

Modulation of Stress in Coho Salmon, *Oncorhynchus kisutch*, using Feed Supplemented with Capsaicin

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Abstract

In this study properties of the nutraceutical capsaicin were assessed in order to determine the effects on several stress bio-markers in teleost fish. Coho Salmon *Oncorhynchus kisutch*, were fed a diet supplemented with capsaicin for 8 weeks, and a thermal stressor was used in order to ascertain capsaicin's ability to modulate said 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 trial. The results of ANOVA comparison indicated that capsaicin produced no statistically significant differences between fish receiving the capsaicin and those which did not. A cluster based analysis using WEKA data mining software confirmed the findings that at 0.02% of the diet, capsaicin produced no statistically significant differences.

Keywords: Coho salmon; Aquaculture; Stress; Nutraceutical

Introduction

Salmon are a commercially important species worldwide. Although the capture fishery is still a major part of salmon production, the culture of several species has become a massive industry. Norway has been a large producer of salmon and trout for decades [1] and countries like Chile [2] have found their climate amicable for salmon production as well. The Pacific northwestern U.S. and Canada have developed large scale production not only for direct consumption but also as the framework in an attempt to support the large scale industrial harvest from coastal waters [3].

Climate has been one of the deciding factors in location when farming Pacific salmon. This is because salmon are considered a cold water fish. In the wild, their behavior has been shown to be effected at temperatures above 12–15°C in juveniles and temperatures in the range of 24–26°C to cause lethality in adults [4]. This limits their geographical range to the higher latitudes in comparison with a warm water species such as tilapia. It is possible to culture salmon and trout outside of their normal climatic regions. For example, Idaho, sits on top of an enormous aquifer of high quality easily accessed cold water. Springs flowing at around 15°C have allowed it to be the United States' top trout producer [5]. Bardach et al. places 15°C as the optimum temperature for culture [6]. When salmon are exposed to even sub-lethal temperatures, the thermal stress induced can lead to increasing risks from a secondary stressor. In the upper temperature ranges dissolved oxygen levels are lowered compounding the effects of temperature on enzymatic activity. Disease risk is markedly increased above 16°C in most species [4]. If the effects of thermal stress could be overcome, salmon culture could be expanded. This would allow it to be cultured in many places where, because of inadequate water temperatures, it is currently not economically feasible. In an attempt to mitigate the effects caused by thermal stress we have evaluated the use of capsaicin as a nutraceutical.

A nutraceutical is a food additive that imparts health benefits to the recipient [7]. 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 [8]. Researchers 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.

Capsaicin has been shown to cause neural desensitization when placed in direct contact with vanilloid receptors of an organism [9]. Capsaicin has also been shown to affect immune response [10], metabolism [11], and have an array of effects on digestion [12]. The effects of capsaicin on thermoregulation in previous studies [13,14], researchers have prompted analysis of its effects on the salmonid model. Because the mouse model used in these previously mentioned experiments is an endothermic organism, the effects of capsaicin in an ectotherm are purely speculative.

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.

Materials and Methods

Fingerling Coho salmon (*Oncorhynchus kisutch*) were obtained from the Richard Clay Bodine State Fish Hatchery located in Mishawaka, Indiana, a presumed disease free facility used to supply both Coho salmon and steelhead trout (*Oncorhynchus mykiss*) to the Great Lakes region. They were transported to the Indiana University-Purdue University Fort Wayne Life Science Resource Center where they were distributed evenly between eight 10 gallon aquaria. Aquaria replacement water was dechlorinated in a large drum by simple evaporation over the course of a week. This process was

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aided by aeration and light exposure. Tanks were part of two distinct recirculating systems which were aerated by gravity turbulence upon return to the central water reservoir where filtration took place.

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. [15]. Water sampling was done bi-weekly in order to monitor dissolved oxygen (DO), pH and total ammonia.

After the acclimation period Coho salmon were subjected to a thermal stressor over the course of the eight weeks. Four groups of 25 salmon were maintained at the hatchery ideal temperatures of approximately $16 \pm 1^\circ\text{C}$ for the course of the experiment. Four other groups of 25 fish each were acclimated over a two week period from 16°C to approximately 24°C in order to create a stressful environment. This was done using Visi-Therm 100 W heaters in each tank of the thermally challenged system. Temperature was monitored daily on floating glass thermometers (Aquarium Systems, Mentor, Ohio). This temperature was maintained until the termination of the experiment. The eight groups received, in four cases a control diet (two at each temperature) and in the opposing groups the capsaicin formulated diet. All other variables remain unchanged across the groups.

