CAPSAICIN SUPPLEMENTED FEED AND ITS EFFECTS ON STRESS MODULATION IN NILE TILAPIA

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Abstract

Properties of the nutraceutical capsaicin were assessed in order to determine its effects on several stress bio-markers in teleost fish. Nile tilapia, *Oreochromis niloticus*, were fed a diet supplemented with capsaicin for eight weeks, and crowding stressor was used in order to ascertain capsaicin's ability to modulate stress in comparison with control animals. Biomarkers-plasma cortisol, blood glucose, spleen somatic index, packed cell volume, plasma protein, condition factor, and macrophage phagocytic activity were assessed bi-weekly during the experimental period. The results indicated that capsaicin produced no statistically significant differences between fish receiving the capsaicin and those which did not. A cluster based analysis using data mining confirmed the findings that at 0.02% of the diet, capsaicin produced no statistically significant differences.

***Key Words*:** Tilapia, conservation, farming, stress, nutraceutical.

INTRODUCTION

The Nile tilapia (*Oreochromis niloticus*) was chosen as a model organism for the investigation of the nutraceutical properties of capsaicin. Tilapia originated in Africa and is farmed all over the globe. These fish are thought to have been cultured by the Egyptians over four thousand years ago (Gupta and Acosta 2004) as their images are depicted in hieroglyphics. Tilapia was chosen for this study for several reasons. The main reasons are their ease of culture and their tolerance to a wide range of conditions. The Tilapia is extremely hardy, attains a large size in a short period of time, and is becoming heavily cultured in the United States (Yi *et al.* 1996). They can survive in temperatures as low as 10ºC and as high as 40ºC (Azaza *et al.* 2008). As well as the attributes already mentioned, tilapia performs well at oxygen saturation levels under 20%. They may function at lower levels of dissolved oxygen (DO) but revert to a non-productive anaerobic metabolism and may begin extracting oxygen by gulping ambient air. They have been shown to survive at levels as low as 0.1 ppm for as long as 6 hours. These levels are far below that of channel catfish (25-49% saturation) and carp (25% saturation) (Teichert-Coddington and Green 1993). It has also been shown tolerant of a wide range of pH, levels of ammonia of varying species, as well as salinities. Due to the natural overall fitness of the species, tilapia is also resistant to many infections.

Tilapia is not immune to the stress causing interactions produced by crowding (Barcellos *et al.* 2001). Although tolerant of varying conditions, the push to capitalize on production places tilapia at high stocking densities. This can sometimes overwhelm the innate resistance to adversity, making the animal susceptible to acute stress and disease. Stress induced by crowding has long been used by researchers to illicit the stress response in many fish species and is easily measurable using standard stress biomarkers.

In order to understand how to counteract the effects of stress, our research has focused on the use of nutraceuticals. A nutrceutical is a food additive that imparts health benefits to the

recipient (Majaz *et al.* 2012). This is a well-known concept. The fortification of cow's milk with vitamin D has been common place since the 1930s when it was found to cure rickets (Holick *et al.* 2008). The overall goal is to seek natural, safe alternatives to traditional chemical disease treatment through prevention. Since the alimentary tract is where primary nutrient absorption takes place, and it has been intricately tied to immune health, nutraceuticals have the ability to bolster overall fitness thereby lowering losses related to stress and disease. In the past similar experiments have been conducted in order to determine the effects of the nutraceuticals garlic, turmeric, genstein, vitamin C, β-carotene and phosphatidylcholine on stress mediation. In this assay we scrutinize the compound, capsaicin, in order to determine how it affects physiological and immunological biomarkers of stress.

Capsaicin has been shown to cause neural desensitization when placed in direct contact with vanilloid receptors of an organism (Szolcsányi 2004). Capsaicin has also been shown to affect immune response (Powell *et al.* 1993), metabolism (Shin and Moritani 2007) and have an array of effects on digestion (Anogianaki e*t al.* 2007).

The biomarkers chosen to analyze dietary capsaicin's effect on stress induction are condition factor (K), cortisol, glucose, plasma protein, spleen somatic index (SSI), packed cell volume (PCV) and macrophage activity. Of these, cortisol is considered an indicator of the primary stress response, while all others save condition factor and macrophage phagocytic activity are used to determine secondary stress response levels. Condition factor and macrophage phagocytic activity are used to indicate the level of tertiary stress response experienced by the organism as a result of the preceding stress stages.

The focus of this study is to determine the effects of dietary capsaicin on Nile tilapia as measured via stress biomarkers such as plasma cortisol, blood glucose, spleen somatic index, packed cell volume, macrophage activity and condition factor.

