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Skin color retention after dietary carotenoid deprivation and dominance mediated skin coloration in clown anemonefish, *Amphiprion ocellaris*

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Abstract. *Amphiprion ocellaris* is one of the most popular marine ornamental species, and aquaculture produces a substantial share of the market. This study examined the effect of dietary carotenoid deprivation on coloration of adequately colored *A. ocellaris*. Furthermore, socially mediated coloration was also investigated. Juveniles of 35 days post hatch (DPH) were fed an unsupplemented base diet and a *Haematococcus pluvialis* powder supplemented diet (19.3 and 75.5 mg kg^{-1} total carotenoids, respectively) for 180 days as negative and positive controls, respectively. A treatment was fed the supplemented diet (75.5 mg kg^{-1}) for 90 days, and then switched to the unsupplemented diet (19.3 mg kg^{-1}) for 90 days. Saturation and luminosity were not significantly different among the treatment and controls, while hue was. The switch treatment hue shifted slightly towards yellow at 215 DPH, and the exhibited hue could still be considered appealing. At younger ages, hue was highly subject to social structure, with hue increasing with decreasing social status. However, the discrepancy in hue became less prominent as fish aged. Additionally, commercial and hobbyist culture conditions are conducive to minimizing the effects of the social structure on hue. In summary, social structure effects were apparent with minimal long term effects, and an acceptable hue can be retained for at least 90 days after appropriate diet deprivation.
Key Words: diet change, astaxanthin, hue, saturation, luminosity, HSL, social structure.

Introduction. Anemonefishes (Pomacentridae: Amphiprioninae) are a staple of the marine aquarium hobby (Wabnitz et al 2003; Rhyne et al 2012). Much effort has been geared towards achieving appropriate coloration in anemonefishes (Tanaka et al 1992; Hoff 1996; Yasir & Qin 2009a, b, 2010; Ho et al 2013a) with the goal of producing fish that are visually appealing to the consumers (Gouveia et al 2003; Gouveia & Rema 2005). Carotenoids, such as astaxanthin and β-carotene, are yellow–red pigments produced by photosynthetic organisms (Kodric-Brown 1985; Hill et al 2002). Animals are unable to produce carotenoids *de novo* and have to acquire them through their diet (McGraw & Ardia 2003; van Nieuwerburgh et al 2005). Dietary astaxanthin has been broadly used in aquaculture to color both food (Wathne et al 1998; Bjerkeng et al 1999) and ornamental species (Tanaka et al 1992; Hoff 1996; Paripatananont et al 1999; Gouveia et al 2003; Wallat et al 2005; Yasir & Qin 2010). It has been shown that the key dietary pigment(s) - especially astaxanthin - can result in the orange-red coloration in anemonefishes (Tanaka et al 1992; Hoff 1996). For *Amphiprion* a dietary concentration of 80–160 mg kg^{-1} esterified astaxanthin is recommended (Ho et al 2013a), while 214 mg kg^{-1} has been suggested for *Premnas* sp. (Ho et al 2013b) to achieve appealing colorations.

Although the dietary carotenoid requirements for anemonefishes is quite well understood (Tanaka et al 1992; Hoff 1996; Yasir & Qin 2010; Ho et al 2013a; Ho et al 2013b), there is a lack of understanding on the ability of anemonefishes to retain coloration if the dietary pigment source is removed. The pattern of long term coloration of anemonefishes may have weighty effects for the industry. A rapid loss of coloration in

the fish after they are purchased by the consumer may lead to dissatisfaction. Furthermore, this can lead to an erroneous association of captive reared ornamentals with lower quality products, and may consequently influence a market shift away from captive reared fishes.

Anemonefishes are protandric sequential hermaphrodites (Moyer & Nakazono 1978; Iwata et al 2008; Iwata & Manbo 2013), and a strict hierarchy is established that control sex change through aggression (Iwata et al 2008). The hierarchical aggression also leads to depressed growth rates in submissive ambisexual specimens and increased growth rate of the dominant putative female (Iwata et al 2008). Coloration is dependent on dietary astaxanthin concentration (Tanaka et al 1992; Hoff 1996; Yasir & Qin 2010; Ho et al 2013a; Ho et al 2013b). The social status of a specimen may influence its ability to garnish resources (Koebele 1985; McCarthy et al 1992), and ultimately lead to discrepancies in coloration for anemonefishes.

Amphiprion ocellaris and its sister taxon, *A. percula* (Jang-Liaw et al 2002; Santini & Polacco 2006), combined are ranked 5th in import numbers into United States, with an excess of 400,000 specimens imported annually (Rhyne et al 2012). Given the high popularity of this species, the goals of this study were to (1) assess how coloration varies after carotenoids are deprived and (2) to investigate any potential social effects on the coloration parameters of *A. ocellaris*.

