Effect of Varying Levels of Astaxanthin on the Orange Color Intensity in *Amphiprion ocellaris*

Cassidy Clark

Governor's School for Science and Technology

2015-2016

I have read this document and approve the content.

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Mentor Signature

Steve Urick

102 S King Street

Hampton, VA 23669

United States

Abstract

Astaxanthin is a carotenoid pigment that brings out the orange and red color on the skin of marine animals. A brighter orange color is desired because of its aesthetic pleasure to consumers. Until recently, the color of aquaculture clownfish *Amphirprion ocellaris* was more of a yellow pigment than it was an orange one. This is because captive bred clownfish were not receiving an adequate amount of carotenoids in their diet. A sample size of 240 *A. ocellaris* clownfish were exposed to varying levels of astaxanthin ranging from 0 ppm (parts per million) to 1020 ppm. The 0 ppm was used as a control. Two methods to interpret results were conducted, a subjective judge panel and an objective RGB and HSL value analysis. It was seen with both the subjective and objective side that a plateau began near the 400 ppm to 640 ppm level. This would suggest that the optimal level would fall somewhere in between these two levels.

Introduction

Carotenoids are responsible for the majority of the red, orange, and yellow pigmentation in nature, as well as some of the blues, greens, purples, and browns when paired with proteins and lipids (Natural Astaxanthin). The different colors are derived from the change in the polyene chain and ring structures (Cyanotech). β-carotene contains nine double-bond pairs and gives off a yellow-orange color, γ-carotene has 10 double-bond pairs, exhibiting a deep orange color, lycopene, a carotenoid that comprises of 11 double-bonds, produces a red color, and the nine double-bond pairs of astaxanthin give the blended color appearance of an orange-red (Figure 1). Both β-carotene and astaxanthin contain the same number of polyene double-bonds in their structure, suggesting they should be similar in color. Instead, it is the additional keto and hydroxyl groups found in the structure of astaxanthin that make its color appear slightly different (Natural Astaxanthin).

Marine animals are unable to make their own carotenoids, so they must consume other organisms as a way of receiving the color pigment (Ho et al. 2014). Phytoplankton are among the top producers of carotenoids, making nearly several hundred million tons in dry weight each year (Natural Astaxanthin). *Haematococcus pluvialis* algae, compared to other sources such as krill and crawfish, is one of the most concentrated natural forms of astaxanthin that can be found, making it very expensive (Cynotech). An issue with captive raised marine fish is that they receive a lower proportion of carotenoids than do species that live in the wild (Natural Astaxanthin). Synthetic astaxanthin varies in chemical composition from natural astaxanthin only slightly, but still enough to produce different colored hues when animals are fed the same amount of each (NatuRose).

Amphiprion ocellaris, also known as the False Percula clownfish, are members of the Pomacentridae family. There are twenty seven different species within this family that take the genus name of *Amphiprion*, but only one that has the genus of *Premnas* (Madhu et al. 2012). Iwata et al. (2008) describes the *Amphiprion ocellaris* as a protandric species, meaning that all of the larvae are born male. As the fish mature, the strongest and most dominant of the population changes sex to become a female. The second strongest fish becomes the dominant male, acting as the mate for the head female, while the rest of the population remains non-reproducing. It has been observed that the social order in *A. ocellaris* is recognizable by an aggressive nature as well as a size increase.

Anenomefish, especially the *A. ocellaris*, are highly desirable for trade in the market because of their exuberant color (Madhu et al. 2012). In the years 1997-2002, *A. ocellaris* accounted for 15.6% of fish that were shipped globally (Yasir and Qin 2009). In European nations alone, they accounted for nearly 25% of exports shipped into the country (Yasir and Qin

2009). Ranked fifth in the amount of imports entering the United States, the *A. ocellaris* and *A. percula* combined brought in a total of around 400,000 fish per year (Ho et al. 2014). The United States has invested about $200-300 million in ornamental marine fish (Wabnitz 2003, 6). While the ornamental fish trade can be satisfactory for the economy, netting a near $963 million globally, it can be highly detrimental to the aquatic ecosystem (Tissot and Hallacher 2003). As Tissot and Hallacher point out, harvesting species at rates faster than they can reproduce leads to serious depletion, which can affect the structure of the underwater community.

Captive bred fish can help improve the numbers of dwindling species by steering commercial companies away from the ocean and towards the methods of aquaculture. Aquaculture is defined as the raising and culturing of both animals and plants, together or separate, in all kinds of aquatic environments, whether it be salt water, fresh water, brackish water, or any salt to water level concentration in between (NOAA Fisheries, What is Aquaculture?). This practice can be traced back to China as far as thousands of years ago (Stickney et al. 2012).

