EFFECTS OF DIETARY THIAMINE AND MAGNESIUM ON LAKE TROUT WITH INDUCED EARLY MORTALITY SYNDROME (EMS)

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Abstract

Multiple stressors contribute to Early Mortality Syndrome (EMS) in salmonid fisheries and its effects on the Great Lakes region, but the factors responsible for the variation of EMS are not clearly understood. EMS is as a characteristic embryonic mortality that affects the offspring of salmonines, and its impact on lake trout has significantly reduced natural recruitment. In this study, adult individuals were collected from Lake Michigan and their progeny were fed experimental diets containing different concentrations of thiamine and magnesium. A protocol was used to stain cartilage and bone separately for the histology portion. An image processing program was used to determine the percentage of bone and cartilage that was present in each head digitized. Color histograms were produced for each fish and determined the percentage of bone and cartilage proportions for each sample. The seventeen fish samples used were divided into two categories. The first category consisted of nine fish that were collected after the ninth week of the feeding experiment which were all fed commercial diet, and the second category was composed of all seventeen fish with commercial and experimental diets. For the first category, correlations were seen when comparing overall fish weight to percentage of bone and cartilage. This suggests that as the fish increased in size, they portrayed more advanced ossification and less cartilage was remaining. However, correlations between the differing diets and ossification were difficult to determine in the second category due to unevenly distributed samples.

Introduction

"Lake trout was considered the native top predator within the deepwater community during the 20th century, but local extinction was occurring due to over-fishing and the parasitic, non-native sea lamprey" ("Strategic Vision" 2001). "Sea lamprey invasions into the upper Great Lakes and the associated collapse of lake trout populations served as a catalyst for action by federal, provincial, and state fishery management agencies. Numerous actions were taken including lake trout stocking, sea lamprey control, fishery regulation, and water-quality management to help restore the fisheries. In response to these actions, a period of remarkable recovery of the fisheries began in the late 1950s" ("Strategic Vision" 2001). However in the 1980's, productivity of the Great

Lakes appeared to be declining due to a reduced input of nutrients. This reduced productivity translated to reduced catches in sport and commercial fisheries.

"From 1968 to the present, early life stage mortality has been documented in lake trout, from Lakes Ontario, Michigan, and to a lesser extent, Huron and Erie" (Brown et al. 2005). "Early mortality syndrome" (EMS) in lake trout, *Salvelinus namaycush*, populations in Lake Michigan is considered an example of an "environmentally born disease" (Lee et al. 2008). EMS is defined as an anorexia related embryonic and alevin mortality that affects the recruitment of salmonines in the Great Lakes (Brown et al. 2005). Preventing the causes and spreading of EMS has become a concern among scientists, hatchery owners, sports fishermen, and everyday consumers.

Recent Work and Justification

In previous research on EMS, pathological lesions observed in the brain and other EMS symptoms in fish might have some connection to thiamine deficiencies, yet these findings were never validated (Lee et al. 2008). Great Lakes Fishery Commission sponsored two workshops to facilitate renewed and more extensive investigations into the causes of early life stage mortalities ("Strategic Vision" 2001). As a result of the two workshops, a consensus was reached that thiamine (vitamin B1) deficiency was implicated as a possible cause of EMS ("Strategic Vision" 2001). It is now apparent that coho salmon, adult steelhead, and adult lake trout have signs of thiamine deficiency, and this thiamine deficiency is projected to impact the adult Atlantic salmon and lake trout in the Great Lakes. Low levels of thiamine have also been associated with two other early life stage mortality syndromes affecting Atlantic salmon in New York Finger Lakes (Cayuga Syndrome) and in the Baltic Sea (M74). Two factors that may impact EMS among lake trout but have not been studied thus far are: the role of magnesium (Mg) and mercury (Hg) toxicity. Magnesium deficiency has exacerbated thiamine deficiency so severely that growth ceased in rats. Lake Ontario is recognized for low Mg levels and constant elevated levels of mercury (Lee et al. 2008).