MATERIAL AND METHODS

Captive bred Nile tilapia were purchased from Troyer Farms, a presumably disease free facility located in Geneva, Indiana. Troyer Farms fish are hybrid all male Nile tilapia produced at AmeriCulture in New Mexico. Four hundred fish were received with an approximate average individual weight of 25 grams at the time of purchase. These Nile tilapia hybrids can develop into two different phenotypes, one which is white or reddish and the more widely recognized gray coloration with black bars. No genotypic differences were assumed to affect the results of this assay and thus no preference was during random selection to either phenotype (AmeriCulture 2013).

Half of the fish were evenly divided and placed into two, 200 gallon tanks that are part of a 2000 gallon full recirculating system. The other half of the fish were divided evenly and placed into an identical system. These systems rely on swirl separation and sand filtration for particulate removal and contain large bio-reactors for denitrification. Water temperature was maintained at a constant 27ºC.

Feeding was suspended until day three in order to allow dissipation of handling stress. Fish were maintained in these conditions for two weeks in order to allow acclimation to the laboratory environment in accordance with Uchida *et al.* (2003). Water sampling was done bi-weekly in order to monitor DO, pH and total ammonia.

After the two week acclimation period, fish were redistributed amongst the four tanks to a density of 70 kg/m^3 for two of the groups which will henceforth be referred to as the stress groups. The density of the other two groups 30 kg/m^3, will be considered unstressed or control groups.

Over the course of eight weeks two groups of tilapia (one from the stressed and one from the unstressed) were fed a diet of 20 mg capsaicin/kg commercial trout feed (Aquamax 300) or 0.02% of the diet. Capsaicin is insoluble in water so in order to ensure consistency pure cacao butter was used to encapsulate the feed. The other two groups (one from the stressed and one from the unstressed) received a control diet consisting of the commercial feed plus the vehicle. All treatments were performed in duplicate. Each of the treatments was fed once daily to satiation. Groups were designated in correlation with their treatment. The Control group was considered to unstressed and received only commercial feed plus the vehicle (cacao butter). The Capsaicin group was unstressed yet received the capsaicin supplemented feed. The Stress group was subjected to the crowding stress, but received the same feed preparation as the Control group. The Capsaicin Stress group was subjected to crowding stress while receiving the capsaicin feed.

Feed was supplemented with capsaicin by first melting 25 ml pure cacao butter, which has a melting point in excess of 32°C, and adding 2 mg Natural Capsaicin (360376-1G, 65% capsaicin, 35% dihydrocapsaicin) attained from Sigma Chemicals. The fluid butter was mixed with 1 kg of feed until homogeneity is perceived. This prevented the elution of the capsaicin into the environment prior to consumption. Similarly, the control feed will received 25 ml of pure melted cacao butter per kilogram in the same procedure with the exception of capsaicin. The mixture was allowed to cool and then stored in a refrigeration unit until its time of use.

Twenty four fishes were sampled on the first day of the experiment and subsequently every two weeks thereafter. Fish are sampled by euthanasia with ethyl 3-aminobenzoate, methane sulfonic acid salt (MS-222) 98%. After being euthanized the fish were each measured for total length (cm) and using a digital scale their weight (g) was determined. These measurements were used to calculate the Fulton's condition factor (K) using the formula K= {weight (g)*100/ (length (cm) ^{3}}. Condition factor was used as a determinant of the fish's overall wellness and the results were recorded for statistical analysis (Froese 2006).

Next, using heparinized hypodermic needles, blood was drawn from the caudal vein. This was done by inserting the needle just ventral of the lateral line and just posterior of the caudal fin. The needle should be inserted to make contact with the vertebral body and then slightly retracted. Withdrawing the plunger on the syringe creates a vacuum and the syringe quickly fills with blood (Perrott *et al.*1991). This blood was used to ascertain the levels of circulating glucose in the animals system. This was done using a standard glucometer (Gensic *et al.* 2004). After calibrating the glucometer, a test strip was inserted into the glucometer. Then a small drop of blood had been placed on the tip of a test strip until the glucometer began the test.

After determining the glucose levels some of the blood remaining in the syringe was used to determine the blood hematocrit (packed cell volume). Blood was drawn into capillary tubes and the ends sealed with Critoseal on one end and capped using Critocaps on the opposing end. The capillary tube was then centrifuged using a micro-centrifuge. Centrifuging

separated the blood into its components of red blood cells and plasma (Siwicki *et al.* 1994). Blood was centrifuged for 5 minutes at 1000 RPMs. The level of packed cells was then determined using a Micro-Hematocrit Capillary Tube Reader.