Brightly colored fish are highly desired by many pet store customers. Raising the quality of color in captive bred fish through the use of astaxanthin will help bring in a major influx of money through marine ornamental trade. This in turn will boost the economy (Natural Astaxanthin). The optimization level determined from this experiment will go on to help both the feed company and the fish raising industry. Reed Mariculture can benefit from this information, using the data to decide whether they would like to alter their current level of 250 ppm to the more desirable range of 400-640 ppm. Bradford Bay Farms can change the food they feed to their fish to match the recommended level of astaxanthin. This could potentially greatly increase the profits of both parties.

Materials and Methods

The Virginia Tech Agricultural Research and Extension Center (AREC) uses an aquatic habitats unit (AHAB) that consists of twenty-four 10 L tanks. These tanks operate on a recirculating aquaculture system (RAS), which means that water used by the fish is run through a filter, or series of filters, and then replenished back into the system. The RAS system at the AREC is composed of four parts: a sump, a bubble wash bead filter, a fine filter, and a UV light. The sump is used as a biological filtration system that cleans out the ammonia and nitrate that has accumulated in the water. The bubble wash bead filter is a mechanical filter that catches a vast majority of solid particles floating in the water. The fine filtration is used to filter out smaller particles and anything else that slipped by the bubble wash bead filter. The UV light is then used to kill off any bacteria or *Vibrio* that may be lurking in the water.

At the AREC, there are standard water quality parameters of which must remain constant to ensure fish health and survival. Over the seven week trial, ammonia was kept lower than 0.5 mg/L, nitrite was under 0.1 mg/L, nitrate was less than 25 mg/L, pH between 7.5 and 8.0, alkalinity within 140 mg/L and 180 mg/L, dissolved oxygen greater than 90% or over 6 mg/L, salinity between 20 g/L and 30 g/L, and temperature remained between 26°C to 28°C. The tank systems were checked at least once a day to make sure the requirements were being met. If not, there were various steps that to fix the issue at hand, such as: increased flushing of the system, decreased feeding, increased or decreased bicarbonate by the addition of baking soda, increased aeration, addition of synthetic sea salt, or adjusted thermostat.

There were 240 fish, all supplied by Bradford Bay Farms, factoring 10 fish per one of the 24 tanks. Each treatment consisted of 40 fish, allowing four tanks for the purpose of replication. Reed Mariculture supplied the six levels of otohime feed, including the astaxanthin color additive in five of them, excluding one for the control. The levels were as follows: 0 ppm, 150 ppm, 250 ppm, 400 ppm, 640 ppm, and 1020 ppm. The current amount of additive used by Reed Mariculture is 250 ppm. The levels chosen were focused on treatments both equally above and below this standard in order to determine if the current amount is the one most people find appealing. The clownfish were fed three times a day until saturation by Zachary Murphy, an intern at the facility. The experiment was conducted as a double blind study where neither the researchers, the intern, nor the judges were aware of which tank was randomly selected to receive what treatment. Only the mentor, Mr. Steve Urick, possessed this information and later revealed the color trail key after the experiment was over for analysis purposes (Table 1).

An objective method and a subjective method of collecting data was both used to determine the optimal level of astaxanthin required to produce the desired orange color. The objective method involved the use of an iPhone 6 camera, Adobe Photoshop CC 2015, and an HSL color algorithm from WorkWithColor.com (Ho et al. 2014). The camera was used to take a picture of all the fish from each of the twenty-four tanks, resulting in twenty-four pictures. The fish were placed in a hexagonal opaque weigh boat with a white paper background that had the numbers one through twenty-four written in the corners to indicate the tank being pictured (Figure 2). The numbering was designed so that odd numbers were placed in the same picture. The same set up was done with the even numbers. This was to minimize confusion, ensuring consecutive numbers were not photographed in the same picture. The tank identification was assumed to be the lowest number photographed, unless there was a slash through the number, in which case the higher number would be the tank identification. A thin film of water was placed in the weigh boat along with the fish to ensure their health and safety. Using Adobe Photoshop CC 2015, the red, green, and blue (RGB) values were extracted from the pictures, excluding any glare, white bands or black pigmentation (fins, tail, and outer portion of white bands). The RGB values were then imputed into the color picker to find the correlating hue, saturation, and luminosity (HSL) values (WorkWithColor.com). Three separate ANOVAs were created, one for hue, one for saturation, and one for luminosity. This was because each value has its own describing factor. Hue relates to the type of color presented on a cylindrical scale, thus explaining its unit of degree. Saturation describes the density and intensity of color perceived, having the unit of percent. Luminosity refers to the amount of light absorbed in a percentage.