Individual groups were named in correlation with their treatment. The Control group was considered to be 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 thermally stressing conditions but received the same feed preparation as the Control group. The Capsaicin Stress group was subjected to thermal stress while receiving the capsaicin feed.

Over the course of eight weeks the two experimental groups of salmon were fed a diet of 20 mg capsaicin/kg commercial trout feed (Aquamax 100) 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 group received a control diet consisting of the commercial feed plus the vehicle. All treatments were performed in duplicate. Each of the treatments were fed once daily to satiation.

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 receive 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.

24 fish 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 (98%) [16].

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 Condition factor (K) using the formula $K = \{\text{weight (g)} \times 100 / (\text{length (cm)})^3\}$. Condition factor was used as a determinant of the fish's overall wellness and the results were recorded for statistical analysis [17].

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 [18].

Blood was used to ascertain the levels of circulating glucose in the animals system. This was done using a standard glucometer [19]. After calibrating the glucometer, a test strip was inserted into the glucometer. Then a small drop of blood was 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 [20]. 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/100 ml [19].

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 -80°C 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 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 $\text{SSI} = (\text{spleen weight} / \text{body weight}) \times 100$.

Lastly, the head kidney was removed using sterile technique and placed in a centrifuge tube in a solution of 2 ml L-15 containing 2% FBS (+100 i.u./ml Pen-Strep, 10 unit ml^{-1} heparin) before maceration on ice. 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 rpm for 15 minutes. The pellet of cells was then resuspended in 1 ml 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 10 mm circle of a labeled double etched Fluoro slide in duplicate. After a drying period of approximately 90 minutes, the excess fluid was removed and 100 μl of formalin killed *Bacillus 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 5 minutes. Upon removal from the methanol, the slides were placed in Wright Giemsa stain for 5 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 5 or more. One hundred cells per slide were counted and the results recorded.

All results were analyzed using Sigmaplot 11. A one way analysis of variance (ANOVA) test for statistical significance ($P < 0.05$) in order to compare the effect of capsaicin feed in both the control and the stressed groups. 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 \pm 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 Figure 2. (The "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 R and index value was calculated as opposed to the R and index in order to determine how significant the differences were between groups.

Results

Plasma cortisol concentration

Due to the fact that the cortisol was pooled from all the fish in each treatment on each sampling, I am unable to statistically analyze the data on each individual sampling date. In these groups, the cortisol levels did reveal a trend. The mean cortisol levels were consistently lower in all groups which received the capsaicin feed. Members of the two stressed groups also had higher cortisol levels the unstressed counterparts (not statistically significant) (Figure 1). When the groups are compared over the course of the experiment the decrease in plasma cortisol levels show an identical pattern, with the Capsaicin group's results being lower than that of the Control and the Capsaicin Stress group being higher than that of the Stress group (Figure 2).

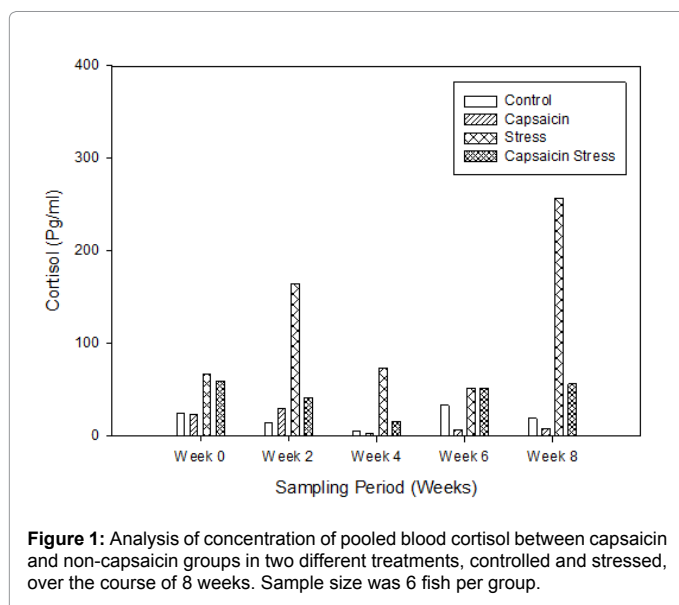


Figure 1: Analysis of concentration of pooled blood cortisol between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of 8 weeks. Sample size was 6 fish per group.