A small amount of the plasma obtained previously was used to determine the plasma protein levels of the blood. A refractometer (VEEGEE Scientific Inc. Kirkland, WA) was calibrated using distilled water before applying one to two drops of plasma. The refractometer was then used to measure plasma protein to the nearest g $100ml^{1}$ (Gensic *et al.* 2004).The remaining blood was placed in a microcentrifuge tube and centrifuged at 1000 RPMs in order to separate red blood cells from the plasma content. The plasma was collected in a separate sterile microcentrifuge tube and labeled for further analysis while the red blood cell pellet was discarded. The plasma was stored in a -80ºC freezer until the conclusion of the experiment. After the samples from week 8 were collected the samples were subjected to the Cortisol Enzyme Immunoassay by Enzo Life Sciences (Plymouth Meeting, PA) in a 96 well plate. The results were determined using a 96 well plate reader.

The spleens of each fish were excised and weighed and the weights recorded. This was used to determine the spleen somatic index (SSI) using the formula SSI = (spleen weight/body weight)*100. Lastly, the head kidney was removed using sterile technique and placed in a centrifuge tube in a solution of two mL L-15 containing 2% FBS (+100 i.u./ml Pen-Strep, 10 unit/ml heparin) and then placed on ice and allowed to macerate. The tissue was then passed through a sterile sieve using a sterile tissue pestle and resuspended in the L-15 solution. The cells were then washed by spinning at 1000 rpms for 15 minutes. These cells when compacted comprise a pellet. This pellet was then resuspended in 1ml of L-15 solution of 0.1% FBS (+100 i.u./mL Pen- Strep). After vortexing, 100 µl of the suspension was placed in the 10mm circle of a labeled double etched Flouro slide in duplicate. After a drying period of approximately 90 minutes, the excess fluid was removed and 100 µl of formalin killed *B. megaterium* was aliquoted in duplicate onto each slide. After two hours of incubation at room temperature, the slides were washed in phosphate buffered saline (PBS) before being fixed in methanol for approximately five minutes. Upon removal from the methanol, the slides were placed in Wright Giemsa stain for five minutes. Slides were then washed with PBS and then deionized water, before allowing drying overnight.

Slides were read by light microscopy. Macrophages were identified and observed for phagocytic activity. A macrophage was identified as exhibiting phagocytic behavior if the number of bacteria observed as engulfed equals five or more. Five/one hundred cells per slide were counted and the results were calculated.

All results were analyzed using Sigmaplot 11. A one way analysis of variance (ANOVA) test for statistical significance ($p<0.05$) was used. In groups showing a non-normal distribution the Kruskal-Wallis ANOVA of Ranks was used. The Holm-Sidak method was used to compare multiple means when differences were detected. Error bars in the graphs represent ± the standard error of the mean (SEM). A cluster analysis was performed using WEKA (Waikato Environment for Knowledge Analysis) data mining software in order to seek statistical differences between dual variable groups based on all variables simultaneously, as opposed to the typical individual comparisons done using ANOVA. The methodology is, in a simple sense, an ANOVA in reverse. The ANOVA seeks statistical significance for between group variability versus within group variability. The K-means

cluster analysis seeks to determine the minimum variability within a cluster and a maximum variability between clusters by moving objects in and out of groups in order to get the most significant ANOVA results. Results were graphed in (Fig. 13). ("X" symbol represents the dominant group in each cluster. Correctly paired clusters would show a uniform color in their respective column indicating significant differences between groups). The adjusted Rand index value was calculated as opposed to the Rand index in order to determine how significant the differences were between groups.

RESULTS AND DISCUSSION

Plasma Cortisol Concentration

Although not statistically applicable due to the pooling of the extracted plasma, cortisol concentrations peaked in all groups during week two. The Capsaicin treatment was consistently lower in comparison than the Control for weeks two through eight. This trend was also seen in the two stressed groups with the Capsaicin Stress group showing decreased cortisol levels in comparison to the Stress group in weeks two through eight. When the levels of cortisol are compared from the groups that were crowded to the groups that were not crowded, levels were consistently higher from weeks two through eight in the crowded groups (Fig. 1).