The subjective method involved a panel of six judges consisting of Pet World associates, Aquariums Unlimited workers, and various other aquaculture specialists. All judges were blind to the treatment level presented to them as well as the color additive used. Each participant was required to complete two triangle tests (Appendix A). A single triangle test involved three randomly selected tanks, two of the tanks containing one treatment level while the third contained a different treatment level. This ensured whether the judges could perceive a visual difference between two similar treatment groups. The first test involved two tanks with the treatment level of 250 ppm and one tank with the treatment level of 400 ppm. The second triangle test involved two tanks with the treatment level of 640 ppm and one tank with the treatment level of 250 ppm. The judges were then asked to rate the overall color of a randomly selected tank from each of the six treatment levels. This was based upon a scale of 0-9, 0 representing very poor color and 9 representing excellent color (Appendix B).

In addition to the two triangle tests and the color preference rating, the judges were given a questionnaire (Appendix C). This information was used to compare the different perspectives on the aquaculture industry from multiple different backgrounds. A discussion was opened up to all participants after each data sheet had been completed and turned in. Although the fourth sheet did not particularly contain any numerical data to be analyzed, it was an interesting component to the study.

Results

Three separate ANOVA tests were conducted for statistical analysis of the orange color of each clownfish. The p-values extracted from these tests were as follows: 0.000 for hue, 0.000 for saturation, and 0.035 for luminosity. This information is very important because each of the p-values for all three of the ANOVAs were below 0.050, meaning each test was statistically significant in nature. It is highly unlikely that the data presented was due to chance, although factors such as poor lighting which may have created shadows, or the water glare could be cause for slight variation.

The boxplot created for hue (Figure 3) presents a steady downward sloping graph, depicting a generic indirect relationship between concentration of astaxanthin and hue of the fish. This graph shows a plateau around the 400-640 ppm mark. This means that even though the concentration of astaxanthin is increasing, the color of the fish is remaining relatively the same. A varied difference between concentration and hue is seen on the other side of the graph where the control of 0 ppm is located. Here it can be seen that numerically, the treatment level of 0 ppm was a different color shade than the treatment level of 150 ppm or 250 ppm.

The saturation boxplot (Figure 4) exhibits a different relationship when relating to the concentrations of astaxanthin. As the concentration increases, the saturation of each treatment level increases as well, establishing a direct relationship between the two. The higher treatment levels showed greater saturation values because they were fed feed that gave them the ability to hold on to the color better than the lower treatment levels.

The luminosity boxplot (Figure 5) did not present a generic relationship between the concentration of astaxanthin and the amount of light found in the color of the clownfish. The first three treatment levels, 0 ppm, 150 ppm, and 250 ppm, all had relatively high luminosity values. This would correlate with the idea that luminosity refers to the brightness of color, and the color additive of astaxanthin usually adds a darker color tone. The first three treatments received a very little amount, if any, of astaxanthin. The other three treatment levels, 400 ppm, 640 ppm, and 1020 ppm all had lower values for luminosity, deeming them a color more closely related to black than to white.

The second portion of the study involved a panel of six judges, all from an aquaculture background, that rated the color of the treatment levels based upon their preference. This data was compiled into a table (Table 2) for ease of comparison. It was seen that the treatment level of 400 ppm was the most preferable pigment of orange among all of the judges. Another commonality witnessed amongst the judges' answers was that the treatment level of 0 ppm was highly disliked. The other treatment levels were liked and disliked in a relatively equal manner, none being considerably more liked than the others, such as the 400 ppm, nor considerably disliked, such as the 0 ppm.

Discussion and Conclusion

Finding an optimal level of astaxanthin to include in fish feed is very important to the aquaculture industry. It not only helps the feed company with understanding how much color additive they should incorporate, making sure they do not over spend and instead make profit, but also the fish farmers because they know the best level to feed their fish in order to attract the most attention and most buyers. Subjectively, the judges unanimously deemed the treatment level of 400 ppm the optimal colorization level for the most attractive clownfish. Objectively, the optimal level was very close between the treatment level of 400 ppm and 640 ppm. The 640 ppm treatment level had the highest average amount of saturation, and a luminosity average that was closest to black than any other treatment level while the 400 ppm level had the more moderate bright orange hue value.