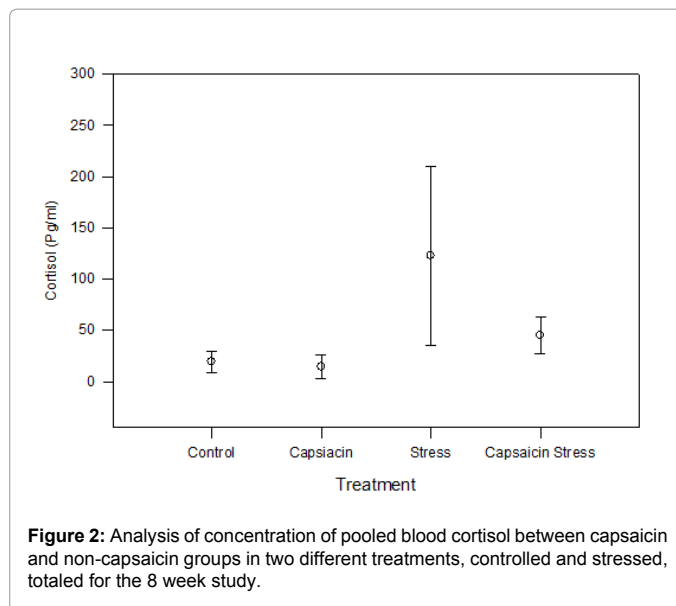


Figure 2: Analysis of concentration of pooled blood cortisol between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the 8 week study.

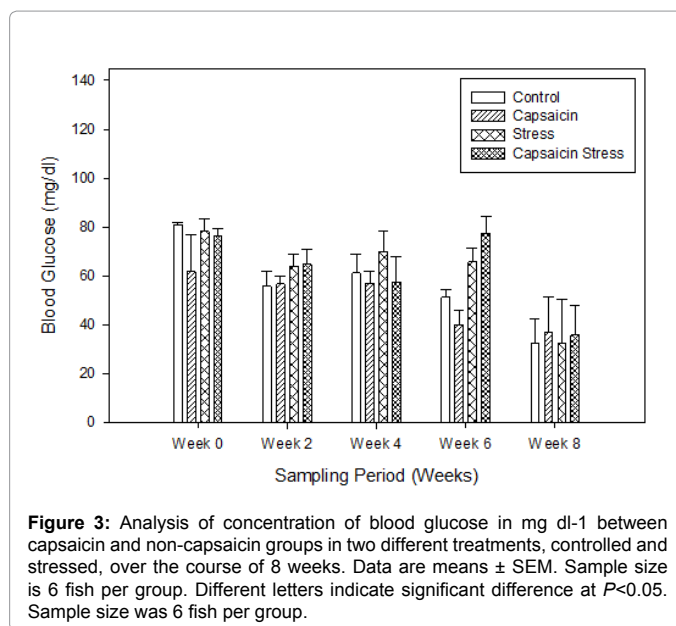


Figure 3: Analysis of concentration of blood glucose in mg dl-1 between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of 8 weeks. Data are means \pm SEM. Sample size is 6 fish per group. Different letters indicate significant difference at $P < 0.05$. Sample size was 6 fish per group.

Blood glucose levels

No significant differences were found when blood glucose levels were compared between the groups administered capsaicin and the control in all weeks (Figure 3). When the results are compiled 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 although the results are not statistically significant (Figure 4).

Spleen Somatic Index (SSI)

The results for the spleen somatic index show the most variation between the stress treatment and the unstressed in week eight. There was still neither a statistically significant difference between stress and unstressed groups and the groups that received the capsaicin and those that did not when compared with controls (Figure 5). In the model of