Thiamine deficiencies can bring about several problems related to fish health and survival. An enzyme in freshwater fish exists that degrade thiamine in muscle during food storage and passing in the gastrointestinal tract (Grosvernor and Smolin, 2002). This enzyme, thiaminase, forms degradation products which display inactive anti-vitamin

activities. Animals that consume prey with thiaminase activity are subject to thiamine (vitamin B1) deficiencies (Wistbacka and Byland, 2007). Thiamine deficiencies also have been shown to display neuropathologies in a variety of vertebrates (Amcoff et al. 2002). In fish, symptoms include anorexia, instability, convulsions, and darkening of skin (Wistbacka and Byland, 2007). Thiamine deficiencies do not seem to be the direct result of inadequate dietary thiamine, but rather several factors reduce the availability of thiamine (Honeyfield et al. 2005b). Factors that have been considered to cause thiamine deficiency include man-made pollutants, low levels of antioxidants, and large scale changes to the food-web with alterations in prey species (Amcoff et al. 2002).

Alewife, *Alosa pseudoharengus*, and rainbow smelt, *Osmerus mordax*, entered the Great Lakes area as invasive species and became well established in the ecosystem (Honeyfield et al. 2005a). Both of these species contain thiaminase. Furthermore, alewives are considered a dominant forage species of salmonids in Lake Michigan (Honeyfield and et al. 2005a). Consequently, lake trout populations are affected by thiaminase-containing prey fish and result in deterioration of reproduction and viability of progenies (Brown et al. 2005b).

The native lake trout capture in Lake Michigan decreased dramatically after 1954 because sea lamprey predation and overfishing reduced the natural population size severely (Bronte et al. 2007). Today, lake trout in Lake Michigan are being restocked due to controlled fishing measures and sea lamprey control but most spawning stocks are too low to support sustainable natural reproduction (Bronte et al. 2007). Several factors have inhibited natural reproduction from taking place. These include sea lamprey predation, increased alewive populations (food based), physical disturbances (hypoxia), and other forms of predation (Bronte et al. 2007).

It seems that many factors responsible for the severity of EMS in the Great Lakes and their interactions are not clearly understood. The goal of this study is to clarify the association between the hindrance of ossification due to differing thiamine and Mg concentrations through histological analysis. It is anticipated that the fish fed diets with higher levels of Mg and thiamine concentrations will display the most accelerated ossification. Likewise, the fish fed diets lacking Mg or thiamine will have hindered development of their skeletal systems. The results from this study will provide insight on

thiamine-Mg interactions in fish nutrition research. Hopefully from this research, methods to help control EMS in a natural setting can be developed, and hopefully new feeding methods will be developed to help prevent EMS from harming lake trout in hatcheries and in natural settings.

Methods

Feeding Experiment

In this study, adult individuals of lake trout were collected from Lake Michigan and the gametes were fertilized at the Lake Michigan site using a controlled experimental fertilization process. This particular process involves using a fixed sperm to egg ratio in order to standardize the conditions. The alevins of the adults collected were separated into several experimental groups and offered diets with two levels of thiamine (0 and 20 mg/kg) and two levels of Mg (0 and 250 mg/kg). Four different experimental diets along with a control diet (commercial fish food) were provided. The four experimental diets were supplemented with thiamine and magnesium (TM ++), added thiamine and deficient in magnesium(TM +-), deficient thiamine and added magnesium (TM -+), and deficient in both magnesium and thiamine (TM --). Each tank began with 99 alevins (33 progenies from 3 different females), and there were four tanks assigned for each diet. The fish were fed twice a day at 2 % body weight initially. Towards the beginning of the experiment, the experimental diets initiated overeating and mortality. The fish were later fed with "wet diets" four times a day at 1.5% fish body weight on March 26, 2008 to correct this problem. The fish were sampled and weighed at the beginning of the experiment to establish the correct amount of food per tank. The fish were routinely sampled and weighed after 4 and 7 weeks to readjust the correct amount of diet fed per tank. The experiment was ended after 10 weeks of feeding. At the beginning and end of feeding experiment, all fish biomass were weighed to calculate weight gain.