Although many of the judges stated that the 400 ppm treatment level had excellent color, there was one judge who believed that the treatment level of 0 ppm had excellent coloration as well. It was later explained that many customers today would probably look at this color fish and see its unusual pigment as desirable. This could be due to a number of different reasons, including that more and more pet owners are moving away from the “mainstream” look, or that the lighter orange color provides a more noticeable contrast with the black of the tails and fins. This information can be supported by the answer to statement four in the questionnaire (Table 3). This judge stated that they disagreed on the fact that brightly colored clownfish could be sold easier than an average colored clownfish.

Hopefully a greater transition from wild-caught fish to captive-bred fish will be seen in the coming years. This new data could help progress the transition, eliminating the issue of captive-bred fish not exhibiting the same exotic coloration as the wild-caught fish due to a lessened amount of carotenoid intake. This shift would cause revolutionary improvements to the environment as well as the marine ornamental fish trade economy. Many of the judges were seen to have purchased both wild-caught and captive-bred fish. It would be miraculous to see a full movement away from the wild-caught fish to the captive-breed fish. One day soon this will be the future, with the help of studies such as this one that are focused on improving the standards and quality of fish raised in captivity.

Acknowledgments

I would like to express great thanks to my mentor, Mr. Steve Urick, for providing the necessary materials required to conduct the experiment and for the vast knowledge base I have accumulated during my time working under him at the facility. He has helped with many aspects of the the trial, both physically with taking down tanks full of water and mentally with brainstorming to come up with solutions to any roadblocks. I would also like to thank Virginia Tech for allowing both my partner, Lauren Cook, and I to work in a facility owned by the college, Bradford Bay Farms, for providing our project with many healthy clownfish, Reed Mariculture, for providing the fish feed dressed with the varying treatment levels, and all of the judges who took time out of their busy schedules to participate in the study. I would like to extend my sincerest gratitude to Zachary Murphy, who fed the fish every day, and Alex Squadrito for providing us with the preliminary learning base needed to begin the mentorship.

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Table 1: Color Trial Key. Each treatment persisted of four tanks, ensuring there were at least three additional tanks undergoing the same conditions for repetition purposes. The treatment assignments for each tank were randomly selected using the software program Excel by Mr. Steve Urick.

Tank	ppm
1	150
2	250
3	400
4	400
5	150
6	640
7	640
8	0
9	400
10	1020
11	150
12	0
13	1020
14	250
15	0
16	250
17	640
18	1020
19	400
20	1020
21	0
22	640
23	250
24	150

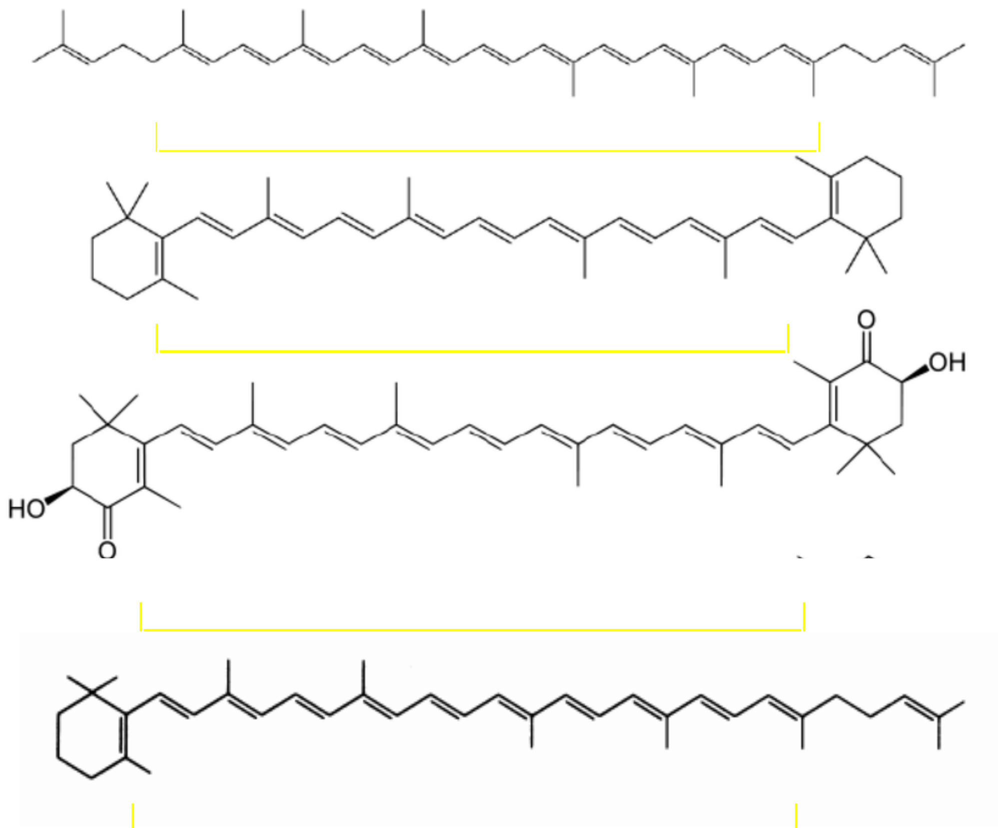
Table 2: Results from the color preference ranking that the panel of judges conducted.