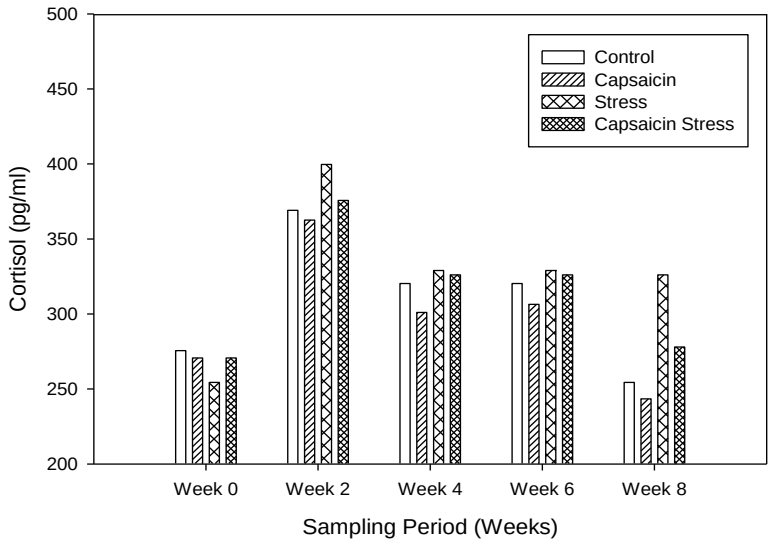

Fig. 1. Analysis of concentration of pooled blood cortisol between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of eight weeks.

When the groups are compared over the course of the experiment the elevation in plasma cortisol levels show an identical pattern, with the Capsaicin group's mean result (296.8 pg/ml) being lower than that of the Control (307.9 pg/ml) and the Capsaicin Stress (315.3 pg/ml) group being lower than that of the Stress group (327.6 pg/ml) (Fig. 2).

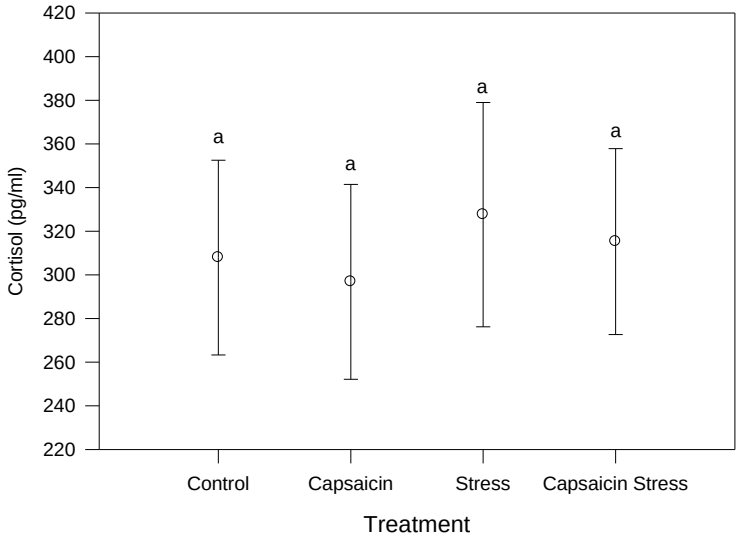

Fig. 2. Analysis of concentration of pooled blood cortisol between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the eight week study.

Blood Glucose Levels

Blood glucose levels were similar between treatments in weeks two and four, but were shown to be consistently lower in the Capsaicin and Capsaicin Stress groups when compared to the Control and the Stress groups in weeks six and eight (Fig. 3). When the results are

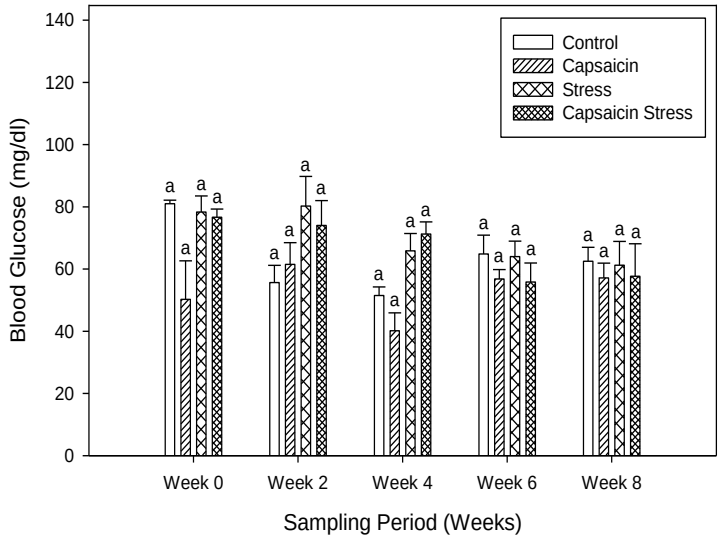

Fig. 3. Analysis of concentration of blood glucose in mgdl-1 between capsaicin and non capsaicin groups in two different treatments, controlled and stressed, over the course of eight weeks. Data are means ± SEM.

for the duration of the experiment, the groups that received capsaicin in their feed were shown to have lower blood glucose levels than the groups that received the feed with just the vehicle (p<0.05) indicated the differences in these groups were statistically significant.