Histological Experimental Design

As the feeding experiment was concluding, specimens were collected on weeks nine and ten for histology analysis. The protocol used to stain cartilage and bone was modified from Song and Parenti's suggested method (Song and Parenti, 1995). Two separate

groups of lake trout alevins were used. Alevins collected on week ten from the feeding experiment had differing experimental diets. Alevins collected on week nine were fed commercial diet and were of different sizes (weights). It was intended to establish the effects of fish size and correspondence to the ossification process.

Step 1: Specimens were fixed in 10% formalin for three weeks.
Step 2: Specimens were washed in cold, tap water for 24 hours. The specimens were then skinned.
Step 3: For cartilage staining specimens were placed in alcian blue solution (80mg alcian blue 8GN, 640 ml 95% ethanol, 160 ml glacial acetic acid (for 800 ml)) for 1-2 days. We were able to identify only nine of the samples because of lost labelling. Samples that were identified were re-weighed, recorded, and placed into alcian blue solution one day later.
Step 4: Specimens were transferred to ethanol and 800 ul of 1% KOH in 800 ml for 1 hour. Then they were immersed in 95%, 75%, 50%, and 30% ethanol. 2-3 hours were allowed between each change. The specimens were kept in ethanol overnight and then the specimens were washed in water in constant flow for a day.
Step 5: For muscle digestion, specimens were placed in trypsin solution for several or weeks. The enzyme solution consisted of 2.7 grams of trypsin in 30% sodium borate (240 ml saturated sodium borate, 560 ml distilled water). The solution was changed every 2-3 days until bone and cartilage were visible. The trypsin solution was changed June 13, 17 and on July 2 due to yellow coloration in larger samples (samples 14, 15, 16, and 12).
Step 6: After the coloration in all specimens was clear, the fish were transferred to 0.5% aqueous KOH for about one hour.
Step 7: For bone staining, the fish were place in alizarin red S solution for about a day. (Alizarin red S: alizarin red S powder was added slowly to 0.5% KOH while stirring until solution turned a deep purple color).
Step 8: The specimens were transferred to 0.5% KOH for 30 minutes.
Step 9: 0.5% KOH with 3 drops of hydrogen peroxide solution was prepared and the specimens were placed in it and left overnight. (300 ml plus 10 drops hydrogen peroxide)
Step 10: To store the specimens, the fish were put into a mixture of 30% glycerin and 70% of 0.5% KOH until they sunk to the bottom of the Petri dish. Then the solution was

switched to 70% glycerin and 30% 0.5% KOH. The fish were stored permanently in 100% glycerine.

After the histological experiment was complete, skeletal and cartilage developments were visible (Figure 1).

Analysis of Methods

Results from the histology portion of the experiment were analyzed and quantified by using ImageJ image processing program. Photos were taken of fish individually with a ruler for a scaled reference. A digital camera attached to a microscope was used to take photos. A sample study area was determined for each fish starting from the front of the jawline to the gill cover and pectoral girdle (Figure 1). The study area encompassed the head region. The photos of the head were uploaded into the ImageJ software, and color histograms were produced to determine the amount of red and blue color in each sample. The color histogram provided the mean amount of red and blue pixels for the study area highlighted. The histogram also provided the study area in pixels^2. From this data, percentage of red and blue pixels was determined for each sample.

