Determining an Optimum Level of Dietary Astaxanthin to Improve Coloration of *Amphiprion ocellaris*

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Abstract

Amphiprion ocellaris (false Percula clownfish) is one of the top-selling species in the marine ornamental fish industry, and is one of many fish species still subject to ecologically harmful wild-catch practices. *A. ocellaris* can also be bred in captivity, but captive-breeding is not popular due to the dull coloration the fish have in captivity. Using six different concentrations of astaxanthin (the main carotenoid responsible for their orange-red coloration) top-dressed onto fish feed, an optimum concentration of astaxanthin to improve coloration was found. Two hundred and forty juvenile *A. ocellaris* were fed fish feed dressed with 0, 150, 250, 400, 640, or 1020 ppm astaxanthin for seven weeks. Immediately following the end of the trial, six judges knowledgeable in aquaria evaluated color quality of the fish. Image analysis was performed on the fish, and three one-way ANOVAs determined statistical significance for the treatment means. Boxplots of hue indicated an optimum concentration of dietary astaxanthin at 640 ppm. Results from the judging panel gave valuable insight on the current state of the aquaculture industry concerning *A. ocellaris,* and the optimum concentration can serve as a parameter for fish feed companies and fish farms to begin placing colorful captive-bred *A. ocellaris* on the market.

Introduction

Anemonefish, one of the most popular kinds of marine ornamental fishes, have caused an increase in activity in the global ornamental and anemonefish markets (Kanokrung et al. 2013). Ten of the top twenty globally traded species were damselfishes or anemonefishes (Rhyne et al. 2012). Their value and overall appeal are determined by brightness, coloration, and pattern (Wang et al. 2006). When cultured in captivity, anenomefishes display dull, faded colors

(Ramamoorthy et al. 2010) due to the lack of dietary carotenoids that they would otherwise find in the wild (Tanaka et al. 1992).

Among the anemonefish available for commercial sale, *Amphiprion ocellaris,* the false Percula clownfish, is one of the easiest species to breed in captivity (Gopakumar 2005), is one of the globally top-selling marine ornamental species (Wabnitz 2003, 19), and is in the top five species imported into the United States (Rhyne et al. 2012). Because captive-bred fish do not have access to dietary carotenoids, false Percula clowns maintain a faded yellow color in captivity as opposed to a brighter orange in the wild, decreasing their sale value. This can be changed by supplementing a carotenoid into their diet. *A. ocellaris* can successfully incorporate astaxanthin (the main carotenoid of the species) into its skin in captivity (Tanaka et al. 1992). Natural sources of astaxanthin come from *Haematococcus pluvalis* algae. This dietary astaxanthin significantly affects coloration, and the longer the fish are exposed to it, the brighter they become (Ho et al. 2012).

Anenomefishes with increased levels of dietary astaxanthin show brighter coloration (Ho et al. 2012; Seyedi et al. 2013). No optimum level of astaxanthin top-dressed onto fish feed has been determined. If an optimum level of astaxanthin could be determined for *A. ocellaris,* captive breeding rates for fish could increase, causing companies to refrain from harmful wild-catching methods and resort to a cheaper, more efficient, and ecologically-friendly way of producing and selling these popular ornamental fish.

Materials and Methods

Twenty-four 10 L tanks ran on a recirculating aquaculture system (RAS). Dissolved oxygen (DO) was kept at a level > 6 mg/L, salinity was kept between 20-30 g/L, alkalinity was kept between 140-180 mg/L using sodium bicarbonate, ammonia was kept at a level < 0.5 mg/L, nitrates was kept at a level < 25 mg/L, pH was kept between 7.5-8.0, and temperature was kept between 26-28 °C. All of these water quality parameters are ideal for marine fin fish, and are standards set by the facility. Each tank contained ten juvenile *A. ocellaris* obtained from Bradford Bay Farms (Quinby, Virginia). Each treatment level of astaxanthin (0 ppm astaxanthin (control), 150 ppm astaxanthin, 250 ppm astaxanthin, 400 ppm astaxanthin, 640 ppm astaxanthin, 1020 ppm astaxanthin) had four tanks. The fish feed consisted of astaxanthin extracted from *Haematococcus* algae top-dressed onto Otohime (Marubeni Nisshin Feed Co. Ltd.) obtained from Reed Mariculture, Inc. (Campbell, California). These levels of top-dressed Otohime (TDO) were fed to the fish over the course of seven weeks. Treatments were randomly assigned an alphabetical letter by Excel to prevent any subconscious bias, and the tanks that received each treatment were also randomly assigned (Table 1). Only one individual not directly associated with the trial knew the treatment assignments. Fish were fed three times per day and feedings were no less than four hours apart. An intern blind to the treatment levels fed the fish when the researchers were not available to feed them during the day. All fish were fed to satiation.

