). Iwama also states that plasma protein however, is not a reliable stress biomarker on its own and needs to be used alongside other biomarkers because of wide variability in results (Iwama, 1998).

Across all the stress biomarkers the control without CBD group (CC0) showed a lower number compared to stress group without CBD (SC0). We see in figure 3.1 that the glucose level was high (~90 mg/dL) for SC0 but low for CC0 (~51 mg/dL), which is a sign of stress for stress control group (SC0). In figure 3.2, packed cell volume, we see that SC0 is higher than CC0 (~22% vs ~16%). The same trend is seen in the case of Protein. This is an indication that the stress groups were indeed stressed by exogenous cortisol compared to the control groups.

Comparing all three of the parameters, there seems to be an underlying trend running in all three of them. The stress groups tend to indicate slightly higher levels of stress in all three parameters than the non-stressed groups, which is to be expected as mentioned previously. SC0 had one of the highest stress levels as it had exogenous cortisol but no CBD was supplemented in the feed. Although CCBD3 had one of the lowest stress levels, there was no significant difference between the different dosages of CBD among the stress groups as well as the non-stress groups. In fact, looking at the hematocrit (figure 3.2) and protein (figure 3.3) results, it looks like SCBD1 and SCBD3 show slight promise in stress reducing ability of CBD when stress is present. Thus, among the two (SCBD1 and SCBD3), the lowest concentration was chosen for further investigation as it would be the most economic option for aquaculturists. A chronic study was designed based on this experiment to test the effect of 0.001% CBD on chronic stress in the context of growth and various physiological and immunological stress biomarkers. In this study over 72 hours, no substantial difference was observed in terms of hematology. However, over 4 weeks of stress and supplementation of CBD should yield to a larger impact and therefore difference in stress biomarkers between groups.

## 3.5 Conclusion

With an ever-increasing population and high demand of protein, farmers are struggling to keep up with the increasing demand for food. Farming of cattle and other domestic animals are well documented to cause significant contribution to methane emissions (Johnson and Johnson, 1995), therefore negatively impacting our environment. While aquaculture has its own issues, fish farming is more environmentally friendly and require less land than cattle. In order to increase profits in aquaculture, farmers are starting to look for more ecofriendly and cheaper alternatives to using commercials drugs to reduce stress in fish. Currently CBD is not a cheap resource, but with increasing acceptance, legalization and farming of marijuana, the price of CBD may decrease in the near future. Today CBD is commonly sold as a "cure all", but can we back the claims with evidence? This is why it is important to know the effects of CBD on the stress physiology of vertebrate organisms, since, as stated before, research done on lower level vertebrates may be applied to humans. However, further investigation into whether CBD is able to modulate the stress response in fish in the long run is needed.

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# CHAPTER 4. DETERMINATION OF FEED UTILIZATION AND GROWTH PERFORMACE OF CHRONICALLY STRESSED AND NON-STRESSED NILE TILAPIA, *OREOCHROMIS NILOTICUS*, FED CANNABIDIOL (CBD) -SUPPLEMENTED DIETS

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## 4.1 Abstract

This study evaluates the effects of Cannabidiol (CBD) on feed utilization and growth of stressed and non-stressed Nile tilapia, reared in recirculating aquaculture system. Tilapia were fed with and without CBD (0.001% of feed weight), and with and without hydrocortisone (0.01% of body weight) every day for four weeks. This experiment compared the Cortisol levels, Absolute Feed Intake, Feed Conversion Ratio, Protein Efficiency Ratio and the Condition Factor of the fish. Supplementing the feed with CBD seems to improve their protein conversion efficiencies in non-stressed condition. However, under stress condition, supplementing the feed with CBD reduced their efficiency of weight gain in comparison to protein fed. All the groups at the end of the study were shown to have the same condition factors despite the discrepancy in result between Feed Conversion Ratio and Protein Conversion Ratio. Therefore, it could be that the fish were gaining their weight in fat instead of protein when stressed and supplemented with CBD. Further research needs to be conducted with a higher dose of CBD for longer than four weeks to investigate their effect on the fat content of these fish in comparison to their protein content.

Keywords. CBD; Growth; Nile tilapia; Stress physiology; *in-vivo*.

## 4.2 Introduction

Stress in fish can be a significant issue in aquaculture due to poor husbandry methods. Husbandry methods such as crowding, handling, and vaccinating can lead to stress which can affect the productivity of the fish. To mitigate the stress response, farmers often use antibiotics and chemical drugs that may lead to creation of superbugs or affect non-target species. Not to mention the potential to affect human health if the chemicals stay in the fish's system and then consumed. In order to solve this issue, we must understand the root of the problem, "stress".

Stress can be deleterious and is a common phenomenon in farming, especially aquaculture. Thousands of dollars are lost every year due to poor stress management and its resultant loss in productivity due to disease and poor health. Stress is a state of decreased fitness or a response to any external agent that challenges the homeostasis and threatens its survival (Colombo, Pickering, Belvedere and Schrech, 1990). Stress responses can be adaptive and maladaptive. Adaptive response occurs when a body learns how to cope with stress and maintain the normal basal state of bodily activities. Maladaptive response is when the prolonged exposure to the stress leads to deleterious effects to the body. There are three hormones that mediate the stress response: adrenaline, noradrenaline, and cortisol. Together, these hormones change the state of the body from "rest and digest" to "fight or flight" state. Fight or flight response puts many vital mechanisms of the body on hold (such as digestion, immune response, growth and reproduction) and enhances the blood circulation to the muscles, increases heart rate, and prepares the body to either fight or flee. This is useful for short periods of time (acute stress) but deleterious in prolonged exposure. Chronic stress weakens the immune and inflammatory response which can make an organism susceptible to various secondary diseases (Eddie and Norman, 2008).

Stress has widespread physiological effects throughout the body as detailed by Moyle et al. (1990). The responses can be categorized into primary, secondary, and tertiary responses. After the stressor is detected by the organism, hormones (cortisol and adrenaline) are released from cells in the anterior kidney following stimulation from the CNS. Thus, in the primary response, cortisol and adrenalin are released into the blood. Cortisol is made after corticotropin releasing hormone (CRH) from the hypothalamus stimulates the pituitary gland to release adrenocorticotrophic hormone (ACTH). ACTH is carried in the blood to the interrenal cells in the head kidney from which the cortisol is released. Adrenaline (epinephrine) is released by the chromaffin tissue of the head kidney after the direct stimulation of the sympathetic nervous system. In secondary response, changes in blood and tissue occur. According to Moyle et al., (1990), Hematological changes such as elevated blood glucose (hyperglycemia) and reduced blood clotting time is observed. Diuresis begins as well as electrolyte losses, leading to osmoregulatory dysfunction. Several tissue changes are also observed such as, depletion of liver glycogen and interrenal vitamin C, hemorrhage of the thymus, and hypertrophy of the interrenal cells. At tertiary response, major physiological changes or whole-body responses are observed. Reduced resistance to infectious diseases, growth, reproductive success, and survival occur. This gets in the way of promotion from one life stage to

another in fish which can lead to decline in population (Moyle and Schreck, 1990). We can measure these changes to determine the level of stress of an organism. In this paper we will focus on the growth response of Nile tilapia which is the most economically relevant point to aquaculturists.

Stress responses can be mitigated with chemical treatments such as drugs and antibiotics. However, as discussed earlier, usage of various chemicals in aquaculture can lead to environmental pollution and affect non-target species. On the other hand, usage of antibiotics can lead to the buildup of bacterial resistance and the emergence of superbugs. Therefore, scientists are on the search for alternative solutions such as nutraceuticals. Nutraceuticals are functional foods (often phytochemicals) that have health giving additives and provide medicinal benefits (Lim and Webster, 2006). Nutraceuticals can potentially alleviate stress and boost immunity in various animals including fish (Mustafa, Randolph and Dhawale, 2011; Ibrahim, Khan, Rinard and Mustafa, 2015; Mustafa, Dhawale, Park and Yoo, 2013).

Cannabidiol (CBD) is a fairly new nutraceutical. It is a derivative of the plant marijuana (*Cannabis sativa*). It is a non-psychoactive compound unlike Tetrahydrocannabinol (THC). Clinical research on CBD includes studies on stress, anxiety, cognition, movement disorders, and pain (Morales, Reggio and Jagerovic, 2017). CBD has shown to have therapeutic potential such as, neuroprotection (Fasinu, Phillips, Elsohly and Walker, 2016), anxiety and psychosis (Morales, Hurst and Reggio, 2017), epilepsy (Anavi-Goffer et al., 2012), depression (Breuer et al., 2016), and pain (Burstein, 2015). It has also shown to have immunological potential such as reducing inflammation (Ben-shabat, Hanuš, Katzavian & Gallily, 2006), cancer (Burch, Mortuza, Mustafa and Blumenthal, 2021; Juknat, Kozela, Kaushansky, Mechoulam and Vogel, 2016), and bacterial infection (Ryberg et al., 2007). Viudez-Martínez et al. in 2018 aimed to assess the effect of CBD on different gene targets of the hypothalamus-pituitary-adrenal axis under control and stress conditions in mice. In order to do so, they administered 5 mg/ kg, 15 mg/ kg or 30 mg/ kg (of mice body weight) of CBD intraperitoneally before exposing the mice to restraint stress. Then, using real time PCR analysis, they measured the relative gene expression of corticotropin-releasing factors. Interestingly, CBD at 5 mg/ kg and 15 mg/ kg were able to block the gene expression caused by acute stress (Viudez-Martinez, Garcia-Gutiérrez and Manzanares, 2018). Expression of stress related genes are certain to have a physiological impact on the whole organism's stress

response. A relevant parameter of stress of economic value would be how mitigating stress using CBD can affect growth of fish in aquaculture.

Despite the widespread therapeutic usage of CBD, not a lot of research has been conducted on the effects of CBD on the stress physiology of vertebrates and whether it could be used to mitigate the stress responses and compensate for growth in high stress conditions of aquaculture. In this experiment, we have worked with a fish model to study its effects on stress physiology and growth performance. We chose Nile tilapia as our fish model as it is one of the most cultivated fish around the world (Gupta and Acosta, 2004) and is also widely used in fish model research. Benefits of using a fish model are that the fish are easily stressed in the presence of low-level stressors as they live under water where the environment does not change as much as terrestrial environments (McCain, Quarrar and Mustafa, 2015). Fish also have a similar physiological and immunological response to stress as other vertebrate animals. Their responses are also controlled by the nervous and endocrine system thus the findings may have human medical applications.