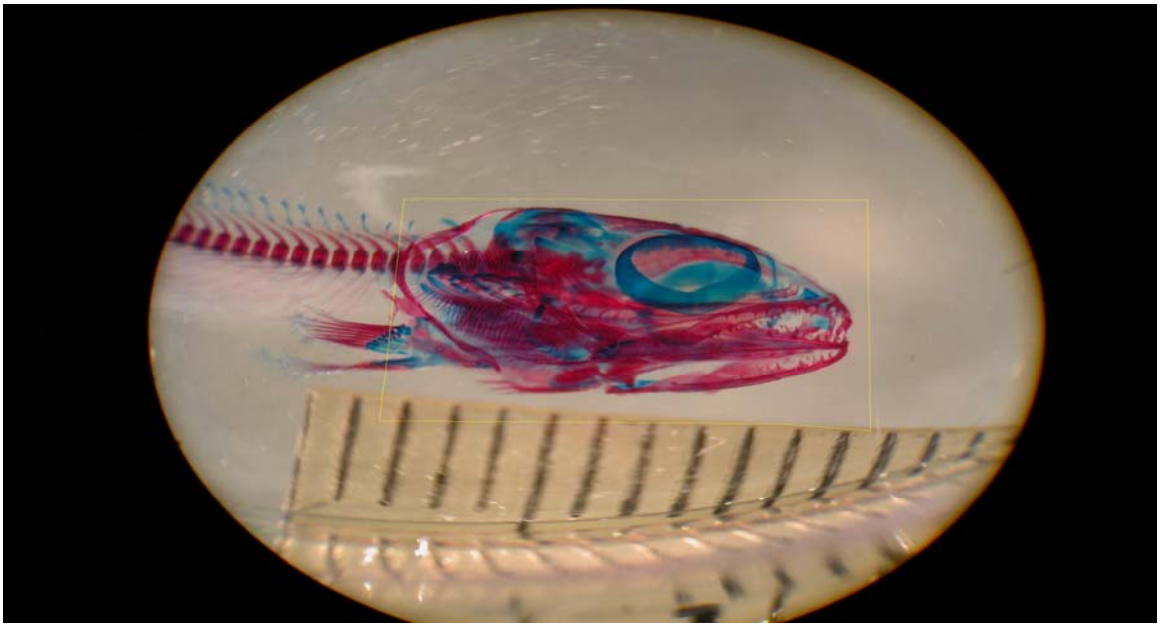


Figure 1. Photo displaying scaled photo of head with study area highlighted in yellow. Bone ossification is displayed in red color, and cartilage is displayed in blue.

Statistics

The influence of the treatments was compared using a Spearman Rank Order Correlation in SPSS (Version 17.0) software. The results were regarded as significant at $P<0.05$ and $R<0.5$. The variables compared in statistical analysis were the percentage red and blue compared to the weights of the skinned fish of different sizes (n=9).

Results

The seventeen samples collected were divided into two different categories. The first category was composed of the nine fish that were collected after the ninth week of the feeding experiment. These fish were all fed the commercial diet, and they differed in weights. Based on their weights, these fish were divided into three classes: small, medium, and large. Each class was composed of three fish. The second category was composed of all seventeen fish with commercial and experimental diets. The percentage of red and blue pixels was determined by the mean red/blue pixel count divided by the total average number of red and blue pixels in the study area of color histogram (Figure 2). The green color was disregarded and assumed to be a blend of red and blue.

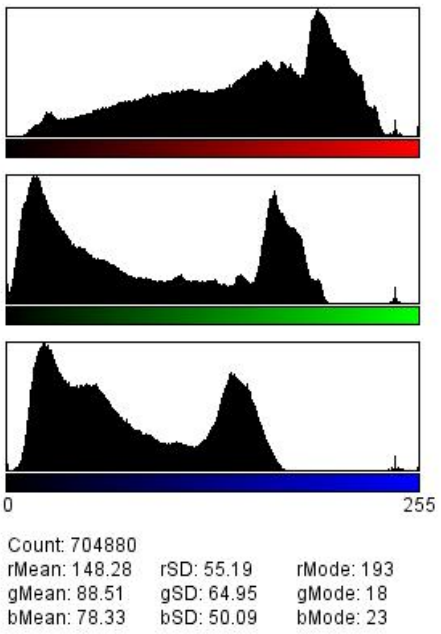


Figure 2. Color histogram of fish 16. The percent of red pixels was determined by rMean (148.28) divided by the total average of red and blue pixels (rMean+bMean). The percent

of blue pixels was determined by bBlue (78.33) divided by the total average of red and blue pixels (rMean+bMean).