Tank number and Astaxanthin Level	Person 1	Person 2	Person 3	Person 4	Person 5	Person 6
Tank 1 (640 ppm)	4	4	7	5	7	4
Tank 2 (1020 ppm)	4	4	8	6	3	4
Tank 3 (150 ppm)	2	3	7	4	2	1
Tank 4 (250 ppm)	6	4	7	7	5	3
Tank 5 (0 ppm)	1	0	5	8	2	1
Tank 6 (400 ppm)	8	9	9	8	9	7

Table 3: Results from the additional questionnaire filled out by the panel of judges.

Statement	Strongly disagreed	Disagreed	Neutral	Agreed	Strongly agreed
Captive bred clownfish currently available on the market are of excellent quality	0	0	3	3	0
Captive bred clownfish quality has gotten better in the last year	0	2	1	1	2
Currently available captive bred clownfish are lower quality than wild caught	2	2	2	0	0
I can sell a bright orange clownfish for more than an average color clownfish	0	1	1	3	1
Over time, clownfish coloration declines after I receive/hold the fish*	0	4	1	0	0
I am willing to pay a premium for the best color clownfish	0	0	1	4	1

*One judge did not answer for this statement, explaining 5 recorded answers rather than the usual 6



Lycopene

β-Carotene

Astaxanthin

γ-Carotene

Figure 1: The differing polyene structures of the four carotenoids are shown, highlighting the area where the double-bond pairs are located. Lycopene gives the appearance of red, β-carotene gives the appearance of a yellow-orange, astaxanthin gives the appearance of an orange-red, and γ-carotene gives the appearance of deep orange.

Figure 2: All ten fish were placed in the hexagonal weigh boat along with a thin film of water to ensure fish safety. The white paper underneath served as a tank identification for each picture as well as a solid background to place the opaque weigh boat on top of. This is a picture of tank 1 because it is the lowest number in the picture and does not have a slash through it.