In our experiment, we aimed to test the effect of CBD in mitigating stress by measuring physiological growth and feed utilization parameters. Parameters measured in this experiment were plasma levels of cortisol, Absolute Feed Intake (AFI), Feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER), and Condition Factor (CF). By measuring these parameters in the presence and absence of CBD in stressed and non-stressed fish, we were able to track whether the fish were stressed, how much food the fish were consuming per day, efficiency of conversion of the feed to fish weight, efficiency of conversion of the protein in feed to fish weight, and how it all ultimately affected the condition factor.

## 4.3 Methods

### 4.3.1 Fish maintenance

Nile tilapia, *Oreochromis niloticus*, fingerlings (average length 20±2 cm; average weight 175±10 g) were purchased from Troyer Aqua Farms, Geneva, IN. The fish were kept in a recirculating aquaculture system (Aquatic habitats ™, Aquatic Eco-Systems, INC). The system was maintained and cared for following an approved animal care protocol (pH: 6.0-7.0, ammonia: <0.05 mg/ L; dissolved oxygen 5.00-7.00 mg/ L, and temperature: 25 ± 2 °C). The fish were fed 1.5% of their body weight twice a day with a compatible commercial fish feed, Purina® AquaMax®

Fingerling Starter 300 (Purina Mills, MO, USA). All fish were taken care of following an approved protocol by Purdue University Animal Care and Usage Committee (PACUC) following the guidelines of the US National Research Council's "Guide for the Care and Use of Laboratory Animals".

### 4.3.2 Experimental design

No previous study was found that tested the effects of CBD on the growth of Nile tilapia in the long run (Chronic stress). Therefore, to determine the appropriate concentration of CBD to use, we had conducted a preliminary acute test. In the acute study, the fish were maintained following similar experimental design and protocol as this study. However, it was done only over a period of 72 hours and the fish were fed with feed supplemented with three different concentrations of CBD, with and without hydrocortisone. At the end of the experiment we collected blood from the fish and evaluated stress biomarkers such as blood glucose, hematocrit, and plasma protein. From the study we found that there was no significant difference in the stress physiology of *O. niloticus* between 0.001%, 0.002%, and 0.003% CBD (% of feed weight) over 3 days. Therefore, in our chronic study, the lowest concentration was tested long term to see its effect on growth as it is the most economic option for aquaculturists.

In this study, fish were divided into four groups. Each group consisted of 4 fish (N=4), each fish in individual tanks (individual replicates). The fish were fed with four different mixture of feed twice a day. Group 1 was fed with standard feed with no supplement (C= Control feed); group 2 was fed with 0.001% CBD (99% pure isolate from Sigma Aldrich®, MO, USA) supplemented feed (CCBD = Control feed with CBD); group 3 was fed with 98% hydrocortisone (at 0.01% body weight from Acros Organics, NJ, USA) supplemented feed (S= Stress feed); and group 4 was fed with 0.001% CBD and hydrocortisone (0.01% body weight) supplemented feed (SCBD= Stress feed with CBD). Hydrocortisone is metabolized in the body into cortisol which is a stress hormone. The amount of hydrocortisone used was determined using a previously established stress protocol of Nile tilapia (Barton, Schreck and Barton, 1987). This allowed us to compare the effects of CBD on stressed and non-stressed Nile tilapia.

### 4.3.3 Feed preparation

The feed was prepared weekly as the food needed was dependent on the fish weight at the beginning of that week. The feed was prepared by dividing the total commercial feed (Purina® AquaMax® Fingerling Starter 300) needed that week in half and putting calculated dosage of hydrocortisone in it and thoroughly mixing to make for a final dosage of 0.01% hydrocortisone of fish body weight. The feed was then left out overnight to dry. Then the CBD supplemented fish feed was prepared by further dividing the feed (with and without hydrocortisone) in half and mixing the one of each half of feed with 99% pure isolate Cannabidiol from Sigma Aldrich® at 0.001% CBD of feed weight. Then the feed was allowed to dry overnight and stored at 4°C. Ethanol was used throughout as a medium to dissolve and thoroughly mix the CBD and hydrocortisone to the feed. In the drying process, the ethanol was allowed to evaporate. A proximate analysis of feed is presented in Table 4.1.

Table 4.1. Proximate composition of feed ingredients used to prepare four treatment groups of fish feed represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups.

| Ingredients (% Feed Weight) | C | CCBD | S | SCBD |
|---|---|---|---|---|
| Crude protein | 50 | 50 | 50 | 50 |
| Crude fat | 16 | 16 | 16 | 16 |
| Crude fiber | 3 | 3 | 3 | 3 |
| Calcium (Ca) | 5.2 | 5.2 | 5.2 | 5.2 |
| Phosphorus (P) | 1.3 | 1.3 | 1.3 | 1.3 |
| Sodium (Na) | 0.6 | 0.6 | 0.6 | 0.6 |
| CBD | -- | 0.001 | -- | 0.001 |
| Hydrocortisone* | -- | -- | 0.01 | 0.01 |

*% Body Weight

### 4.3.4 Fish sampling

To calculate the growth parameters, the fish (4 fish/ group) were sampled at the end of a four-week period. The length and weight of the fish were measured. The fish were euthanized using tricaine methane sulfonate (MS-222) (Western Chemicals, WA) at 400 mg/ L. This allowed us to see how four weeks of exposure to CBD and hydrocortisone had affected their physiology

(cortisol) and growth performances [length (cm) and weight (g)] in relation to how much food the fish were given (g) and how much protein was contained in the food (calculated according to the ingredients list of Purina® AquaMax® Fingerling Starter 300) they consumed (g).

Cortisol is one of the major stress hormones that are released by the interrenal cells of the head kidney tissue of the fish and thus indicative of stress (Barton et al., 1987). The blood collected from the caudal vein of the fish. Then the it was centrifuged at 5000 rpm for 10 minutes to collect the plasma. The supernatant plasma was collected and preserved at -80°C for future use. Due to the small amount of serum that was able to be isolated, the serum were pooled together from all the fish within a treatment group (n=1). To measure the cortisol level in the plasma, Cayman Chemical Cortisol ELISA kit (Item No. 500360) (Ann Arbor, MI, USA) was used, following manufacturer's protocol.

Using the aforementioned parameters, the following growth performance parameters were calculated using the formulae:

$$\text{Absolute Feed Intake (AFI): } \frac{Feed\ intake\ _{Total}\ per\ tank\ (g)}{fish\ per\ tank * days}$$

$$\text{Feed Conversion Ratio (FCR): } \frac{feed\ given\ (g)\ per\ fish}{weight_f - weight_i\ per\ fish}$$

$$\text{Protein Efficiency Ratio (PER): } \frac{weight_f(g) - weight_i(g)\ per\ fish}{feed\ protein\ intake(g)\ per\ fish}$$

$$\text{Fulton Condition Factor (CF): } \frac{weight(g) * 100}{length(cm)^3}$$

### 4.3.5 Statistical analysis

The data collected were analyzed using SigmaPlot® 14.0, Systat Software Inc. A one-way analysis of variance (ANOVA) followed by Tukey's test was performed to determine whether the differences between the samples were significant ($P < 0.05$). The analyzed data is presented in the form of means ± standard errors of means (SEM) throughout the paper.

## 4.4 Results and discussion

Cortisol ELISA assay was done to see whether the hydrocortisone fed to the fish was able to stress the fish effectively. In order to interpret the results, we must first understand how cortisol is generated in the body and how long it remains active. In fish, stressors stimulate the

hypothalamic-pituitary-interrenal axis (HPI) which prompts the release of adrenocorticotrophic hormones (ACTH). This in turn prompts the interrenal cells of the head kidney tissue to generate cortisol in response. This is similar to the cortisol production in mammals who have hypothalamic-pituitary-adrenal cortex (HPA) and are also stimulated by the adrenocorticotrophic hormones (ACTH) (Barton and Iwama, 1991). The cortisol release is controlled by a negative feedback loop at the HPI axis (Fryer and Peter, 1977). The plasma cortisol levels are kept in check by regulating how much of it is produced endogenously. In fish, the plasma cortisol levels come down to the basal level 24 hours after an acute stress is perceived. Cortisol is rapidly metabolized in the liver due to its action on it and are filtered and excreted by the kidney (Laberge, Yin-Liao and Bernier, 2019).

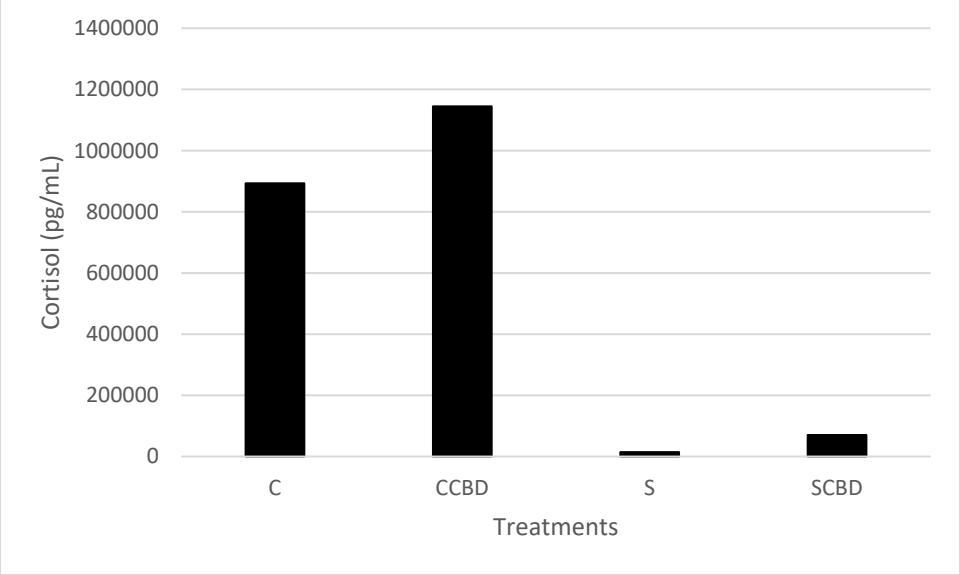


Figure 4.1. Plasma cortisol concentrations of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means and no standard error bar is presented as blood serum were pooled together from all the sample fish per treatment (N=1).