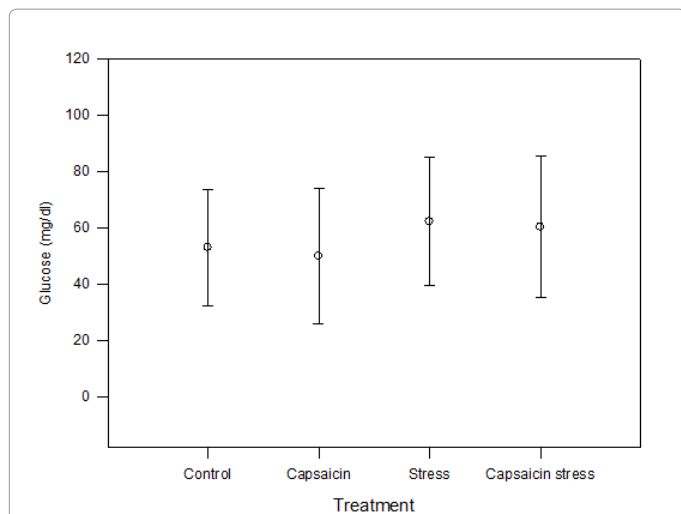


Figure 4: Analysis of concentration of blood glucose in mg dl-1 between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the 8 week study.

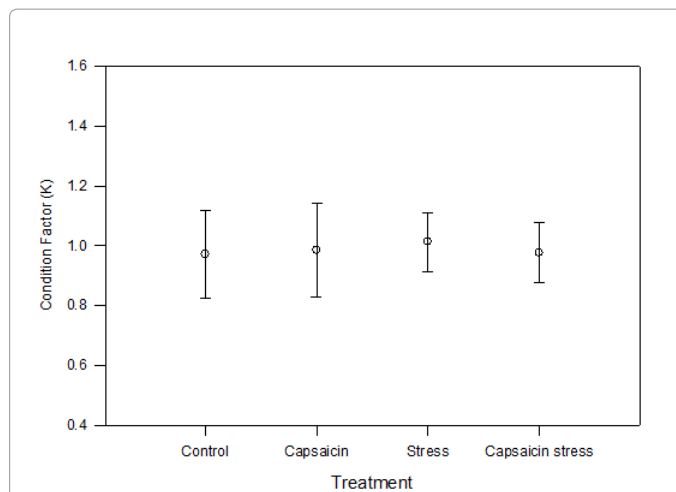


Figure 6: Analysis of the condition factor (K), between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the 8 week study.

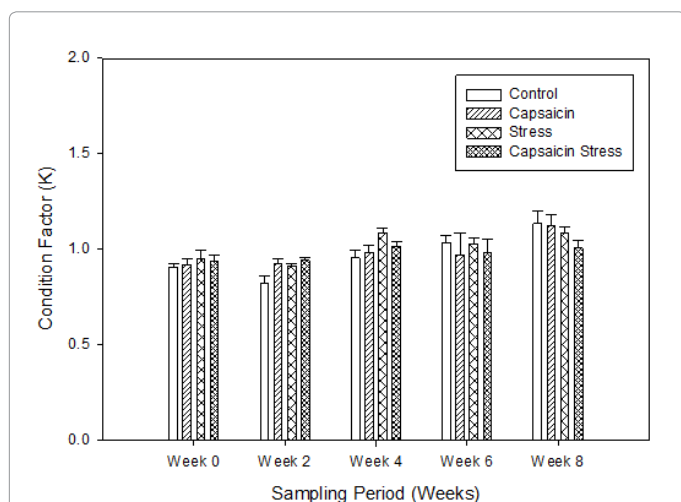


Figure 5: Analysis of the condition factor (K), between capsaicin and non-capsaicin groups in two different treatments (controlled and stressed), over the course of 8 weeks. Data are means \pm SEM. Sample size is 6 fish per group.

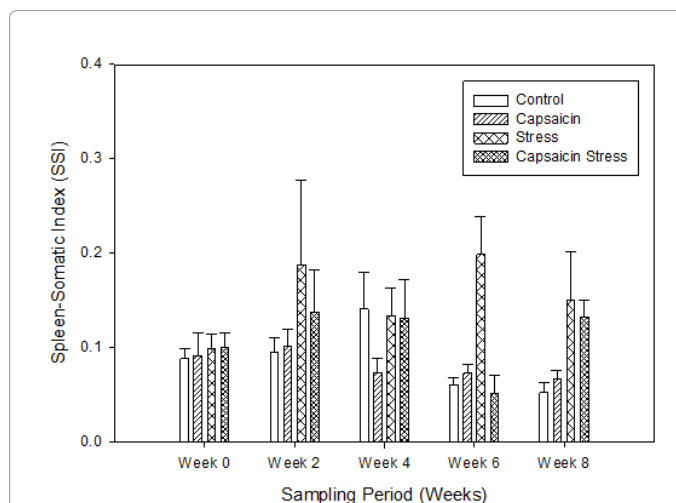


Figure 7: Analysis of the Spleen Somatic Index (SSI) between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of 8 weeks. Data are means \pm SEM. Sample size is 6 fish per group.

overall results the Stress group had highest index when compared all other treatments (not statistically significant) (Figure 6).