Analysis shows a statistically significant difference between Capsaicin group and the Capsaicin Stress and Stress group but no differences from Control (Fig. 4).

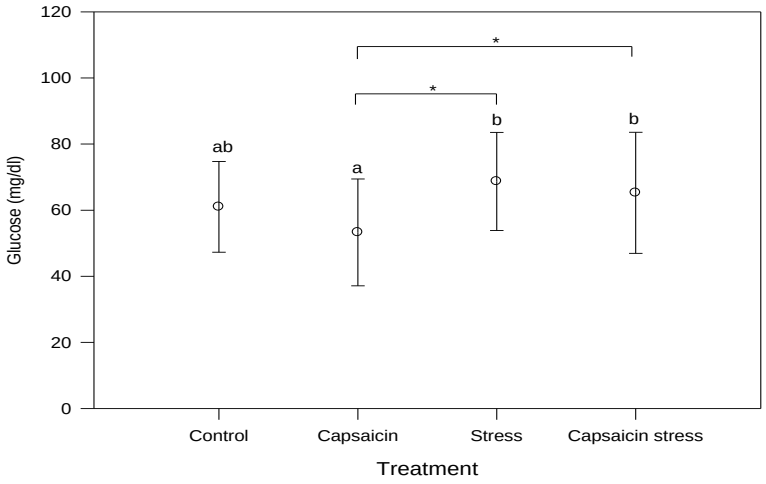

Fig. 4. Analysis of the concentration of blood glucose in mgdl-1 between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the eight week study.

Spleen Somatic Index (SSI)

The spleen somatic indices show variation between the groups in weeks two and four. By week six the indices were larger in the Capsaicin compared to the Control and the Capsaicin Stress when compared to the Stress group. This shifted in week eight is resulting in Capsaicin and Capsaicin Stress reading lower than the Control and Stress groups respectively (Fig. 5).

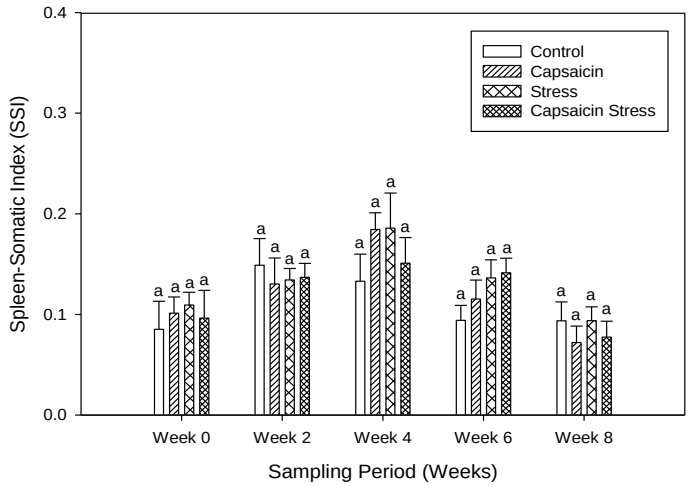

Fig. 5. Analysis of the Spleen Somatic Index (SSI) between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of eight weeks. Data are means ± SEM.

In the model of overall results the Capsaicin and Capsaicin Stress group had higher indices when compared to Control and Stress treatments respectively. This group was found

to have a non-normal distribution. Using the Kruskal-Wallis ANOVA on the Ranks there were no significant differences (p>0.05) between these groups (Fig. 6).

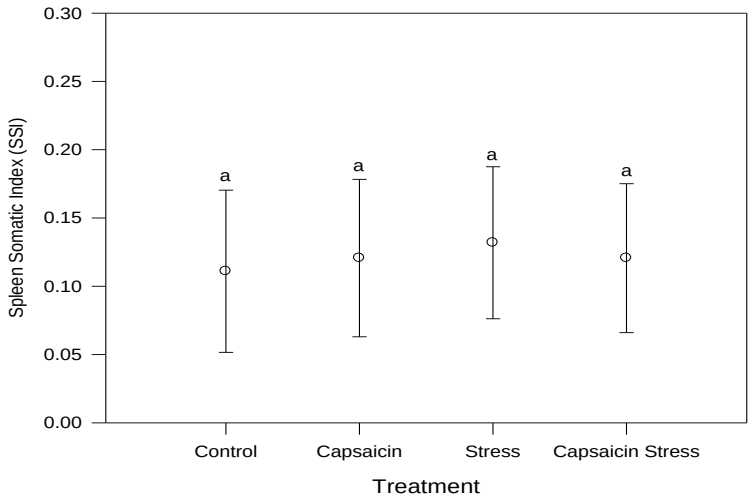

Fig. 6. Analysis of the Spleen Somatic Index (SSI), between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the eight week study.