According to Figure 4.1, the stress groups (S and SCBD) had a lower plasma cortisol level in comparison to the control groups (C and CCBD). Statistical analysis is not possible as the serum were pooled together from all the sample fish within the treatment group before running ELISA assay (N=1). Our result is an indication that the exogenously supplemented cortisol via fish feed were getting absorbed into the blood of the fish, thus, stressing the fish. The increased level of

exogenous plasma cortisol in turn was shutting down the endogenous cortisol production due to the negative feedback loop at the HPI axis. Inhibition of the HPI axis would shut down the production of ACTH and thus inhibit the production of cortisol from the interrenal cells of the head kidney tissue. Then, the exogenous cortisol in the plasma were being metabolized rapidly by the liver and excreted completely within 24 hours. Blood cortisol is cleared faster when fish are administered with exogeneous cortisol in the blood to stimulate stress due to the increased level of cortisol in the blood (Barton et al., 1987). The combined action of these systems lowered the plasma cortisol levels of the hydrocortisone supplement fed fish (S and SCBD) while keeping the endogenous cortisol levels of the control fed fish groups (C and CCBD) up. Since the control fish were not stressed using exogenous cortisol, they were able to maintain a basal level of endogenous cortisol throughout the 4 weeks of this experiment due to the HPI axis not shutting down via negative feedback loop and regular rate of clearance of cortisol from the blood. Due to our dosage of hydrocortisone, each time after feeding, the fish would have had a spike in plasma cortisol which would manifest itself by affecting secondary or tertiary stress biomarkers down the line (Barton et al., 1987). These findings are supported by previous studies where similar phenomena were observed, such as in tilapia in high density stress conditions (Khan, 2016), cortisol fed catfish (Davis, Torrance, Parker and Suttle, 1985) and *in-vitro* tissue culture of Coho salmon (Bradford, Fitzpatrick and Schreck, 1992).

As we have established that the stress feed treatment fish groups (S and SCBD) were in fact stressed, let us look at the feed conversion and growth performance parameters and how they were affected by CBD. There was no significant difference in the feed intake of fish per day across the different treatments (Figure 4.2; N= 4, P= 0.642, F= 0.576, DF= 3). Feed was equally consumed whether the fish were stressed/ not stressed or supplemented with CBD. Since majority of the research done on CBD currently are on mice and rats and not fish, most of the literature to support our findings are, therefore, from mice or rat models of research. In a research done by Farrimond et al. (2012), rats were dosed with Cannabinol and Cannabidiol, where Cannabinol was found to increase and Cannabidiol was found to decrease consumption of feed over the experimental period (Farrimond, Whally and Williams, 2012). In another study conducted by Riedel et al. (2009), THC (a cannabinoid) was found to have an effect in reducing feed intake in fasted and non-fasted mice (Riedel et al., 2009). Contrary to popular belief, both groups found cannabinoids to have a consumption reducing effect in warm-blooded vertebrates.

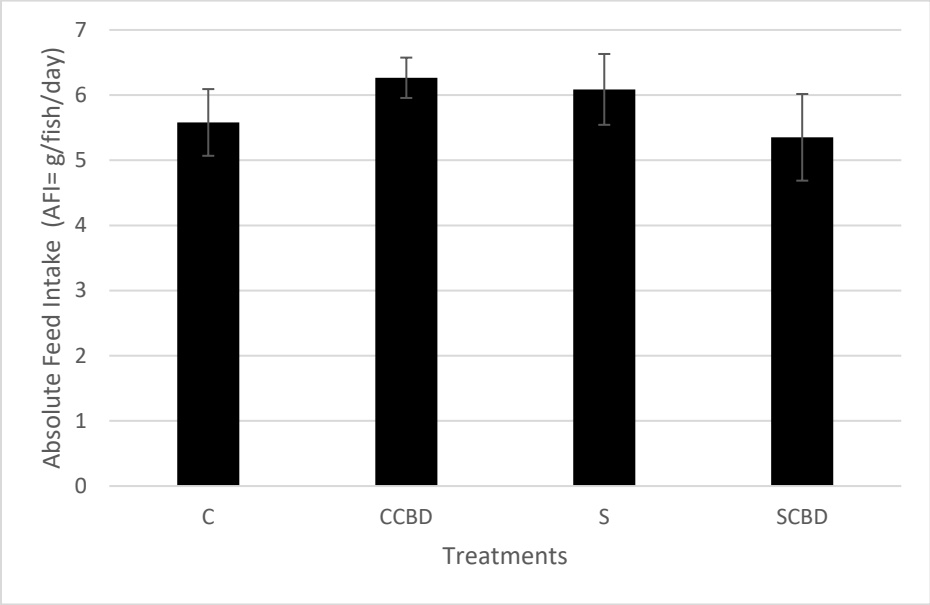


Figure 4.2. Absolute Feed Intake (AFI) of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. N= 4, P= 0.642, F= 0.576, DF= 3.

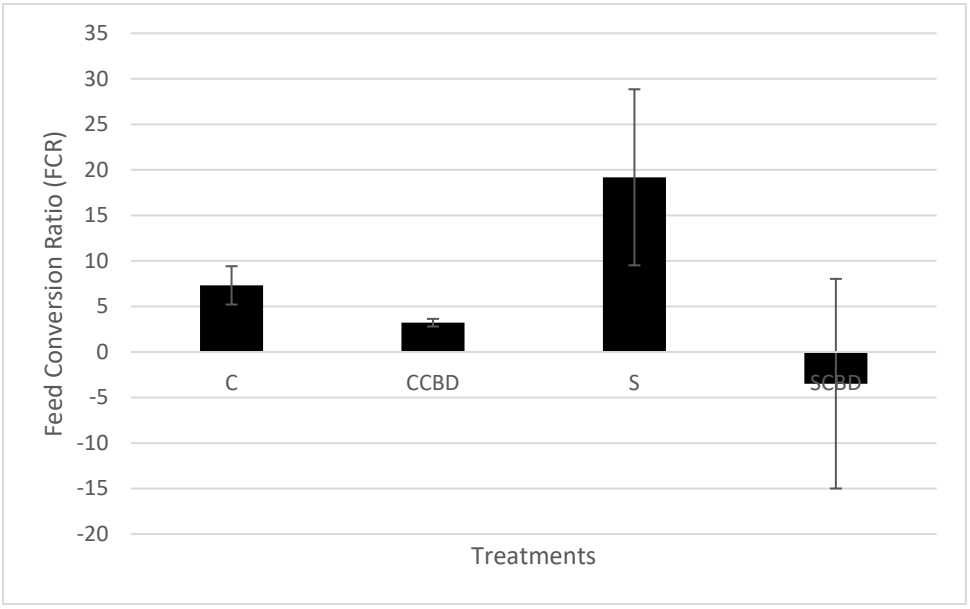


Figure 4.3. Feed Conversion Ratio (FCR) of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. N= 4, P= 0.244, F= 1.629, DF= 3.

Feed conversion ratio (FCR) is the ratio of food given, to the weight gain in fish. Higher the FCR, the lower the weight gain in comparison to feed given to fish. A high FCR value means the efficiency of feed conversion was poor. There was no significant difference in FCR among the treatment groups in our experiment (Figure 4.3; N= 4, P= 0.244, F= 1.629, DF= 3). However, the stressed (S) group had the highest FCR value, suggesting that they had the lowest weight gain in comparison to how much food they were fed. SCBD had the lowest FCR and thus the highest efficiency of conversion. Comparing between C and S groups, S had the lower efficiency of converting feed weight into body weight. It seems that stress not only reduces the consumption of feed (Martins et al., 2011) but also could potentially reduce its efficient conversion to body weight. In the case of both C vs CCBD and S vs SCBD, the fish had a higher conversion of feed weight to body weight in the presence of CBD compared to in the absence of CBD. This would have important implications for the aquaculture industry. This is consistent with the findings of Khan et al. (2010). They had found that supplementing feed of broilers with various concentrations of *Cannabis sativa* seeds led to a higher efficiency of conversion of feed weight to body weight while reducing overall consumption of feed (Khan, Durrani, Chand and Anwar, 2010).

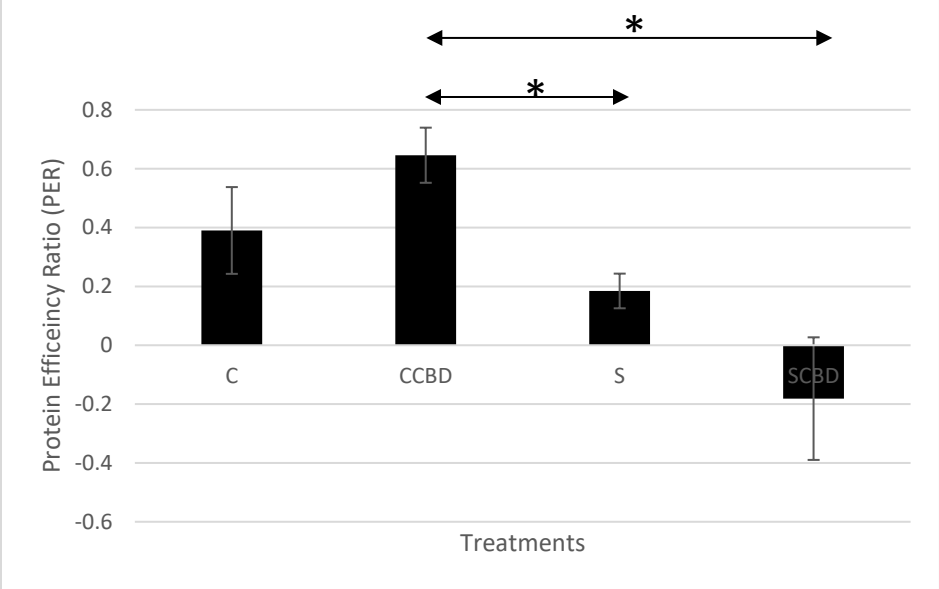


Figure 4.4. Protein Efficiency Ratio (PER) of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. *= significant difference at P≤0.05. N= 4, F= 6.072, DF= 3.