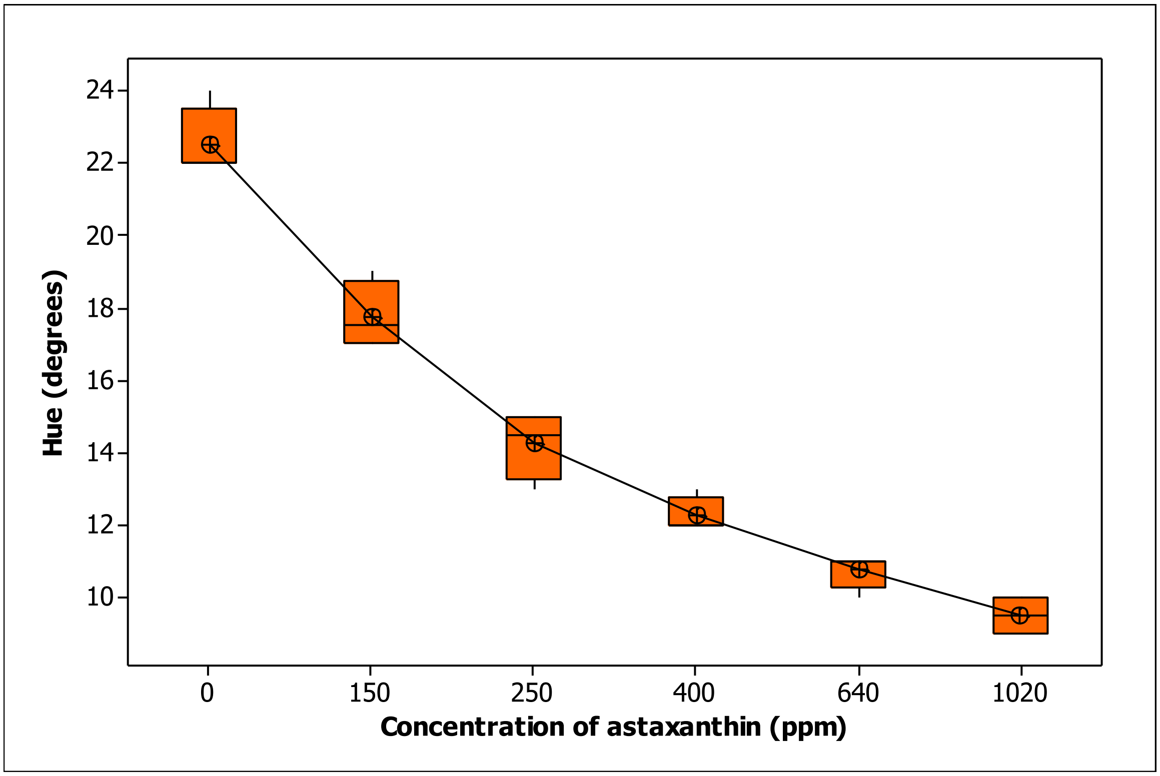


Figure 3: A steady decline of the graph shows that as the concentration of astaxanthin increased, the hue value decreased. It is seen that as the graph approaches the higher end of the treatment levels, there appears to be the presence of a plateau.

One-way ANOVA: Hue versus [Astaxanthin]

Source	DF	SS	MS	F	P
[Astaxanthin]	5	475.000	95.000	155.45	0.000
Error	18	11.000	0.611		
Total	23	486.000			

S = 0.7817   R-Sq = 97.74%   R-Sq(adj) = 97.11%

```
                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
   0   4  22.500  1.000                                  (-*-)
 150   4  17.750  0.957                        (-*-)
 250   4  14.250  0.957                (-*-)
 400   4  12.250  0.500           (-*-)
 640   4  10.750  0.500       (-*-)
1020   4   9.500  0.577  (-*-)
                         --------+---------+---------+---------+-
                              12.0      16.0      20.0      24.0
```

Pooled StDev = 0.782

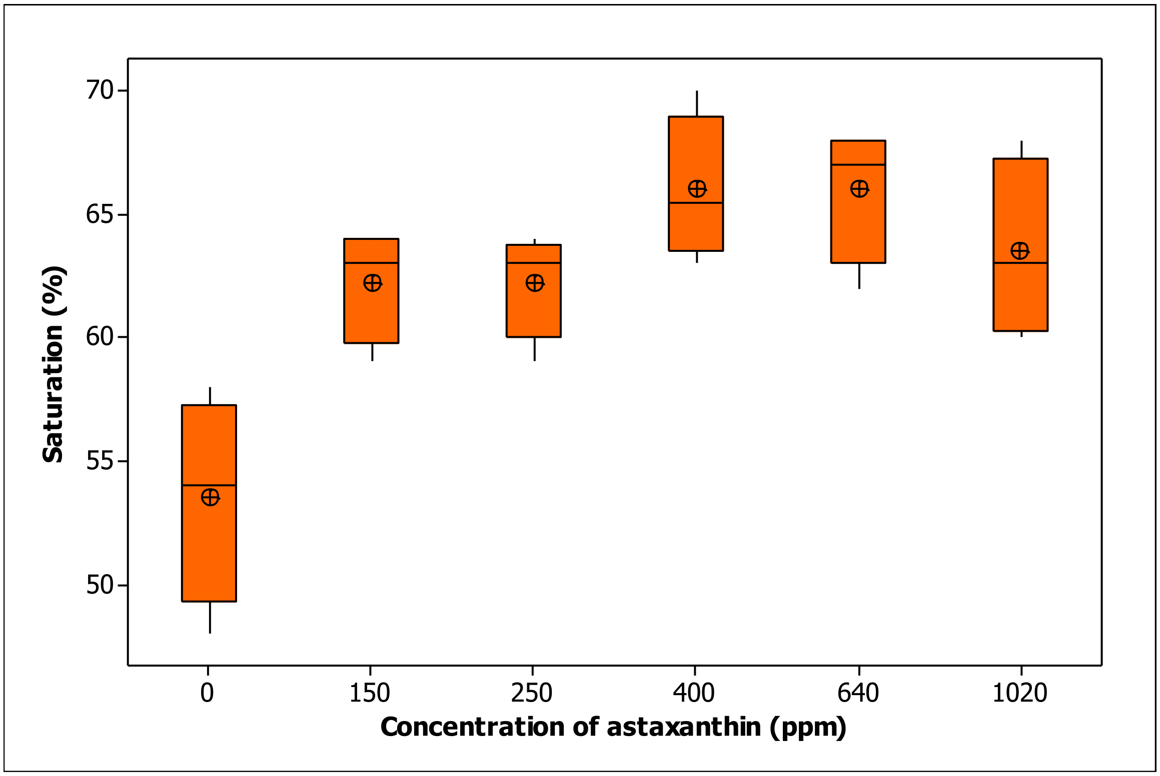


Figure 4: The graphs shows that as the concentration of astaxanthin increases, so does the saturation value. This means these two have a direct relationship with one another. It is interesting to note that the highest treatment level, 1020 ppm, has slightly lower saturation values than the 400 ppm and the 640 ppm.