Packed Cell Volume (PCV %)

The packed cell volume was shown be highest overall in week two. There were no noticeable trends between groups when comparing Capsaicin to Control and Capsaicin Stress to the Stress group (Fig. 7).

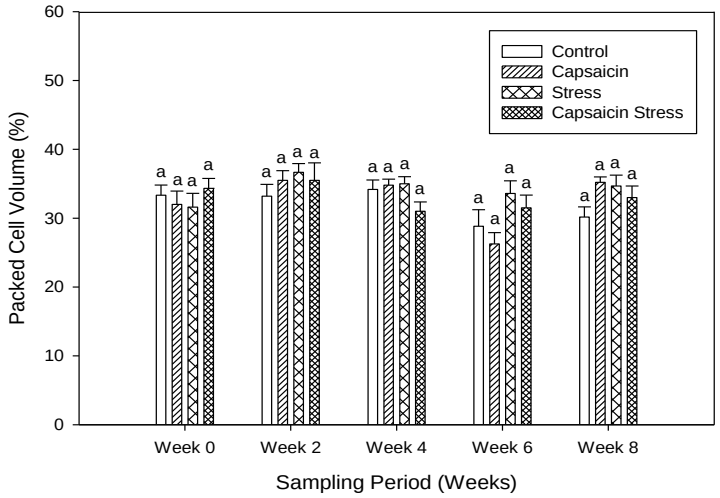

Fig. 7. Analysis of the packed cell volume percentage between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of eight weeks. Data are means ± SEM.

When analyzed from compiled data for the duration of the experiment the Stress group was shown to have the highest packed cell volume when compared to all other groups. This was not shown to be a statistically significant difference based on p>0.05 (Fig. 8).

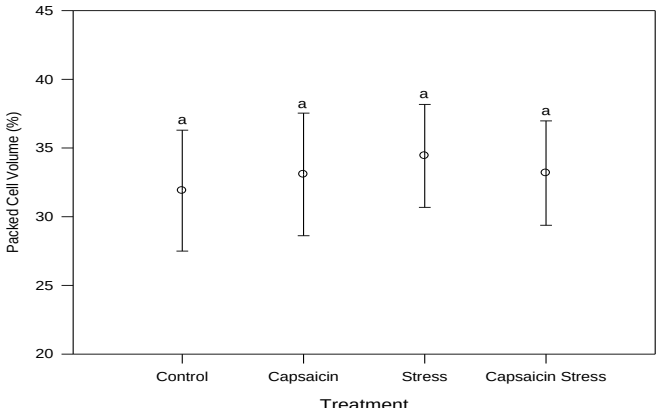

Fig. 8. Analysis of the packed cell volume percentage between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the eight week study.

Plasma Protein Levels

Plasma protein levels were analyzed with the results showing no significant differences or trends over the course of the experiment (results not shown).

Macrophage Activity

After compiling the results of the macrophage phagocytosis assay it was shown that the Capsaicin group displayed enhanced activity in weeks two, four, and eight when compared to the Control. The Capsaicin Stress Treatment also showed enhanced phagocytosis in all weeks when compared with the Stress group (Fig.9).

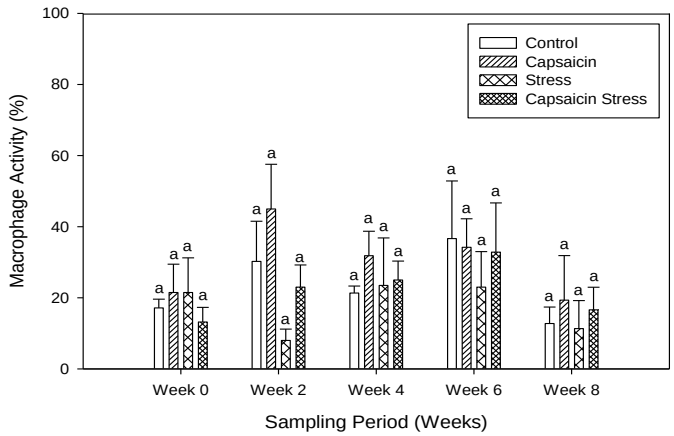

Fig. 9. Comparison of macrophage phagocytosis of formalin killed *B. megaterium* between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of eight weeks. Data are means ± SEM. Sample size was 6 fish per group. "*" indicate significant difference at $p<0.05$.