Packed Cell Volume (PCV %)

The packed cell volume analysis shows no statistically significant differences on any of the weeks when the groups are compared to control (Figure 7). There were no statistical differences when compiled capsaicin groups' results were compared with controls over the course of the experiment (Figure 8).

Plasma protein levels

Plasma protein levels were analyzed with the results showing no significant differences or trends over the course of the experiment.

Macrophage activity

After compiling the results of the macrophage phagocytosis assay,

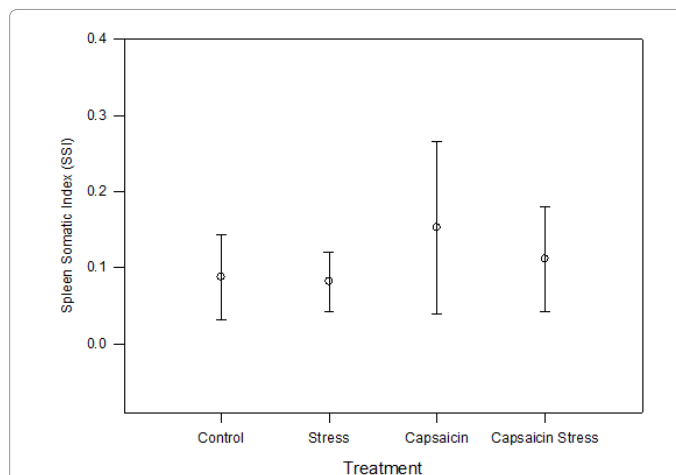


Figure 8: Analysis of the Spleen Somatic Index (SSI), between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the 8 week study.

in week two the Control group's macrophage activity was highest. It was shown that the two capsaicin groups displayed the highest mean level of macrophage activity in weeks four and six when compared to the groups that did not receive capsaicin. In week eight the Capsaicin group had a prominently higher mean than all other groups (Figure 9). When the data is compiled over the course of the experiment capsaicin groups had higher mean macrophage activity when compared to groups which did not receive capsaicin feed (Figures 10 and 11).

Condition factor

The analysis of the condition factor (K) has shown that the capsaicin did not produce a statistically significant difference when compared with controls in all weeks (Figure 12). There were no statistically significant differences between groups when condition factor is analyzed from the compiled results of the experiment. The mean condition factor was lowest in the Control when compared to all groups and highest in the Stress group (Figure 13).

Cluster analysis

The cluster based analysis was performed on data from week 8 due

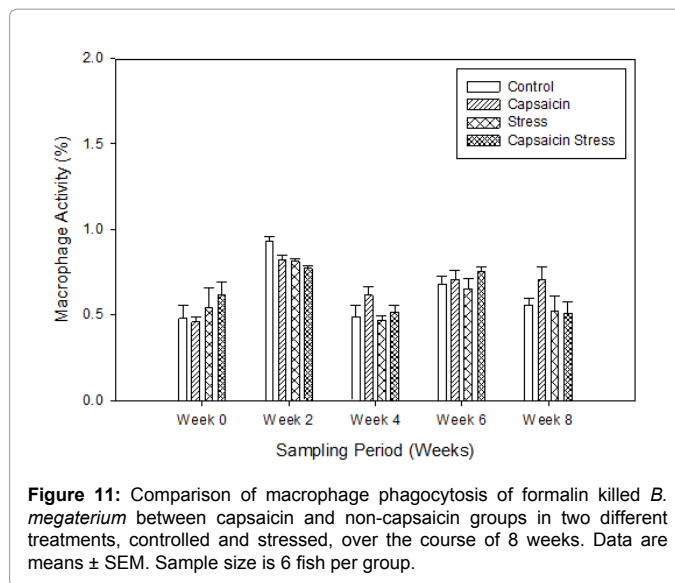


Figure 11: Comparison of macrophage phagocytosis of formalin killed *B. megaterium* between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of 8 weeks. Data are means \pm SEM. Sample size is 6 fish per group.