One-way ANOVA: Saturation versus [Astaxanthin]

```
Source          DF      SS     MS     F      P
[Astaxanthin]    5  425.00  85.00  8.72  0.000
Error           18  175.50   9.75
Total           23  600.50

S = 3.122   R-Sq = 70.77%   R-Sq(adj) = 62.66%


                         Individual 95% CIs For Mean Based on Pooled
                         StDev
Level  N    Mean  StDev    +---------+---------+---------+---------
   0   4  53.500  4.203    (------*------)
 150   4  62.250  2.363                      (------*-----)
 250   4  62.250  2.217                      (------*-----)
 400   4  66.000  2.944                             (------*------)
 640   4  66.000  2.828                             (------*------)
1020   4  63.500  3.697                        (------*------)
                           +---------+---------+---------+---------
                         50.0      55.0      60.0      65.0

Pooled StDev = 3.122
```

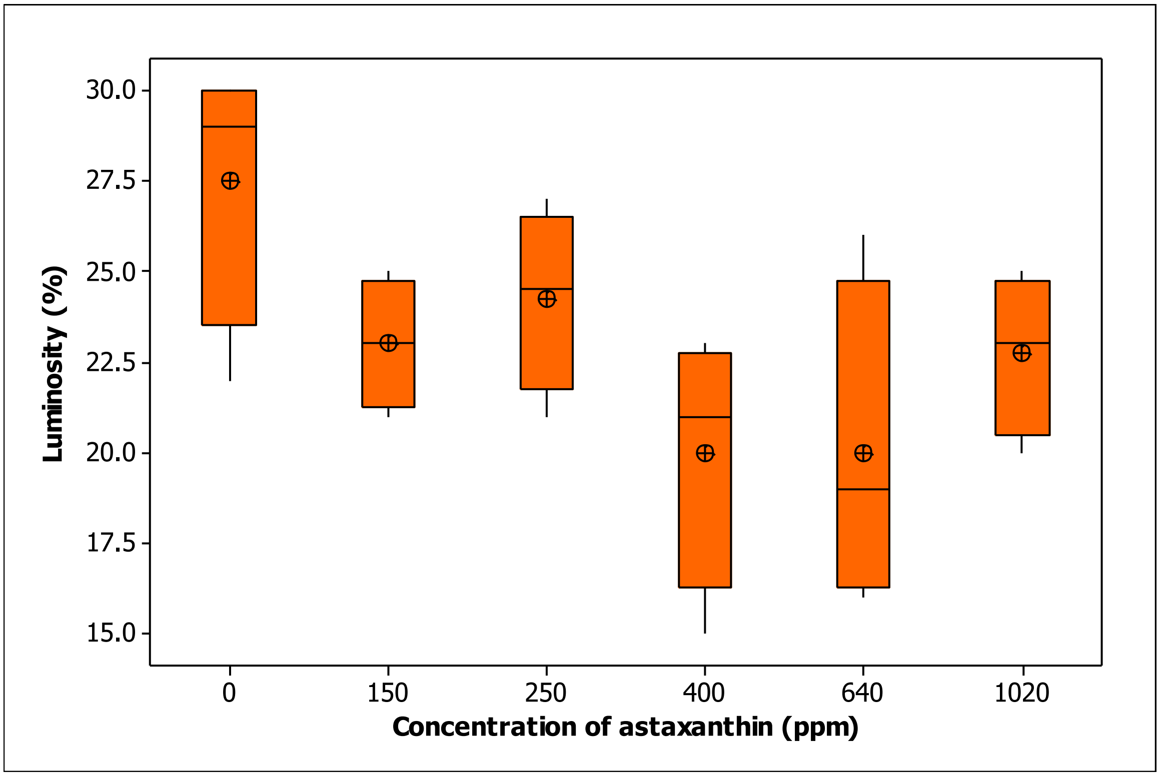


Figure 5: The relationship between the concentration of astaxanthin and the percent luminosity is not as easily seen. 150 ppm and 250 ppm are relatively the same in value, as are the treatment levels of 400 ppm and 640 ppm.