To determine the coloration of the fish, photos of the fish from each tank were taken (iPhone 6 camera) on a white background (white, opaque weigh boat) with a thin water layer on top before the trial started. Fish were placed back in their tanks and were not handled until the end of the trial period. After the trial was complete, pictures were taken of the fish again; these images were used for data collection. Using Adobe Photoshop CC 2015, red, green, and blue values (RGB) for each fish were found. These RGB values were put into an online color algorithm (http://www.workwithcolor.com/hsl-color-picker-01.htm) to determine hue, saturation, and luminosity (HSL) for the fish (Ho et al. 2014). A one-way ANOVA was performed for each HSL value, because each value describes a different aspect of color.

The subjective method involved a panel of six judges (local pet store owners knowledgeable about aquaria) come to the facility to view the fish and answer questions about them. Judges were blind to the treatment levels. Judges were first asked to take two triangle tests to determine how well they could tell apart different colors (Appendix A). All triangle tests consisted of five randomly selected fish respective of their levels of astaxanthin in each tank, and all tanks had white sides and backs. The first triangle test consisted of two 250 ppm tanks and one 400 ppm tank. The second triangle test consisted of two 640 ppm tanks and one 250 ppm tank.

The judges were then asked to fill out a color preference rating chart (Appendix B), and were asked to judge six tanks of fish based on their color quality. Each tank consisted of five randomly selected fish respective to each concentration of astaxanthin. Afterwards, the judges responded to a questionnaire (Appendix C). This questionnaire was used to determine current local opinions on the state of the aquaculture industry with respect to clownfish. Judges were also encouraged to participate in an open discussion about clownfish and the ornamental industry afterwards.

Results

ANOVAs performed on hue, saturation, and luminosity values generated 0.000, 0.000, and 0.035 p-values, respectively; all of these were less than 0.050, indicating statistically significant differences among treatment means. Saturation and luminosity have a higher level of variation due to other environmental factors, such as lighting, gloss, and shadows.

The boxplots for hue (Figure 1) indicate an optimum level of astaxanthin at 640 ppm. At this concentration, there is a small difference between 640 and 1020 ppm; though the p-value was 0.000, the disparity is small enough to declare 640 ppm the optimum level of astaxanthin. The boxplots also describe a continual decreasing trend as astaxanthin concentrations increase. In terms of color, this means that the color of the fish began at a lighter orange with no astaxanthin (approx. 24°) and shifted to a darker orange (approx. 18°) before leveling off at a dark red-orange (approx. 10-12°).

The boxplots for saturation (Figure 2) describe a difference between fish treated with no astaxanthin versus fish treated with astaxanthin. Fish treated with 0 ppm astaxanthin had the lowest saturation, and all fish treated with astaxanthin had significantly increased saturation, with no overlap with the control.

The boxplots for luminosity (Figure 3) describe the decreasing trend of the lightness of color, or how closely the color was approaching a pure white. Fish treated with 0 ppm astaxanthin has the highest luminosity average, which correlates with the fact they were given no pigment to darken their coloration. At 150 and 250 ppm, luminosity decreases slightly, but sees the greatest decrease at 400 and 640 ppm. One thousand and twenty ppm has a slight increase and its average is closer to 150 and 250 ppm, but that can most likely be attributed to uneven lighting or gloss.

Responses to the color preference rating chart (Table 2) and questions (Table 3) in subjective data collection varied, but certain parts of them contained general trends. For the color preference rating test, the judges thought 400 ppm and 250 ppm were both considered as "quality" coloration; 1020 ppm and 640 ppm were not as appealing as 400 ppm and 250 ppm.

Though 400 ppm garnered the highest scores, the sample size of judges was too small, and their responses cannot be statistically analyzed for significance.

Notable responses from the questionnaire include that four of the six judges purchased both captive-bred and wild-caught fish; two exclusively bought captive-bred fish; and judges that bought wild-caught fish justified their reasoning due to coloration or convenience (e.g. availability). Overall, judges thought captive-bred clownfish are of relatively good quality, are not much different than wild-caught fish, disagreed that coloration declines after holding the fish, and are willing to pay a premium to receive the best colored clownfish.