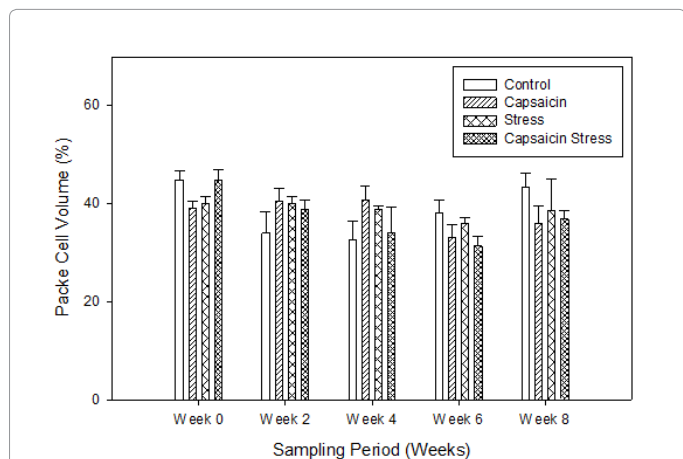


Figure 9: Analysis of the packed cell volume percentage between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, over the course of 8 weeks. Data are means \pm SEM. Sample size is 6 fish per group.

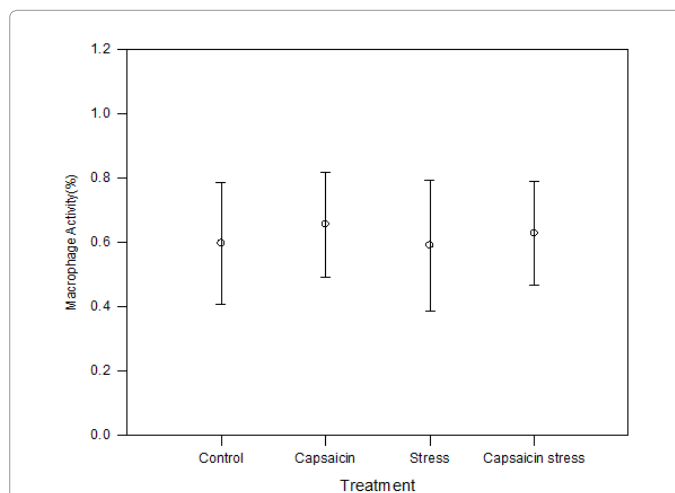


Figure 12: Comparison of macrophage phagocytosis of formalin killed *B. megaterium* between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the 8 week study.

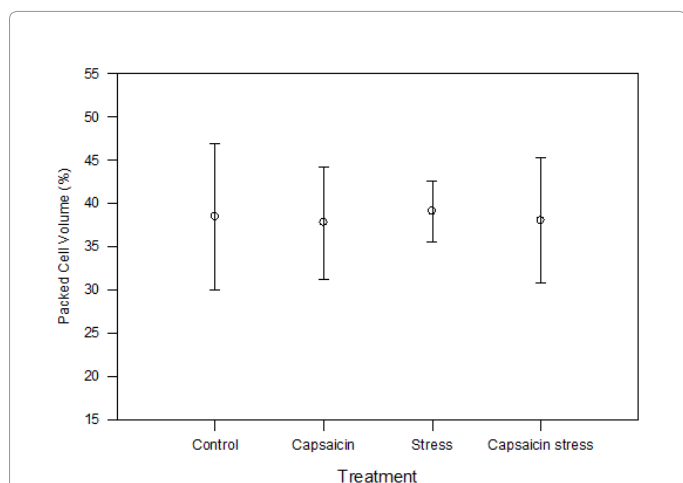


Figure 10: Analysis of the packed cell volume percentage between capsaicin and non-capsaicin groups in two different treatments, controlled and stressed, totaled for the 8 week study.

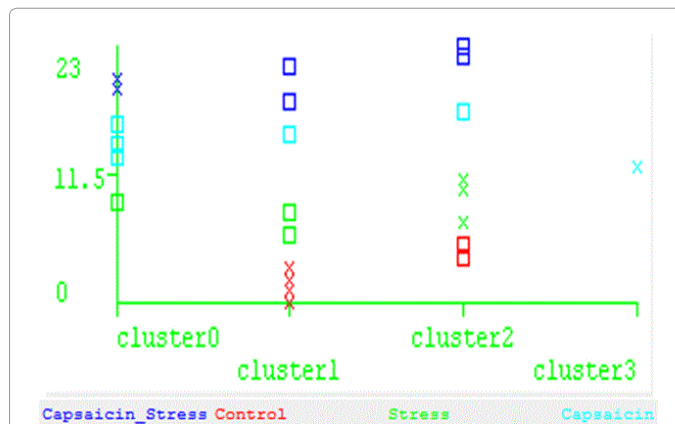


Figure 13: Visualization of cluster analysis created using WEKA data mining software. Salmon_Week8_all_K-Means_K=4 Incorrectly clustered instances: 14.0.

to the largest degree of differences between groups. The calculation led to an adjusted R and index (R_a) of -0.1554. This result makes us unable to reject the null hypothesis.