Percentage of red and blue compared with weights of fish (Category 1)

Of the nine fish samples with differing sizes, there was evidence of a negative correlation between the percent of blue and weight (Table 1). The P value was 0.005 and the R value was -0.833. There also was a negative correlation between the percent of red compared to the percent of blue in each fish (Table 1). The p value was 0.000 and the R value was -0.983. As the pixel count of red increased, the pixel count of blue decreased. There was a positive correlation between the percentage of red and weight of the fish. The R value and p values were 0.850 and 0.004 respectively. As the fish increased in weight, the percentage of red pixel increased.

Table 1. Spearman Rank Order Correlation for nine fish of different sizes (n=9). Significant findings are highlighted in green.

Spearman's rho			Weight without skin (g)	Percentage red	Percentage blue
	Weight without skin (g)	Correlation coefficient Sig. (2 tailed) N	1.000 9	.850 .004 9	-.833 0.005 9
	Percentage red	Correlation coefficient Sig. (2 tailed) N	.850 .004 9	1.000 9	-.983 0.000 9
	Percentage blue	Correlation coefficient Sig. (2 tailed) N	-.833 0.005 9	-.983 0.000 9	1.000 9

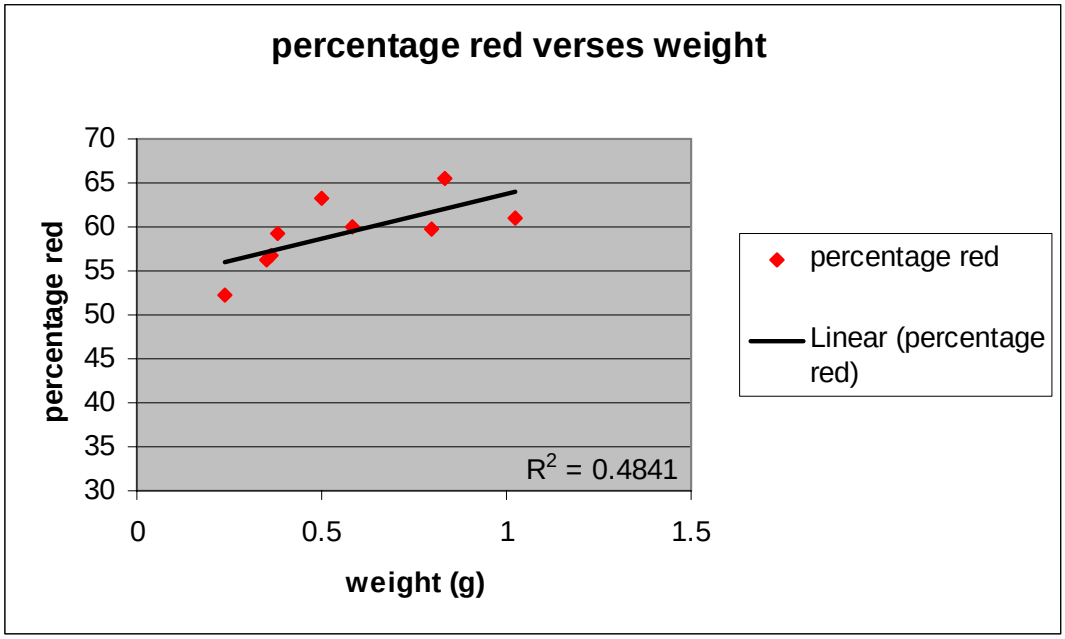


Figure 3. Trend of percentage red compared to weight of fish.