This trend was confirmed when the data is compiled over the course of the experiment. This group was found to have a non-normal distribution. Using the Kruskal-Wallis ANOVA on the Ranks there were no significant differences ($p>0.05$) between these groups (Fig. 10).

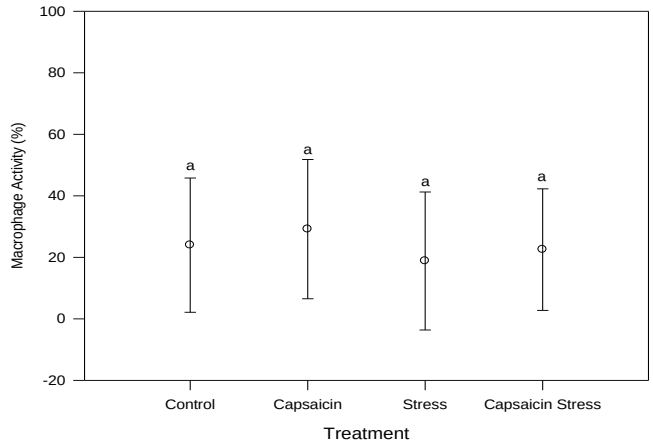

Fig. 10. Comparison of macrophage phagocytosis of formalin killed *B. megaterium* between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the eight week study.

Condition Factor

The analysis of the condition factor (K) has shown that the Capsaicin group was highest in all weeks when compared to Control. The Capsaicin Stress group was higher in week two than the Stress group (Fig.11).

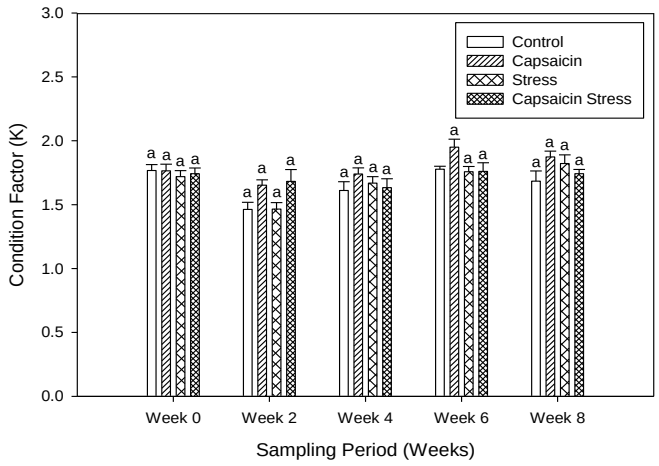

Fig. 11. Analysis of the condition factor, (K), between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of eight weeks. Data are means ± SEM.

The condition factor was highest in the Capsaicin group when the results are compiled. The Capsaicin Stress group was also shown to be higher than the Stress group analyzed overall. $p<0.05$ indicated the differences in these groups were statistically significant. Analysis shows a statistically significant difference between Control group and the Capsaicin groups, and a difference between the Capsaicin group and the Stress group. Since there is no difference from the Control group or significant differences between corresponding treatments, this data simply indicates that blood glucose was lowest in the Capsaicin group and highest in the groups subjected to stress (Fig. 12).

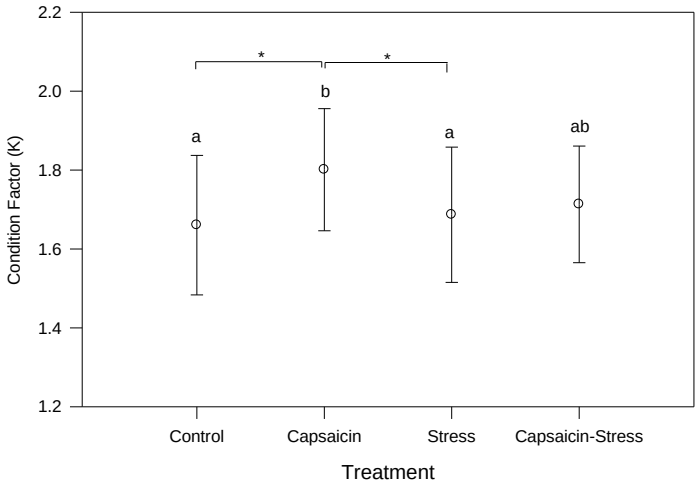

Fig. 12. Analysis of the condition factor (K), between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the eight week study.