Protein efficiency ratio (PER) (Figure 4.4; P≤0.05, N= 4, F= 6.072, DF= 3) is the ratio of weight gain to the feed protein intake. A higher PER value suggests that there was a higher gain in weight in comparison to the amount of protein in grams present in the food consumed, thus higher efficiency of protein to body weight conversion. In our experiment, CCBD group had the highest efficiency of conversion of protein to body weight compared to all the other groups. CCBD group had significantly higher PER compared to both the stress groups (S and SCBD). CCBD group was not only supplemented with CBD but also was not exposed to stress that could bring this efficiency down, thus the result is not unusual. Comparing C to S and CCBD to SCBD, in either case, stress had decreased the weight gain in comparison to the feed protein which is a sign of stress reducing the protein efficiency ratio. With CCBD vs SCBD showing a significant difference. This is to be expected as in stress conditions, organisms focus their energy on stress modulation and survival rather than growth and reproduction (Barton, 2002).

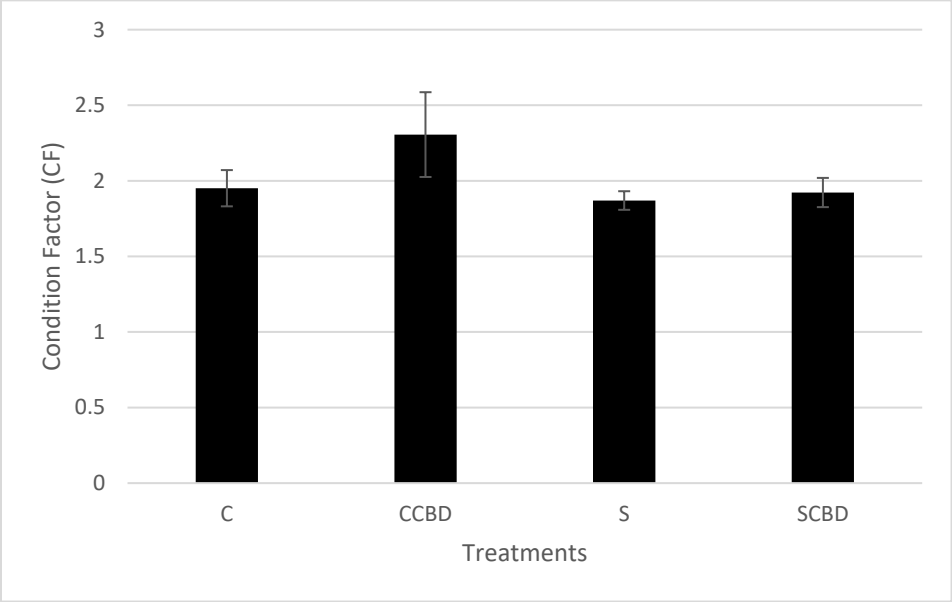


Figure 4.5. Condition Factor (CF) of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. N= 4, P= 0.505, DF= 3.

Last but not least and perhaps the most important parameter for aquaculturists, condition factor (CF) is an indicator of a fish's overall health in terms of weight and length. Analysis and discussion of fish weight and length data was not included in this paper as the condition factor is

a better indicator of fish growth. A fish could be heavy but small or large but thin. To make quality fish products, you need to make fish with good condition factors. So how did the amount of feed consumed, feed conversion ratio and protein efficiency ratio result in the fish's overall health? Control group fed CBD supplemented diet (CCBD) had the highest condition factor (Figure 4.5; N= 4, P= 0.505, DF= 3). All the other groups had the same condition factors and there was no significant difference between all the groups. It is possible that the stress was so significant that any supplemented CBD did nothing to alleviate the stress. It is good to note that all the fish had condition factor higher than 1, an indicator of good health in terms of weight to length ratio.

Comparing FCR and PER and the condition factor, CBD increased the efficiency of conversion of feed weight to body weight in stressed conditions the most (Figure 4.3), while it did not translate to a high efficiency of feed protein to body weight conversion (Figure 4.4). Moreover, the condition factor of all the groups were the same despite stress and CBD supplementation. So, could the fish have gained weight in fat in stressed conditions in the presence of CBD? No external research was found to have investigated the matter. Further research to quantify the protein and fat content of the fish postmortem might solve the mystery. As we do not know how much of the weight gained in our research was protein/ fat.

Overall, looking at all the results, supplementing the feed with CBD improves the protein conversion efficiency in non-stress conditions significantly more so than stressed group and stressed group supplemented with CBD. However, under stress condition, supplementing the feed with CBD reduced their efficiency of weight gain in comparison to protein fed. All the groups at the end of the study were shown to have the same conditions factors despite the discrepancy in result between Feed Conversion Ratio and Protein Conversion Ratio. Therefore, it could be that the fish were gaining their weight in fat instead of protein when stressed and supplemented with CBD. Further research needs to be conducted that investigates the fat content of these fish in comparison to their protein content. Also, perhaps a higher dose of CBD supplementation for longer than four weeks may result in a larger difference in data among the treatment groups.

## 4.5 Conclusion

In a world of ever-increasing population and high demand of protein, farmers are struggling to keep up the pace. Farming of cattle and other domestic animals are well documented to have significant contribution to methane emissions (Johnson and Johnson, 1995). To increase profits,

farmers are looking for more ecofriendly and cheaper alternatives to using commercials drugs to reduce stress in fish. While CBD may not be cheap as of now, but increasing acceptance, legalization and farming of marijuana should ultimately result in its lowering of price. Either way, it is important to know of the effects of CBD on the stress physiology and growth of vertebrate organisms in a world where CBD is widely sold as a cure all, where it may or may not be the case. As this study demonstrates, CBD shows promise in increasing productivity through improved protein efficiency ratio of fish. Further investigation into the matter may not just be useful in stress mediation in aquatic organisms but may also have implications in human medicine as well.

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# CHAPTER 5. EFFECTS OF CBD (CANNABIDIOL) ON THE PHYSIOLOGY OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*) AS A CHRONIC STRESS MITIGATING AGENT *IN-VIVO*.

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## 5.1 Abstract

This study evaluates the effects of Cannabidiol (CBD) on the physiology of stressed and non-stressed Nile tilapia, reared in a recirculating aquaculture system. Tilapia were fed with and without CBD (0.001% of feed weight) and with and without hydrocortisone stress hormone (0.01% of body weight) every day for four weeks. This experiment compared the plasma cortisol, blood glucose and protein levels, liver and spleen somatic indices (HSI and SSI, respectively), and lysozyme activity of the fish. Stress group (S) had a significantly higher value than the control group (C) in two of the parameters, glucose and lysozyme activity, this is an indication of stress. CBD had a stress reducing effect under stressed conditions in lysozyme activity. Although not significant, the stress reducing effect of CBD on stress biomarkers such as glucose and HSI also seemed promising. Further investigation into the matter may not just be useful in stress mediation in aquatic organisms but may also have implications in human medicine as well.

Keywords. CBD; Physiology; Nile tilapia; Stress physiology; *in-vivo*.

## 5.2 Introduction

Due to extensive husbandry methods used in aquaculture, stress in fish is a significant issue. Crowding, handling, and vaccinating are common husbandry methods that can stress the fish, leading to lower productivity (Hough, Glaze, Blumenthal and Mustafa, 2016). To mitigate the stress response, farmers often use antibiotics and chemical drugs. When antibiotics and other chemical drugs are released into the environment it can lead to the creation of superbugs or it may affect non-target species. The exposure of antibiotics and other chemicals also have the potential

to affect human health if the chemicals remain within the fish when consumed. In order to diminish the use of harmful substances in aquaculture, we must understand the root of the problem, "stress".

Stress, unfortunately, is very common in every form of animal farming, especially aquaculture. Poor stress management causes a loss in productivity due to disease and poor health, this in turn causes farmers to lose thousands of dollars every year. Stress is known as a state of decreased fitness or as a response to any external stimulus that disrupts homeostasis and therefore threatens survival (Colombo, Pickering, Belvedere and Schrech, 1990). There are two responses to stress known as the adaptive response and the maladaptive response. Adaptive stress response occurs when the body is able to maintain homeostasis due to its ability to adapt to the exposed stressor. Maladaptive stress responses occur when the body experiences deleterious effects due to the body's inability to adapt to a prolonged exposure to a stressor. There are three endogenous hormones that the body produces to mediate the stress response: adrenaline, noradrenaline, and cortisol. These three hormones have the ability to transition the body from the "rest and digest" to the "fight or flight" state. While the body is in the "fight or flight" state many vital mechanisms of the body are put on hold (such as digestion, immune response, growth, and reproduction), blood circulation to the muscles is enhanced, and the heart rate is increased. This prepares the body to either fight off what has triggered this response or to flee. This tactic is useful in short bouts (acute stress) but can become deleterious when exposure is prolonged, this is known as chronic stress. Chronic stress can make an organism susceptible to various secondary diseases since prolonged exposure to stress has been shown to weaken immune and inflammatory responses (Eddie and Norman, 2008).

According to Moyle et al. (1990), widespread physiological changes occur within an organism when it is exposed to stress. These responses are known as the primary, secondary, and tertiary stress responses. The primary response is triggered after a stressor is detected by the organism, causing the central nervous system to signal for the release of cortisol and adrenaline into the blood. The release of cortisol is a cascading effect that produces a negative feedback loop. Once the brain has perceived a stressor, it signals the hypothalamus to release corticotropin-releasing hormone (CRH); this then stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH) into the blood. ACTH travels through the circulatory system until it reaches its target, the interrenal cells. The presence of ACTH causes the head kidney to release cortisol. Unlike cortisol, the release of adrenaline (epinephrine) is controlled by a

positive feedback loop. Direct stimulation of the sympathetic nervous system causes the chromaffin tissue within the head kidney to release adrenaline.