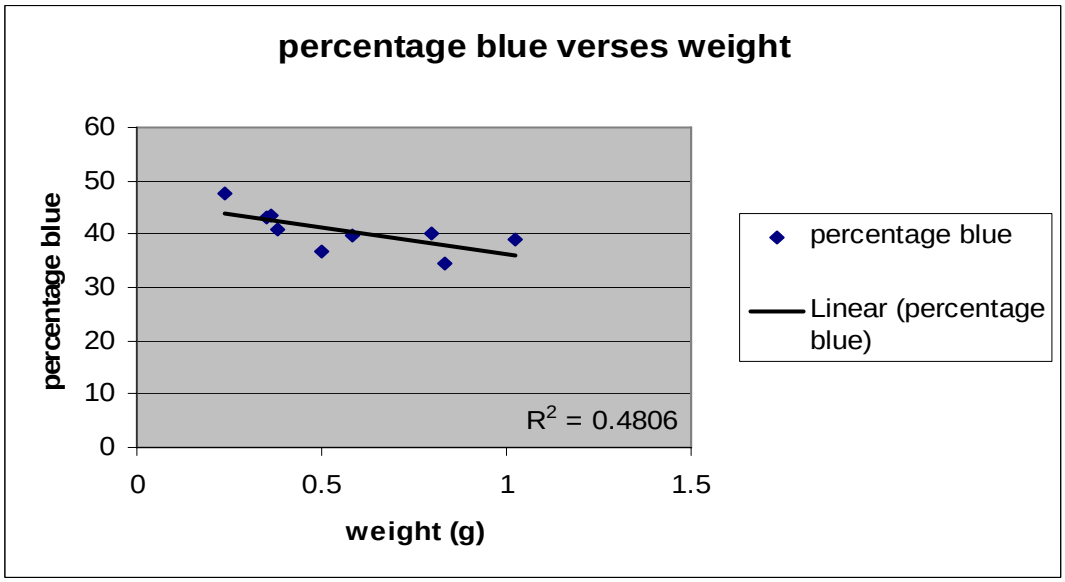


Figure 4. Trend of percentage blue compared to weight of nine fish.

Figures 3 and 4 depict general trends between the weight of the fish compared to the percentage of red and blue. Generally, as weight increased, the percentage of red increased and the percentage of blue decreased.

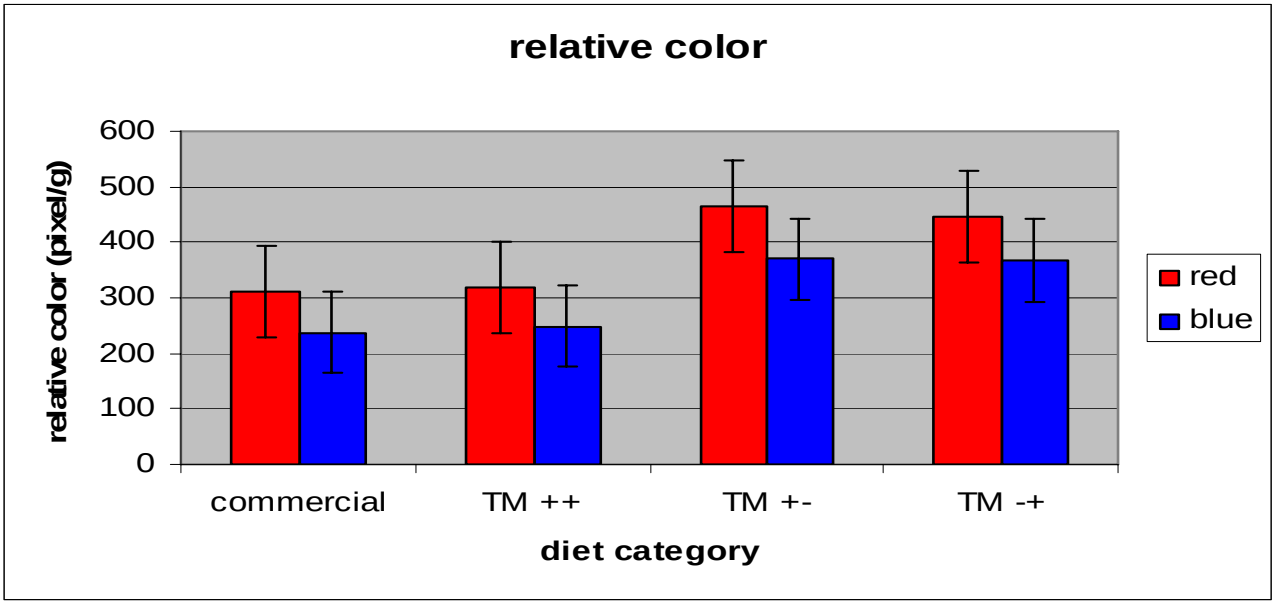
Percentage of red and blue compared to the diet of fish(Category 2).