One-way ANOVA: Luminosity versus [Astaxanthin]

```
Source          DF     SS    MS     F      P
[Astaxanthin]    5  159.3  31.9  3.08  0.035
Error           18  186.5  10.4
Total           23  345.8

S = 3.219   R-Sq = 46.07%   R-Sq(adj) = 31.09%


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
   0   4  27.500  3.786                    (---------*--------)
 150   4  23.000  1.826         (--------*---------)
 250   4  24.250  2.500            (---------*--------)
 400   4  20.000  3.559  (--------*--------)
 640   4  20.000  4.546  (--------*--------)
1020   4  22.750  2.217        (---------*--------)
                         --------+---------+---------+---------+-
                              20.0      24.0      28.0      32.0

Pooled StDev = 3.219
```

Appendix A: Triangle Test

1-1

Two of these samples are identical, the third is different.

1. View the samples and identify the odd sample.

Code/Tank I.D. #	Put an X after the odd sample.
245	______
826	______
413	______

2. Indicate with a X the degree of difference between the duplicate samples and the odd sample.

Slight	______
Moderate	______
Much	______
Extreme	______

3. Acceptability: Check with an X

Odd sample more acceptable`	______
Duplicate sample more acceptable	______

4. Comments:

1-2

Two of these samples are identical, the third is different.

1. View the samples and identify the odd sample.

Code	Put an X after the odd sample.
314	______
626	______
542	______

2. Indicate with a X the degree of difference between the duplicate samples and the odd sample.

Slight	______
Moderate	______
Much	______
Extreme	______

3. Acceptability: Check with an X

Odd sample more acceptable`	______
Duplicate sample more acceptable	______

4. Comments:

Appendix B: Color Preference Rating

1-3

COLOR PREFERENCE

	VERY POOR COLOR		POOR COLOR		GOOD COLOR		VERY GOOD COLOR		EXCELLENT COLOR	
TANK #1	0	1	2	3	4	5	6	7	8	9
TANK#2	0	1	2	3	4	5	6	7	8	9
TANK#3	0	1	2	3	4	5	6	7	8	9
TANK#4	0	1	2	3	4	5	6	7	8	9
TANK#5	0	1	2	3	4	5	6	7	8	9
TANK#6	0	1	2	3	4	5	6	7	8	9

Appendix C: Questionnaire

1-4

Please circle your answer for questions 1-8

1. **Captive bred clownfish currently available on the market are of excellent quality.**

Strongly Disagree Disagree Neutral Agree Strongly Agree

2. **Captive Bred clownfish quality has gotten better in the last year.**

Strongly Disagree Disagree Neutral Agree Strongly Agree

2B. Why? Size Increase or Decrease More or Less Disease/Loss
Other____
__________________________________.

3. **Currently available captive bred clownfish are lower quality than wild caught.**

Strongly Disagree Disagree Neutral Agree Strongly Agree

4. **I can sell a bright orange clownfish for more than an average color clownfish.**

Strongly Disagree Disagree Neutral Agree Strongly Agree

5. **Over time clownfish coloration declines after I receive/hold the fish.**

Strongly Disagree Disagree Neutral Agree Strongly Agree

6. **I am willing to pay a premium for the best colored clownfish.**

Strongly Disagree Disagree Neutral Agree Strongly Agree

7. **How long do you typically hold clownfish before they are purchased by customers (sell out)?**

One week Two weeks Three weeks Four Weeks More than four weeks

8. **What do you feed the clownfish in the sales tanks?**

New Life Spectrum Ocean Nutrition TDO Chroma Boost LRS frozen foods
Other____
_______________.

9. Do you purchase Wild Caught Clownfish? YES NO
WHY?____
_________________________________.

10. Do you purchase Captive Bred Clownfish YES NO
WHY?____
____.

Appendix D: Raw Data

Tank	[Astaxanthin]	Hue	Saturation	Luminosity
1	150	17	59	21
2	250	14	59	24
3	400	13	65	22
4	400	12	70	15
5	150	17	64	22
6	640	11	68	16
7	640	11	68	17
8	0	22	55	22
9	400	12	63	20
10	1020	10	60	24
11	150	18	62	25
12	0	24	58	30
13	1020	9	61	25
14	250	15	63	25
15	0	22	48	28
16	250	13	63	21
17	640	10	62	26
18	1020	9	65	22
19	400	12	66	23
20	1020	10	68	20
21	0	22	53	30
22	640	11	66	21
23	250	15	64	27
24	150	19	64	24