In the secondary stress response, hematological changes start to occur such as, elevated blood glucose (hyperglycemia) and reduced blood clotting time (Moyle and Schreck, 1990). Diuresis and electrolyte loss are also a part of the secondary response, causing osmoregulatory dysfunction. The secondary stress response can also cause numerous tissue changes. These changes can be characterized by the depletion of liver glycogen and interrenal vitamin C, hemorrhaging of the thymus gland, as well as hypertrophy of the interrenal cells. The tertiary stress response is characterized by major physiological changes within the body such as, reduced resistance to infectious diseases, reduced growth, reduced reproductive success, and the reduction of survival. We can measure the changes that occur within each stage to determine the level of stress an organism has endured. In this paper we will measure the concentration of cortisol in the plasma (primary), glucose in the blood, protein in the plasma, the proportion of liver (hepatosomatic index) and spleen (spleen somatic index) weight to total body weight (secondary), and the activity of lysozyme within the blood (tertiary stress response) of Nile tilapia, *Oreochromis niloticus*.

As mentioned previously, the most common way to mitigate stress responses in aquaculture is to treat the fish using antibiotics and other chemicals. As previously mentioned, this method is harmful to our environment as well as potentially dangerous to the humans via consumption of tainted product. To help diminish the use of these harmful substances to lessen the effects of stress in aquaculture, scientists are on the search for alternative solutions such as nutraceuticals. Nutraceuticals are known as functional foods (often phytochemicals) that have been shown to have medicinal properties (Lim and Webster, 2006). Nutraceuticals have repeatedly been shown to potentially alleviate stress and boost the immunity in many different animals including fish (Mustafa, Randolph and Dhawale, 2011; Ibrahim, Khan, Rinard and Mustafa, 2015; Mustafa, Dhawale, Park and Yoo, 2013).

Cannabidiol (CBD) is a fairly new nutraceutical that may have the potential to mitigate the stress response. CBD, like tetrahydrocannabinol (THC) is a derivative of the marijuana plant, *Cannabis sativa*. However, unlike THC, CBD is a non-psychoactive compound of the plant. Extensive research has been conducted on the effects of CBD on stress, anxiety, cognition, movement disorders, and pain (Morales, Reggio and Jagerovic, 2017). CBD has been shown to be

a neuroprotectant (Fasinu, Phillips, Elsohly and Walker, 2016), alleviate anxiety, psychosis (Morales et al. 2017), epilepsy (Anavi-Goffer et al., 2012), depression (Breuer et al., 2016), and pain (Burstein, 2015). Research has also shown that CBD may also benefit the immune system by reducing inflammation (Ben-shabat, Hanus, Katzavian and Gallily, 2006), cancer (Burch, Mortuza, Mustafa and Blumenthal, 2021; Juknat, Kozela, Kaushansky and Mechoulam, 2016), and bacterial infections (Ryberg et al., 2007). In 2018, Viudez-Martínez et al. studied the effect of CBD on different gene targets of the hypothalamus-pituitary-adrenal (HPI) axis of mice under control and stress conditions. They did this by administering CBD intraperitoneally in dosages of 5 mg/ kg, 15 mg/kg, or 30 mg/kg (of mice body weight), before the mice were exposed to restraint stress. Using real time PCR analysis, they were able to measure the relative gene expression of corticotropin-releasing factor. Their research showed that when CBD is given in the 5 mg/kg and 15 mg/kg doses, it was able to block the gene expression caused by acute stress (Viudez-Martínez, García-Gutiérrez and Manzanares, 2018). Expression of stress related genes are certain to have a physiological impact on the whole organism's stress response

A lot of research has been conducted on the widespread therapeutic usage of CBD but there is a lack of research looking at the effect of CBD on the stress physiology of vertebrates. Would CBD have the ability to mitigate stress responses? Would it be able to compensate for the reduction in growth that is seen in fish exposed to high stress conditions in aquaculture? In this experiment, we chose Nile tilapia, *Oreochromis niloticus* as our fish model. Nile Tilapia is one of the most common fish used in aquaculture (Gupta and Acosta, 2004). Benefit of using aquatic organisms is that aquatic environments do not seem to change as much as terrestrial environments. Therefore, fish can be easily stressed in the presence of low-level stressors (McCain, Quarrar and Mustafa, 2015). Another benefit is that the overall physiological and immunological responses of fish are similar to that of other vertebrate animals. Their physiological and immunological responses are controlled by the nervous and endocrine system, also comparable to other vertebrate animals, making them a good model to study human medical applications.

In our experiment, we tested the effect CBD on mitigating the chronic stress response of Nile tilapia. To determine its effect, we measured plasma cortisol (primary stress biomarker), blood glucose levels plasma protein levels, liver and spleen somatic indices (secondary stress biomarker), and lysozyme activity (tertiary stress biomarker) in both stressed and non-stressed *O. niloticus*.

## 5.3 Methods

### 5.3.1 Fish maintenance

Nile tilapia, *Oreochromis niloticus*, fingerlings (average length 20±2 cm; average weight 175±10 g) were purchased from Troyer Aqua Farms, Geneva Indiana. The fish were kept in a recirculating aquaculture system (Aquatic habitats ™, Aquatic Eco-Systems, INC). The system was maintained and cared for following an approved animal care protocol (pH: 6.0-7.0, ammonia: <0.05 mg/ L; dissolved oxygen 5.00-7.00 mg/ L, and temperature: 25 ± 2 °C). The fish were fed 1.5% of their body weight twice a day with a compatible commercial fish feed, Purina® AquaMax® Fingerling Starter 300 (Purina Mills, MO, USA). All fish were taken care of following an approved protocol by Purdue University Animal Care and Usage Committee (PACUC) following the guidelines of the US National Research Council's "Guide for the Care and Use of Laboratory Animals".

### 5.3.2 Experimental design

No previous studies were found showing the potential effects of CBD on chronic stress physiology of *O. niloticus*. Due to this void, the therapeutic dosage for CBD for fish is unknown. To determine the appropriate concentration of CBD, we conducted a preliminary acute study for 72 hours. In the acute study, the fish were maintained following similar experimental design and protocol as this study. However, it was done only over a period of 72 hours and the fish were fed with feed supplemented with three different concentrations of CBD, with and without hydrocortisone. At the end of the experiment we collected blood from the fish and evaluated stress biomarkers such as blood glucose, hematocrit, and plasma protein. From the study we found that there was no significant difference in the stress physiology of *O. niloticus* between 0.001%, 0.002%, and 0.003% CBD (% of feed weight) over 3 days. Therefore, in our chronic study, the lowest concentration was tested to see its effect on chronic stress physiology as it is the most economic option for aquaculturists.

For the chronic study, the fish were divided into four different groups. Each group consisted of 4 fish, each fish in individual tanks (individual replicates). Each group was fed with a different diet and were fed twice a day. Group 1 was fed a diet the standard commercial feed (C= Control feed); group 2 was fed with 0.001% (feed weight) CBD (99% pure isolate from Sigma

Aldrich®, MO, USA) supplemented commercial feed (CCBD = Control feed with CBD); group 3 was fed with 98% hydrocortisone (at 0.01% body weight from Acros Organics, NJ, USA) supplemented commercial feed (S= Stress feed); and group 4 was fed with 0.001% CBD and hydrocortisone (0.01% body weight) supplemented commercial feed (SCBD= Stress feed with CBD). Once hydrocortisone is ingested, the body metabolizes it into cortisol, which is responsible for the stress response. The amount of hydrocortisone used was determined using the findings of previously done research at this concentration (Barton et al., 1987). The addition of hydrocortisone in the feed gave us the ability to compare the effects of CBD on stressed and non-stressed *O. niloticus*.

### 5.3.3 Feed preparation

Since the amount of feed needed depended on the weight of the fish, the feed was prepared weekly. The feed was prepared by dividing the total amount of commercial feed (Purina® AquaMax® Fingerling Starter 300) needed that week in half. One half of the feed would get the calculated dosage of hydrocortisone (0.01% of fish body weight) and thoroughly mixed. The feed was then left out overnight to dry. Then the CBD supplemented fish feed was prepared by further dividing the feed (with and without hydrocortisone) in half. One half of the feed without hydrocortisone and one half of the feed containing hydrocortisone would get 0.001% (of feed weight) of 99% pure isolate Cannabidiol from Sigma Aldrich®. All of the feed were allowed to dry overnight and then stored at 4°C. Drying overnight was needed since ethanol was used throughout as a solvent to dissolve and thoroughly mix the CBD and hydrocortisone to the feed. The drying in an unsealed container allowed the ethanol to evaporate out of the feed. A Proximate composition of feed ingredients is presented in Table 5.1.

Table 5.1. Proximate composition of feed ingredients used to prepare four treatment groups of fish feed represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups.

| Ingredients (% Feed Weight) | C | CCBD | S | SCBD |
|---|---|---|---|---|
| Crude protein | 50 | 50 | 50 | 50 |
| Crude fat | 16 | 16 | 16 | 16 |
| Crude fiber | 3 | 3 | 3 | 3 |
| Calcium (Ca) | 5.2 | 5.2 | 5.2 | 5.2 |
| Phosphorus (P) | 1.3 | 1.3 | 1.3 | 1.3 |
| Sodium (Na) | 0.6 | 0.6 | 0.6 | 0.6 |
| CBD | -- | 0.001 | -- | 0.001 |
| Hydrocortisone* | -- | -- | 0.01 | 0.01 |

*% Body Weight

### 5.3.4 Fish sampling

To calculate the stress biomarkers, the fish were sampled at the end of a four-week period. The fish were euthanized by mixing tricaine methane sulfonate (MS-222) (Western Chemicals, WA) with water at 400 mg/ L. This concentration allows immediate immobilization of the fish to reduce any stress from handling (all within two minutes of catching the fish). The fish were weighed, length was measured (results not included in this paper) and blood samples were collected from the caudal vein using heparinized syringes to prevent blood clot. The blood was placed in a 1.5 mL Eppendorf tube and immediately placed on ice until needed.

The fish were then dissected using aseptic techniques to remove the liver and the spleen. Upon removal, the liver and the spleen were weighed. Then the following parameters were measured using the collected blood and the tissue samples.

#### *Plasma cortisol*

Cortisol is one of the major stress hormones that are released by the interrenal cells of the head kidney tissue of the fish and thus indicative of stress (Barton et al., 1987). The blood was centrifuged at 5000 rpm for 10 minutes to collect the plasma. The supernatant plasma was collected and preserved at -80°C for future use. Due to the small amount of serum that was able to be

isolated, the serum were pooled together from all the fish within a treatment group (n=1). To measure the cortisol level in the plasma, Cayman Chemical Cortisol ELISA kit (Item No. 500360) (Ann Arbor, MI, USA) was used, following manufacturer's protocol.