Discussion

Capsaicin has been shown to have effects on thermoregulation in several endothermic models. In Hori's experiment [13], capsaicin injection in rats caused stimulation of the anterior hypothalamic nuclei. Minutes after injection a 2-5 hour hypothermic period began, followed immediately by pyrexia lasting 1-10 days. This was in part due to heat sensitive thermo receptors firing. Chronic treatment with capsaicin was shown to decrease sensitivity to some chemical irritants, innocuous heat and neurogenic inflammation while touch and cold receptors remain responsive [21]. Based on these studies we hypothesized that capsaicin as a nutraceutical may be capable of creating a desensitized state to in order to reduce the stress effect created by thermal exposure in the Coho salmon model.

The elevated cortisol levels in the salmon groups exposed to thermally elevated conditions were expected. This insinuated that a stress condition was initiated; however the coping mechanisms in the secondary phase of the stress response sufficiently overcame this challenge. The lack of significant differences between the groups that received capsaicin and those that did not lead us to the conclusion that capsaicin at 0.02% of the diet in Coho salmon was insufficient in reducing the stress response biomarkers: blood glucose, and SSI, packed cell volume.

Previous studies have demonstrated the advantages of capsaicin in the diets of the mammalian model using rats, mice and humans [22,23]. Patel and Srinivasan [24] demonstrated capsaicin's effects on reducing the transit time of feed in rats. A reduction of transit time (19%) while maintaining nutrient absorption allows an increase in potential nutrient absorption based on the increased feed intake. The reduction of transit time may also prevent pathogen proliferation in undigested feed substrate by reducing the amount of in-gut incubation time. One of the factors that may affect the increase in nutrient absorption is the increase in amylase production associated with capsaicin intake [25]. An increase in nutrient absorption may reduce the amount of substrate in the gut, thereby complimenting the effect of transit time reduction on pathogen proliferation. This tends to cause a stimulated appetite and protein synthesis. We hypothesize that an increase in nutrient absorption and a decrease in transit time may be used to accelerate growth and increase fitness.

Based on the observation of tertiary condition of the Coho salmon throughout the experiment, it is our understanding that the lack of significant difference between groups receiving capsaicin in comparison with those which received a diet void of capsaicin, capsaicin was not effective in an increase of fitness or in bolstering nutrition. This may correlate to the method of feeding. Because feeding took place once daily, to satiation, the amount of nutrient intake necessary to bolster nutrition and enhance growth may have been insufficient.

Capsaicin has also been shown to enhance immune function [10]. In this study the eosinophilic granule cells (EGCs) of rainbow trout, analogous to mammalian mast cells, were shown to degranulate in response to capsaicin peritoneal injection. These cells not only aid in tissue repair but also have cytotoxic properties as well. Because these cells are primarily found in fish intestine, skin, gills, and central nervous system it was our hypothesis that digestion of capsaicin may trigger their degranulation as well.

In our assay of the non-specific immune response in which macrophage activation was determined based on their phagocytic activity, we found no differences between groups receiving capsaicin and those that did not. This also leads us to conclude that capsaicin was unable to produce a statistically significant change in macrophage activity in our experiment.

The cluster based analysis performed also has 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.

We do not excluded the possibility that capsaicin at higher concentrations cannot produce significant changes. Also, the encapsulation process used may have prevented capsaicin from freely entering the water supply, limiting it to exposure during the digestive process and reducing the effect of capsaicin on external thermal receptors of the fish. It may also be reasonable to assume that capsaicin is not as easily processed in the salmon gut as in that of the mammalian studies observed prior.

Due to our findings that capsaicin did not produce a statistically significant response in thermally stressed Coho salmon in the biomarkers; blood glucose, SSI, packed cell volume, macrophage activity or condition factor, it can be concluded there was no significant effect of capsaicin at this level of dietary intake. This finding agrees with the findings of Eckroth et al. observed in Atlantic cod [26].

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