Discussion and Conclusion

Finding an optimum level of astaxanthin for *A. ocellaris* proves useful for the ornamental fish industry. Fish farms and feed companies can use this information to cut costs of astaxanthin; feed companies can use it to market a product that offers consumers an option to make their clownfish a darker red-orange, and fish farms can use this information to offer the brightest orange clownfish to businesses involved in aquaria.

Overall, the trial was a success, as the goal of finding an optimum level of astaxanthin was reached, and the optimum level was what was expected: 640 ppm. The data describe the "levels" of color that each concentration of astaxanthin creates in the fish, with 0 ppm being the lightest orange and least in saturation of color, 150 and 250 ppm offering an intermediate range of orange and heavier saturation, and 400, 640, and 1020 ppm astaxanthin offering the darker, brighter colors and heavier saturation. Feed companies and fish farms can use these "levels" to customize what color clownfish they want to market to the public. Six hundred forty and 1020 ppm astaxanthin offer very similar coloration; however, and it is not recommended that fish feed companies or farms use astaxanthin concentrations any higher than 640 ppm, because 640 ppm is the beginning of a plateau region where the color begins to remain the same, and use of a concentration higher than 640 ppm would not generate a darker color, and would be a waste of resources.

Though the size of the judge panel responses were not large enough to perform statistical analysis on, they offer insight on local opinion on captive-bred clownfish and coloration. For example, 250 and 400 ppm were both seen as generally the “best” colored fish; this might be due to how 250 ppm creates an attractive, lighter orange-red color, and 400 ppm creates an attractive, darker orange-red color, but not as dark as 640 ppm, which was not seen as attractive as 400 ppm. Both concentrations might offer equally pleasing colors, one lighter, and one darker.

Most of the judges purchased both captive-bred and wild-caught clownfish, and said they bought wild-caught fish because they are more readily available, sometimes are cheaper, and sometimes have better coloration than captive-bred fish. This indicates there is still a need for cheaper, more readily-available captive-bred clownfish in the ornamental fish industry. The optimum level of astaxanthin found in this experiment can serve as the first step towards making that a possibility by introducing captive-bred clownfish into the market that have excellent coloration.

During the open discussion, judges gave interesting insight to what is going on in industry today. One judge mentioned that the 0 ppm fish would have actually sold out in his store the fastest because, though they were not orange like a “classic” clownfish, they were different and could be considered special or unique by a consumer. The lack of brightness also made the clownfishes’ black fin coloration stand out. This comment makes an important point: consumers like color, but they also like uniqueness. Consumerism in aquaria is often based on collections, and what fish comprise collections are often based on comparisons to other collections, and on showing off what people have to others. Consumers, therefore, want unique fish in their collections; even if they are not colorful, they might have some other aspect to them that makes them desirable simply because they are different from "the rest".

Finding the optimum level of astaxanthin for *A. ocellaris* is hopefully the beginning of a shift from wild-catch to captive-breeding practices in the ornamental fish industry, and the end of the destruction of wild fish populations. It is important to remember, however, the key to successfully selling ornamental fish to consumers is uniqueness. Fish can be bright, but fish can also be unique without being colorful. Ending wild-catch practices, then, does not only depend on the brightness of a captive-bred fish, but an awareness of what is popular in the minds of the consumers, and a catering to the desire of the consumer.

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Tables

Table 1. Tank assignment key. Treatment levels were randomly assigned by Excel.

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 color preference rating chart. These are the scale designations that the judges selected (for color) for each tank (0 being poor color, 9 being excellent color).

Tank # and Astax. 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. Responses to questionnaire. For the fifth statement, one judge did not answer.

Statement	# that strongly disagreed	# that disagreed	# that were neutral	# that agreed	# that 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