***Blood glucose***

Blood glucose is another measure of stress as changes in blood glucose occur due to increased respiration, decreased metabolic activity and decreased immunity, all indicative of stress (Ulrich-Lai and Herman, 2009). A Glucometer (Freestyle, Abbott Diabetes Care, Inc., Alameda, CA, USA) was used to measure the blood glucose according to the manufacturer's protocol (Hossain, Blumenthal and Mustafa, 2013).

***Total plasma protein***

Total plasma protein is a good indicator of health and stress levels (Asmathulla, Bidhan and Papa, 2001). As part of the stress response, increased synthesis of certain proteins is induced to act as molecular chaperones to reconstruct damaged cells (Himmelfarb and Ellen, 2001). A protein refractometer was used to measure the refractive index of all the dissolved solid materials in solution. The refractive index scale was 1.333 to 1.360 and the refractometer was calibrated using PBS. To take a reading, a few drops of plasma was placed on the prism and read from the far-right scale in g/ 0.1 L.

***Hepatosomatic index (HSI)***

Energy in fish is stored in the muscles as well as the liver in the form of glycogen. During growth and high stress conditions, glycogen is mobilized in the presence of glucagon released by the pancreas. The glycogen storage of the liver is broken down to glucose and released in the blood, affecting the overall size and mass of the liver. Thus, hepatosomatic index (HSI) is a good indicator of nutrition, growth and stress levels of fish (Wedemeyer, Barton and McLeay, 1990). Hepatosomatic index compares the weight of the liver of the fish in proportion to its body weight. The collected liver from the fish were weighed and the following formula was used to measure the HSI of the fish.

$$\text{HSI} = (\text{Liver Weight (g)}/\ \text{Body Weight (g)}) \times 100$$

***Spleen somatic index (SSI)***

Lymphocytes and blood cells are stored in the spleen and mobilized to immunomodulate (Bronte and Pittet, 2013). Therefore, the spleen somatic index is a good indicator of overall health of fish. Spleen somatic index compares the weight of the spleen of the fish in proportion to its body weight. The collected spleen from the fish were weighed and the following formula was used to find the SSI of fish:

$$SSI = (\text{Spleen Weight (g)}/ \text{Body Weight (g)}) \times 100$$

***Lysozyme activity assay (LAA)***

Lysozyme is an enzyme that lyses bacterial cells which changes the opacity of a given bacterial solution. Thus, this assay measures the ability of the endogenous lysozyme in the blood to clear the color of the bacterial solution using a spectrophotometer (Shugar, 1952). The more bacteria are killed, the clearer the solution becomes which gives a higher transmittance (T) reading on the spectrophotometer. Transmittance is the ratio of light that falls on a substance to the light that pass through it. Higher transmittance means a larger amount of the light passed through the substance. To conduct the assay, the collected blood samples were centrifuged for ten minutes at 5000 RPM to collect the plasma. The supernatant was collected from each sample and put into Eppendorf tubes and set aside. Then a suspension of *Micrococcus lysodeikticus* was made at a concentration of 0.2 mg/ mL in 0.05 M (pH = 6.2) sodium phosphate buffer. Then 1 mL of the suspension was added to an Eppendorf tube. 50 µL of the plasma was then added to the Eppendorf tube and vortexed. 1 mL of this solution was put into a cuvette to measure its transmittance at 540 nm using a spectrophotometer (Spectronic 601 spectrophotometer, Milton Roy Company, PA). Readings were taken at 1 minute and 5-minute mark. This procedure was repeated for each and every one of the sera collected from the fish. To calibrate the spectrophotometer, an uninoculated sodium phosphate buffer was used. We used the following formula to calculate the lysozyme activity assay,

$$LAA = (\text{Final transmittance} - \text{Initial transmittance})/ \text{Total elapsed time}$$

Thus, the lysozyme activity assay is an indicator of rate of increase in transmittance per minute due to the lysozyme clearing bacteria.

### 5.3.5 Statistical analysis

The data collected were analyzed using SigmaPlot® 14.0, Systat Software Inc. A one-way analysis of variance (ANOVA) followed by Tukey's test was performed to determine whether the differences between the treatment groups were significant ($P < 0.05$). The analyzed data is presented in the form of means ± standard errors of means (SEM) throughout the paper.

## 5.4 Results and discussions

Due to lack of research on the effects of CBD on the stress physiology of fish, we have reviewed literature that has tested the effects of CBD on mice models whenever fish model research was unavailable. At the moment, the effects of CBD on the stress physiology of fish is a novel area of research. This makes our research one of the first look at the potential use of CBD as a stress mitigating agent in fish, namely, *O. niloticus*. Whereas CBD on mice models have been well explored in this area. When discussing the effects of stress on animal physiology, we review the stress physiology of fish as it is a well-reviewed and known area of research.

Firstly, cortisol ELISA assay was done to see whether the hydrocortisone fed to the fish was able to stress the fish effectively. In order to interpret the results, we must first understand how cortisol is generated in the body and how long it remains active. In fish, stressors stimulate the hypothalamic-pituitary-interrenal axis (HPI) which prompts the release of adrenocorticotrophic hormones (ACTH). This in turn prompts the interrenal cells of the head kidney tissue to generate cortisol in response. This is similar to the cortisol production in mammals who have hypothalamic-pituitary-adrenal cortex (HPA) and are also stimulated by the adrenocorticotrophic hormones (ACTH) (Barton and Iwama, 1991). The cortisol release is controlled by a negative feedback loop at the HPI axis (Fryer and Peter, 1977). The plasma cortisol levels are kept in check by regulating how much of it is produced endogenously. In fish, the plasma cortisol levels come down to the basal levels 24 hours after an acute stressor is perceived. Cortisol is rapidly metabolized in the liver due to its action on it, and then filtered and excreted by the kidney (Laberge, Yin-Liao and Bernier, 2019).

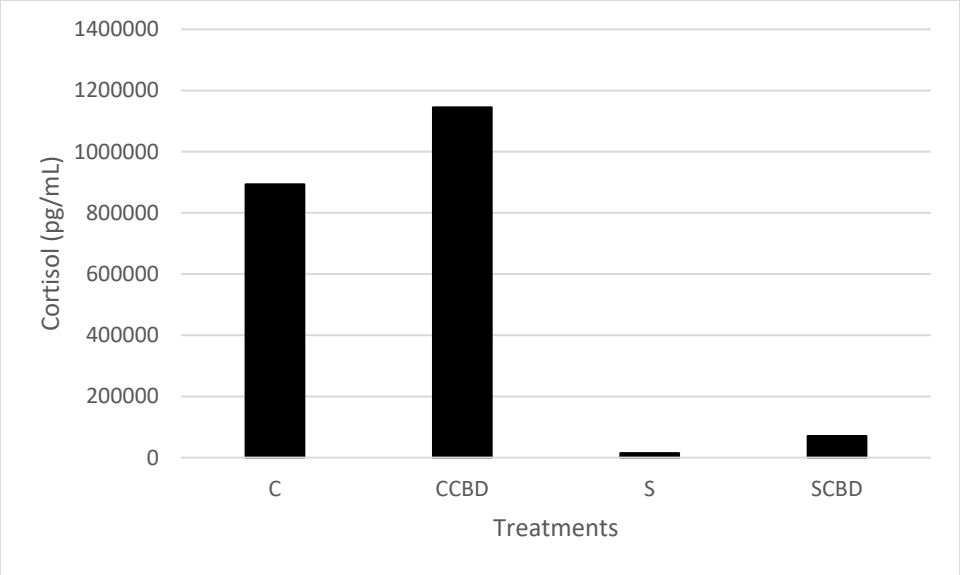


Figure 5.1. Plasma cortisol concentrations in pg/ mL of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means and no standard error bar is presented as blood serum were pooled together from all the sample fish per treatment (N=1).

According to Figure 5.1, the stress groups (S and SCBD) had a lower plasma cortisol level in comparison to the control groups (C and CCBD). Statistical analysis is not possible as the serum were pooled together from all the sample fish within the treatment group before running ELISA assay (N=1). It may seem counterintuitive; however, our result is an indication that the exogenously supplemented cortisol via fish feed were getting into the blood of the fish and thus were stressing the fish. The increased level of exogenous plasma cortisol in turn was shutting down the endogenous cortisol production due to the negative feedback loop at the HPI axis (Fryer and Peter, 1977). Inhibition of the HPI axis would shut down the production of ACTH and thus inhibit the production of cortisol from the interrenal cells of the head kidney tissue. Then, the exogenous cortisol in the plasma were being metabolized rapidly by the liver and excreted completely within 24 hours. Blood cortisol is cleared at a faster rate when fish are administered with exogeneous cortisol in the blood to stimulate stress due to the increased level of cortisol in the blood (Barton et al., 1987). The combined action of these systems lowered the plasma cortisol levels of the hydrocortisone supplement fed fish (S and SCBD) while keeping the endogenous cortisol levels of the control fed fish groups (C and CCBD) up. Since the control fish were not stressed using

exogenous cortisol, they were able to maintain a basal level of endogenous cortisol throughout the 4 weeks of this experiment due to the HPI axis not shutting down via negative feedback loop and the regular rate of clearance of cortisol from the blood. Due to our dosage of hydrocortisone, each time after feeding, the fish would have had a spike in plasma cortisol which would manifest itself by affecting secondary or tertiary stress biomarkers down the line (Barton et al., 1987). It is evident in our secondary and tertiary biomarkers. These findings are supported by previous studies where similar phenomena were observed, such as in *O. niloticus* in high density stress conditions (Khan, 2016), cortisol fed catfish, *Ictalurus punctatus* (Rafinesque, 1818; Davis, Torrance, Parke and Suttle, 1985) and *in-vitro* tissue culture of Coho salmon, *Oncorhynchus kisutch* (Walbaum, 1792; Bradford, Fitzpatrick and Schreck, 1992).