Cluster Analysis

The cluster based analysis was performed on data from week 8 due to the largest degree of differences between groups. The calculation led to an adjusted Rand index (R) of -0.00332. This result makes us unable to reject the null hypothesis (Fig. 13).

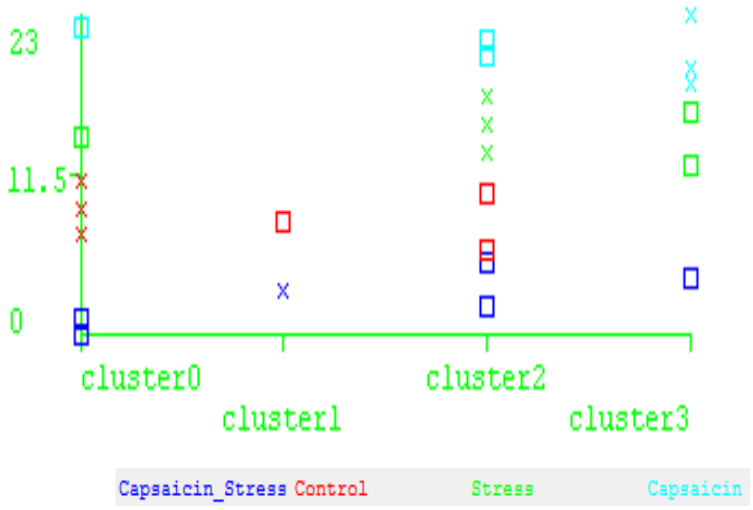

Fig. 13. Visualization of cluster analysis created using WEKA data mining software. Tilapia Week 8 K-Means. K (number of clusters) = 4 (Incorrectly clustered instances: 14/24, 58.3333 %) X represents dominant treatment in each cluster.

The main focus of this study was to determine the effects of dietary capsaicin on several stress parameters in Nile tilapia. The parameters used to quantify the results have been applied from several other previous studies (Froese 2006, Gensic *et al.* 2004, Perrott *et al.* 1991, Uchida *et al.* 2003) and have been proven reliable in their respective measurements of the stress response.

In our study, the capsaicin supplemented feed did not produce any statistically significant results. There was however a trend between the means of the results in several assays. The mean cortisol levels were lower in both groups which received capsaicin in their feed. The same was true of blood glucose, condition factor, and macrophage activity.

Because of the lack of statistical significance it cannot be said with certainty that these results are attributable to the effects of capsaicin. The elevation of cortisol observed in stressed versus non-stressed treatments indicates that the stress response was induced due to crowding. The lack of statistical significance observed in the other biomarkers may be an indication of the innate ability of tilapia to adapt itself to adverse conditions quickly and efficiently, owing to the reputation as a hardy species.

The cluster based analysis performed has also shown that there are no statistically significant differences between groups. This finding confirms our previous conclusion that we were unable to see a difference based on capsaicin in this study.

There is the possibility that the amount of capsaicin (0.02% of the diet), which shown to be more than sufficient to produce a result in the rat model (Lee *et al.* 2003), was not taken up in a sufficient amount to produce a substantial result in Nile tilapia. In many of the studies in which capsaicin was used to produce a result in fish, intraperitoneal injection (Powell *et al.* 1993) was used. It should also be noted that even though the encapsulation process has been proven effective (Poncelet *et al.* 2011), it could potentially restrict the digestibility of capsaicin (EP Patent 2010) in the fish model. The cacao butter, which prevents the capsaicin from eluting into the environment, may also limit the digestibility of the capsaicin dissolved in it. This could be due to simple isolation of the compound by the encapsulant preventing its contact with receptors, or by means of chemical sequestration preventing receptors from binding with the compound.

Although the cacao butter was used as a vehicle, it was also administered to the control groups in order to isolate the effects of capsaicin. There was however, no study performed as to the combinatory effects produced by this vehicle and capsaicin. It was simply chosen for its high degree of palatability, its high melting point (≈35ºC) and, to a lesser degree, its antioxidant properties (Liendo *et al.* 1997).

It is proposed that in order to substantiate our findings, capsaicin be administered in varying doses, from 0.01% to 0.1% of the diet, in the absence of the cacao butter vehicle. We also recommended that the degree of stress be elevated from 70 to 80 kgm-3 in order to pronounce the effects of crowding in the stress parameters thereby accentuating any changes to a higher degree. It is also recommended that tilapia fed a supplemented diet of capsaicin be challenged with disease in order to determine without question the effects of capsaicin on the non-specific immune response. In this suggested trial, capsaicin should be administered prior to disease challenge in order to determine its properties as an immune activator as well as following to determine its use for chemotherapy.

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