Figures

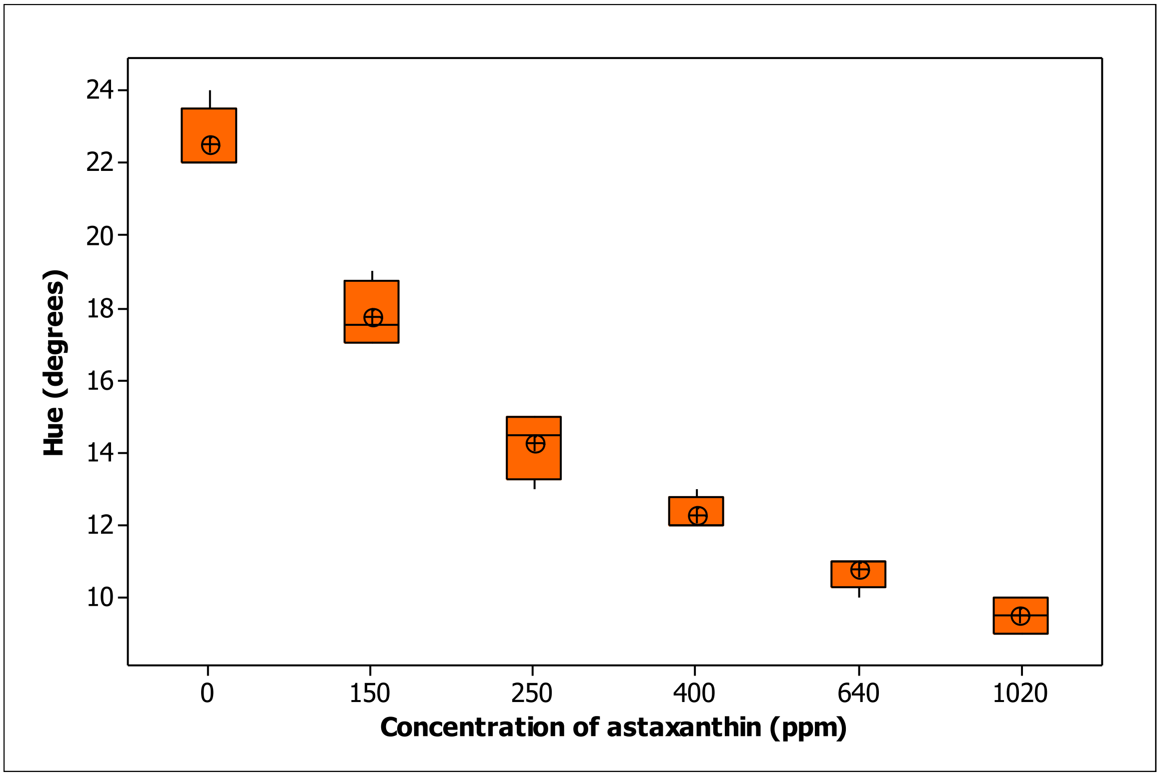

Figure 1. Decline of hue values as astaxanthin levels increase. At 0 ppm, the color is a lighter orange, and as astaxanthin concentration increases, color shifts from orange to more progressively red. Very dark orange-red is around 640 ppm.