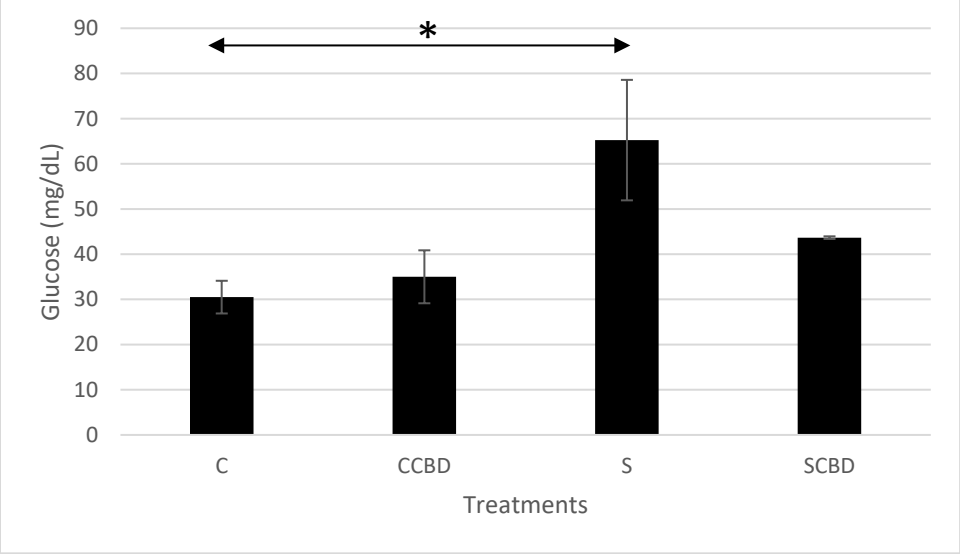


Figure 5.2. Blood glucose levels in mg/ dL of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. *= Significant difference (P< 0.05). N= 4, F= 3.709, DF= 3.

As we have now established that the stress fed treatment groups (S and SCBD) were in fact stressed, let us look at the physiological stress biomarkers and how they were affected by CBD. One of the most important secondary stress biomarkers is glucose. Stress increases the energy demands of the organism to fuel the fight or flight response and maintain homeostasis. This in turn demands for an increase in blood glucose to supply the energy demand. In order to do so, the pancreas releases glucagon which acts on the liver to break down glycogen into glucose (Taborsky,

2010). Therefore, increased blood glucose is a sign of increased stress. In Figure 5.2 (N= 4, F= 3.709, DF= 3), the stressed group (S) had a significantly higher glucose level (more than double) than the control group (C; P= 0.046). Similar results have been reported on the blood glucose levels of fish under stress in various studies outlined by Martinez-Porchas et al. (2009). According to the paper, glucose and cortisol, along with a few other biomarkers of stress such as packed cell volume and protein can be good indicator of stress (Martinez-Porchas, Martinez-Cordova and Ramos-Enriquez, 2009). Also, in the case of stress groups (S vs SCBD), we see that the glucose level was lowered from 65 mg/ dL to 43 mg/ dL when CBD was mixed into the feed, although the results were not significantly different. Thus, CBD was somewhat effective in reducing blood glucose in stressed condition. The effect of CBD on mice blood glucose have been tested by Romero-Zerbo et al. in 2020. In the study, no significant difference was found in blood glucose between groups treated with CBD vs without CBD (Romero-Zerbo et al., 2020).

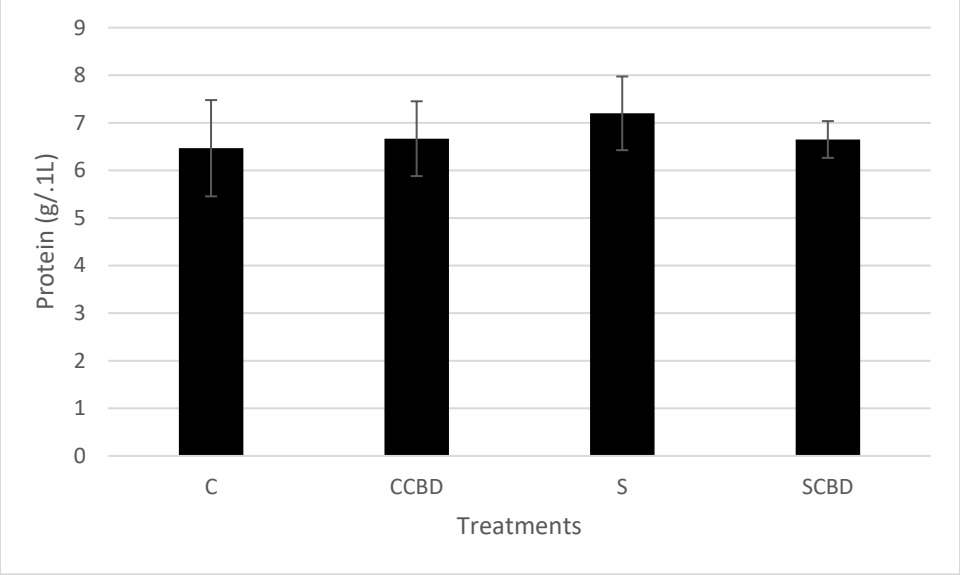


Figure 5.3. Plasma protein levels in g/ 0.1L of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. N= 4, P= 0.910, F= 0.176, DF= 3.

Another important secondary stress biomarker is the plasma protein level. As mentioned earlier, under stress, organisms increase their plasma protein levels to supply proteins around the body to repair any damage done to various tissues due to increased activity from stress (Himmelfarb and Ellen, 2001). So, under stressed conditions, higher protein levels are expected in

the blood. In figure 5.3 (N= 4, P= 0.910, F= 0.176, DF= 3), we see that all the protein levels across the treatments were at the same level (6.5-7.2 g/ 0.1 L). George Iwama (1998) in his paper titled "Stress in Fish" reviews among other things, the effect of stress on plasma proteins. In this paper, he reviews studies that show that stress does in fact increase blood plasma protein and this could be used as a stress biomarker. Plasma protein however, is not a reliable stress biomarker on its own and needs to be used alongside other biomarkers because of wide variability in results (Iwama, 1998).

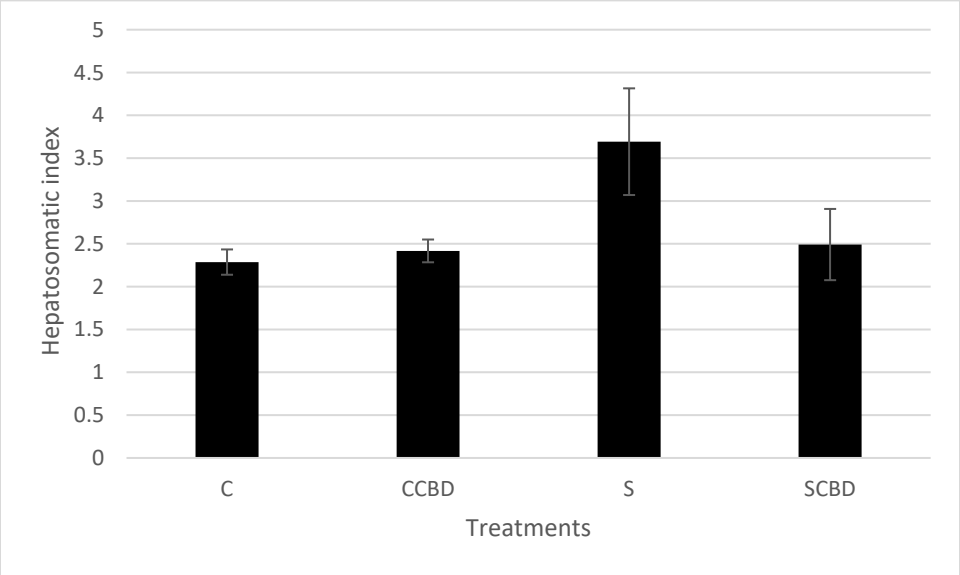


Figure 5.4. Hepatosomatic index of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. N= 4, P= 0.106, F= 2.589. DF= 3.

Continuing to look at the secondary stress biomarkers, hepatosomatic index (HSI) is the weight of the liver in proportion to the fish body weight. Hepatosomatic index should be low in organisms under acute stress as the glycogen stores in the liver get mobilized to form blood glucose under stress (Barton et al., 1987). This glucose is then used to meet the high energy demands of a stressed condition as mentioned earlier. However, as stress is prolonged for a long time (chronic stress), the body stores energy as glycogen in the liver to deal with stress and this becomes the new basal HSI level. Higher metabolic activity in the liver due to stress can lead to higher HSI in chronic stress. This phenomenon is observed in previous research (Barton, 2002). This phenomenon has also been observed in previous chronic stress studies (6 and 8 weeks) conducted

in our lab on *O. niloticus* (Furnas, 2019; Saillant, 2019). In Figure 5.4 (N= 4, P= 0.106, F= 2.589. DF= 3), it can be seen that stressed group (S) had the highest Hepatosomatic index at 3.65. This is much higher than all the other treatment groups (mean HSI of 2.25-2.5); however, this was not significantly different. This is a sign that the cortisol with no CBD supplemented fish were stressed. Another important observation to make is that CBD was able to lower the HSI in stressed condition from 3.65 (S) to 2.5 (SCBD) which are close to the control groups (C and CCBD) HSI level (2.25-2.4). This is indicative of the ability of CBD to reduce stress. To our knowledge, no other study was found to have looked into the matter.

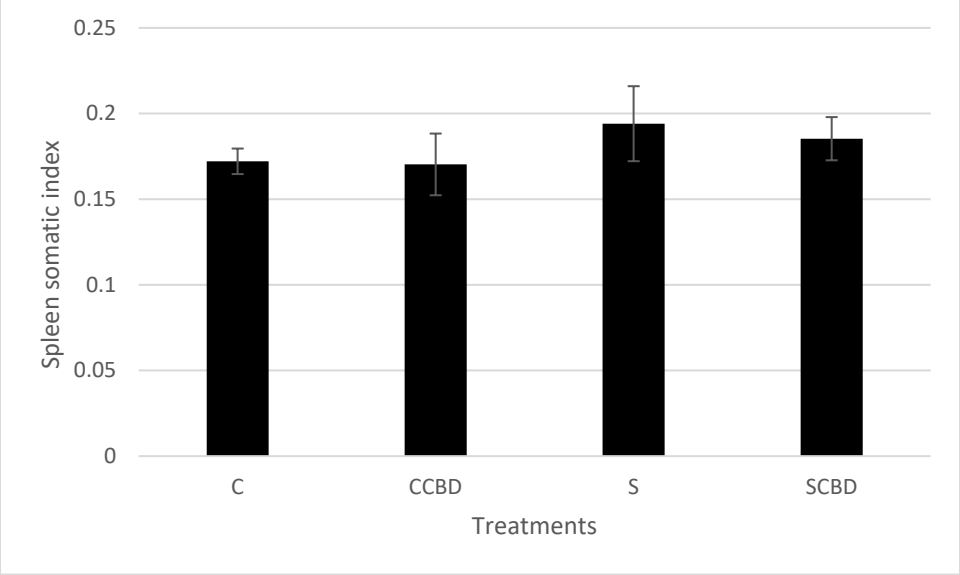


Figure 5.5. Spleen somatic index of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. N= 4, P= 0.695, F= 0.494, DF= 3.