Due to the uneven sample distribution and small sample size, no statistical analysis was performed for this group (Table 2). General trends were seen, but no conclusions or correlations can be drawn from this data set. Figure 5 displays general trends shown in Group 2. The relative proportion color of red and blue adjusted for size was determined by dividing the amount of pixels (red or blue) by the weight of each individual fish. This calculation portrays an amount of red and blue pixel per unit of weight. Since the pixels were only measured on the head, and the weight was taken of the whole body, this calculation should be treated as a proportion of red and blue verses unit of weight.

Table 2. Distribution of diets between seventeen samples. There are 11 fish with commercial diet, two fish with TM (++) diet, three fish with TM (+-) diet, and one fish with TM (-+) diet.

Sample Number	Diet Type
13	Commercial (round tank)
23.5	Commercial
2.6	++
4.5	++
5.5	+-
5.6	+-
8.6	+-
10.5	-+
8	Commercial (round tank)
9	Commercial (round tank)
10	Commercial (round tank)
11	Commercial (round tank)
12	Commercial (round tank)
13	Commercial (round tank)
14	Commercial (round tank)
15	Commercial (round tank)
16	Commercial (round tank)

Figure 5. Absolute color of red and blue for each diet category.



According to Figure 5, TM +-diets and TM -+ fed fish have more absolute color for red and blue. The commercial and TM ++ diets have significantly lower amounts.

Discussion

Strong correlations were seen when comparing overall fish weight to percentage red and blue color. This suggests that as the fish increased in size, they demonstrate advancement in ossification and less cartilage was apparent. The larger fish were more developed compared to the smaller sized fish. However, when the fish were compared against different diets, correlations were more difficult to determine. It is expected that the fish with the more complete nutritional diets (commercial and TM ++diet) would also show more signs of ossification and development. Therefore, a larger area of red color would be apparent for the fish with these diets. According to Figure 5, this is not the case. The fish with less complete diets have shown more relative color for red and blue, which is opposite of what would be expected. This may be to due unevenly distributed sample size (Table 2). These interpretations should be taken with caution due to errors present in the experiment before the result analysis.

Due to insufficient number of fish in the histological experiment design, the sample sizes became unevenly distributed between the different diets. The rest of the histological experiment design proved very effective, and clear results were apparent

after that portion of the experiment was completed. The specimens had a vibrant color, and were not overstained, and the colors were easy to decipher. Previous protocols combined bone, cartilage, and nerve staining for preparing specimens (Song and Parenti, 1995). Monitoring ossification processes have been effective in past research in explaining nutritional deficiencies, and these processes have successfully compared dietary vitamin levels with the influences of ossification in European sea bass (Mazurais et al. 2008). This experiment proved effective for combined cartilage and bone staining based on a few modifications of the Song and Parenti protocol, and the staining protocol can be used as a tool to further compare ossification influences in future samples.

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