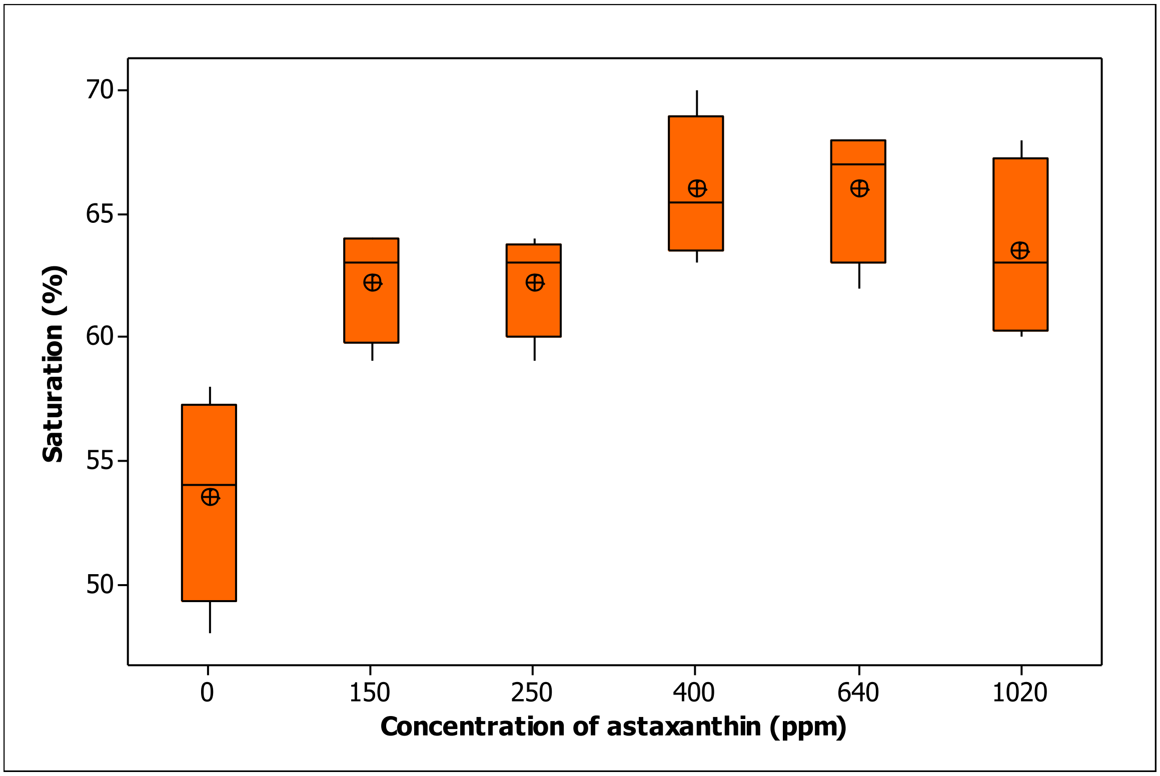

Figure 2. Gradual increase of saturation as astaxanthin concentrations increase. The lower average of 1020 ppm could be attributed to uneven lighting. Otherwise there are two distinct groups: 0 ppm (least saturated); 150, 250, 400, 640, and 1020 (saturated).

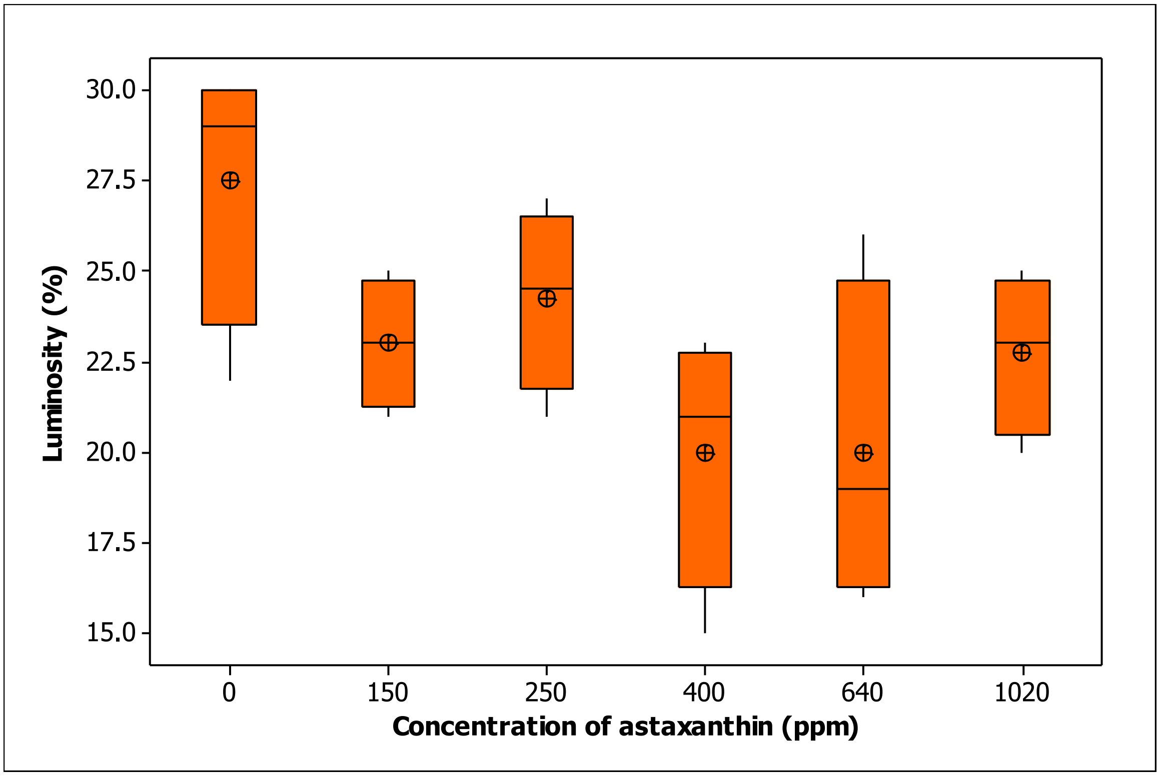

Figure 3. Gradual decrease of luminosity as astaxanthin concentration increases. Slight increase of average in 1020 ppm could be attributed to uneven lighting.

Appendix A: Triangle Tests

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:

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 Chart

	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

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?____.