Just like the HSI, the spleen somatic index (SSI) is the weight of the spleen in proportion to its total body weight. It is a good stress biomarker since the spleen stores immune cells and red blood cells. Stress prompts the release of blood cells and spleen cells (T and B cells, macrophages) from the spleen to supply for the increased respiratory demands and to fight the perceived threat (Bronte and Pittet, 2013). This results in a decrease in weight of the spleen in the short term (Pulsford, Lemaire-Gony, Tomlinson, Collingwood and Glynn, 1994). However, in prolonged stress (chronic), just like in the case of the HSI, the spleen becomes larger to hold larger quantity of immune cells ready to be released in the case of another infection (Saillant, 2019). In a study

published in 2017, it was found that mice exposed to chronic stress from crowding were more likely to physically bite each other. This in turn led to an increase in spleen weight over a period of 19 days due to the activation of spleen immune cells to fight off any invading pathogens from such bites (Foertsch et al., 2017). However, an increased production of immune cells may also affect the overall quality of the immune cells and their ability to fight infections (Renata et al., 2020). Figure 5.5 (N= 4, P= 0.695, F= 0.494, DF= 3) shows that all the treatment groups had similar SSI levels between 0.175 and 0.19. Unsurprisingly, stressed group (S) had the highest SSI.

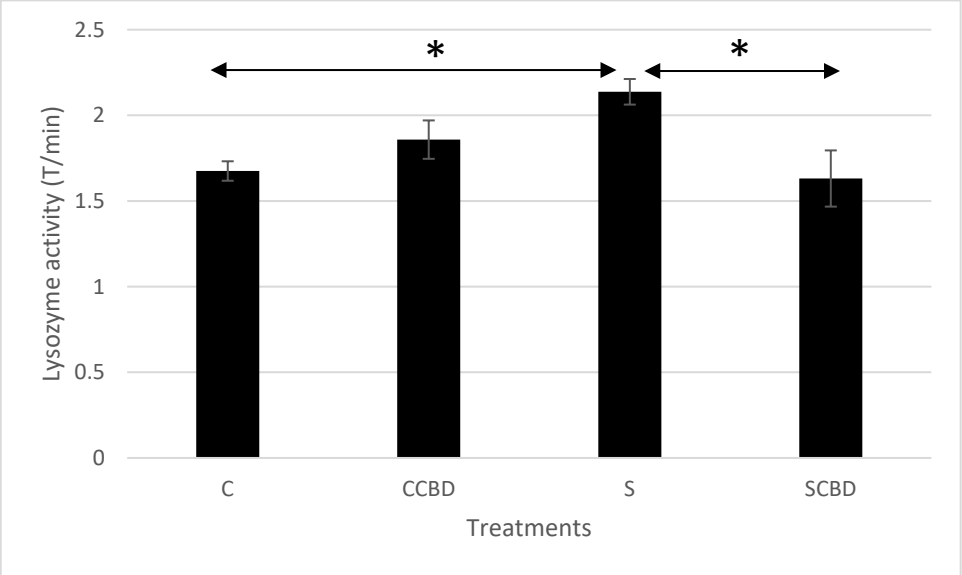


Figure 5.6. Lysozyme activity in Transmittance (T)/ minute of plasma of Nile tilapia fed with four different treatments represented by C (Control feed), CCBD (Control feed with CBD), S (Stress feed= feed with hydrocortisone) and SCBD (Stress feed with CBD= feed with hydrocortisone and CBD) groups. Results are presented as means± SEM. *= Significant difference (P<0.05). N= 4, F= 4.613, DF= 3.

Finally, the tertiary stress biomarker, lysozyme activity demonstrates the ability of blood lysozyme to clear out bacterial solutions. Clearer the solution, higher the amount of light that passes through it in comparison to the light emitted by the spectrophotometer. Therefore, higher the transmittance (T). In stressed conditions, organisms produce increased amounts of lysozyme to fight off any perceived infectious threat (Ragland and Criss, 2017). This gives a higher transmittance value. Such is seen in figure 5.6 (N= 4, F= 4.613, DF= 3), where the stressed group (S) had the highest transmittance value (2.1 T/ min), significantly higher (P= 0.046) than the control group (C) at 1.7 T/ min. This is indicative of stress. In another study it was demonstrated

that handling stress in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), led to an increase in lysozyme activity of the fish (Demers and Bayne, 1997). Notably, between the stressed groups (S vs SCBD), CBD was able to reduce the lysozyme activity significantly (P=0.029) from 2.1 to 1.6 T/ min to the control (C) level; this is indicative of a reduction in stress. This is not surprising as CBD is known to down regulate the immune system and act as an anti-inflammatory agent (Cabral, Rabron and Ferreira, 2014). What is unknown is, whether the lowering of lysozyme activity seen in our data is due to CBD reducing stress by acting on the HPI axis or by directly affecting the immune system. Looking at all the other results alongside lysozyme activity, it is most likely a combination of both.

Lysozyme and SSI are both physiological and immunological parameters as they play a role in fish immunology. Lysozyme is responsible for clearing blood pathogens and spleen somatic index is tied in with the release of spleen cells responsible for immunomodulation. Comparing both figure 5.5 and 5.6, we see an overlap in the trends. However, the differences among the treatment groups are much more profound in the lysozyme activity assay (figure 5.6.). Lysozyme secretion is much more sensitive to stress and is a well-established biomarker of stress. SSI is still important as it allows for the measurement of spleen weight compared to body weight and spleen health is important to the overall immune system, which is affected by stress.

Overall, we see that stress group (S) had a significant higher value than the control group (C) in two of the parameters, glucose and lysozyme activity, which is an indication of stress. CBD had a stress reducing effect under stressed conditions in lysozyme activity. Although not significant, the stress reducing effect of CBD on stress biomarkers such as glucose and HSI also seemed promising. Perhaps a higher dose of CBD supplementation for longer than four weeks may result in a larger difference in data among the treatment groups.

## 5.5 Conclusion

In a world of ever-increasing population and high demand of protein, farmers are struggling to keep up the pace. Farming of cattle and other domestic animals are well documented to have significant contribution to methane emissions (Johnson and Johnson, 1995) known to contribute to global warming. To increase profits in aquaculture, farmers are looking for more ecofriendly and cheaper alternatives to using commercials drugs to reduce stress in fish. While CBD may not be cheap as of now, but with increasing acceptance, legalization and farming of marijuana should

ultimately result in its lowering of price. Either way, it is important to know of the effects of CBD on the stress physiology and immunology of vertebrate organisms in a world where CBD is widely sold as a cure all, where it may or may not be the case. As this study demonstrates, CBD shows promise in reducing stress in fish. Further investigation into the matter may not just be useful in stress mediation in aquatic organisms but may also have implications in human medicine as well.

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# CHAPTER 6. OVERALL CONCLUSION

The *in-vitro* T and B cell proliferation assay results indicated that for mouse spleen T cells, 11.49 µg/mL of CBD was effective in increasing the T cell proliferation when the cells were stimulated by a mitogen. For mouse B cells, CBD seems to have had an inhibitory effect on the cells by reducing the proliferation caused by the mitogen. Similar results were seen in the case of fish spleen cells applied with mitogen, where the inhibition of spleen cells was seen at the final CBD concentration of 0.46 µg/mL. This inhibition of spleen cells can be useful in immunomodulation, thus CBD exhibited immunomodulatory effects. In the acute stress study, among the non-stressed groups, fish fed with the highest concentration (0.003%) of CBD showed one of the lowest stress in terms of the stress biomarkers, while one of the highest stress was detected in the stressed group supplemented with no CBD. However, among the various concentrations of CBD used, no significant difference in stress biomarkers were detected in the hematological parameters. Thus, the lowest concentration (0.001%) of CBD was tested long term in the chronic stress study as a more economic option for aquaculturists. In the chronic stress study, supplementing the feed with CBD improved the fish protein conversion efficiencies in non-stress condition. This has implications of leading to higher protein content in fish per weight of food fed with CBD. Stressed groups showed a significantly higher value than the non-stressed groups in two of the parameters, glucose and lysozyme activity, indicative of stress. CBD had a stress reducing effect under stressed conditions, such as glucose, Hepatosomatic index and lysozyme activity. However, only significant impact was seen in lysozyme activity. Lysozyme activity is indicative of the ability of blood in clearing pathogens which is important in the overall immune health of the fish.

From our current research, CBD shows potential in stress and immune modulation. It may have different effects based on the species, whether they need to enhance their immune response or reduce it to be healthy. It also seems to have had different effect on different parts of the immune system. Hematological parameters were not significantly affected by acute stress. CBD did not make any substantial difference in growth; however, the lysozyme activity was brought down to the non-stress level by CBD in chronic stress. By administering the proper dosage of CBD on a case by case basis, health benefits can be achieved.

In a world of ever-increasing population and high demand of protein, farmers are struggling to keep up the pace. To increase profits in aquaculture, farmers are looking for more ecofriendly and cheaper alternatives to using commercials drugs to reduce stress in fish. While CBD may not be cheap as of now, but with increasing acceptance, legalization and farming of marijuana should ultimately result in its lowering of price. Either way, it is important to know of the effects of CBD on the stress physiology and immunology of vertebrate organisms in a world where CBD is widely sold as a cure all, where it may or may not be the case. As this study demonstrates, CBD shows promise in stress and immune modulation in fish. Further investigation into the matter may not just be useful in stress and immune modulation in aquatic organisms but may also have implications in human medicine as well. Further research needs to be conducted with a higher dose of CBD for longer than four weeks to investigate their effect on the stress physiology and immunology of fish.