Material and Method

Diet. Two diets were prepared - following Ho et al (2013a; 2013b) - from a special blend of Gelly Belly Mix™ that lacked pigment enhancers (Florida Aquafarms Inc., Dade City, FL, USA) and an esterified astaxanthin rich *Haematococcus pluvialis* powder (spray-dried *H. pluvialis* procured from Reed Mariculture, Inc., Campbell, CA, USA).

Given the estimated total carotenoid concentration of the *H. pluvialis* powder, the treatment diet was produced by supplementing the modified gel diet with 1% powder on a dry mass basis to target the recommended minimum 80 mg kg^{-1} in the final diet (Ho et al 2013a). The control diet consisted of only the base diet, no supplementation. Total carotenoid concentration was determined in triplicate (n = 3) by acetone extraction and measurement on a Hewlett Packard Model 8453 UV/Vis Diode Array Spectrophotometer (Hewlett Packard/Agilent Technologies Inc., Santa Clara, CA, USA). Samples were read at 480 nm wavelength (λ) in 1.0 cm quartz cuvettes and expressed using the Beer-Lambert Law and total carotenoid concentration estimated using the astaxanthin extinction coefficient of $E^{1\%}_{1cm}$ = 2200 (Chen & Meyers 1984). Total dietary esterified carotenoid concentration of the jellified (wet) treatment diet was determined to be (mean ± SD) 75.5 ± 3.2 mg kg^{-1}, which was slightly short of the target of 80 (-5.6%) and was deemed acceptable. Total dietary esterified carotenoid concentration of the jellified (wet) control diet was determined to be (mean ± SD) 19.3 ± 0.5 mg kg^{-1}. The control and treatment diet will be referred to as the unsupplemented and supplemented diets, respectively.

Experimental protocol. The experiment was carried out at the Florida Institute of Technology's Aquaculture Laboratory, Melbourne, FL, USA for a duration of 180 days between November 2012 and May 2013. Seventy two *Amphiprion ocellaris* juveniles were acquired from a marine ornamental fish farm (Proaquatix Inc., Vero Beach, FL, USA). The fish averaged 35 days-post-hatch (DPH) in age (range 34 to 36 DPH) and originated from three broods of unrelated broodstock pairs.

The experiment was conducted in a recirculating system consisting of a 430 L main tank fitted with a 120 L sump. The experimental tanks were black plastic round containers with an effective volume of 4.20 L (radius: 10 cm, depth: 17.5 cm, and capacity of 5.25 L) that rested in the main tank. The system was lit by two fixtures fitted with two 4,100K fluorescent tubes (F32t8/Sp41/Eco by GE Lighting, General Electric Company, Fairfield, CT, USA) with a mean (± SD) output of 1.28×10^3 ± 42.3 lx at the surface of the water and set to a 14L:10D photoperiod for the duration of the experiment.

Three fish were randomly assigned into each of the 24 experimental tanks and the tanks were randomly allocated into three treatment sets (eight replicates [n = 8] each). The experiment consisted of three (3) dietary regimes. Two of the dietary regimes served as positive and negative controls, while the third as the experimental treatment. Fish in the negative control were fed solely the unsupplemented diet (19 mg kg^{-1}) and fish in the positive control were fed solely the supplemented diet (75 mg kg^{-1}) for the duration of the experiment (180 days). The fish in the experimental treatment were fed the supplemented diet for 90 days, and subsequently switched to the unsupplemented diet (switch regime) for 90 days. The fish were fed to apparent satiation twice a day. The 90 day period was selected as it was previously shown to result in market appropriate coloration in the amphiprioninids (Ho et al 2013a; Ho et al 2013b).

Fish coloration, size, and mass analysis. Three fish from each tank were measured at ages 35 (baseline), 65, 95, 125, 155, 185, and 215 DPH. The fish were placed on white background and with a white balance-correcting palette in the field of view and photographed for digital color analysis under standardized external light and camera parameters using a Sony model DSC-W560 digital camera (Sony Corporation of America, New York, NY, USA). The fish were then returned to their respective tanks.

The images were corrected for white balance and the mean red, green, and blue values (of the RGB color mode) were determined for the yellow-orange areas (excluding the fins) using Adobe® PhotoShop® CS3 (Adobe Systems Inc., San Jose, CA, USA). Acquired mean RGB values were converted to hue, saturation, and luminosity (HSL) mode using a web based algorithm (HSL Color Schemer: http://www.workwithcolor.com) for analysis following Yasir and Qin (2009a). For details on color space and application in anemonefishes color see Yasir & Qin (2009a), and Ho et al (2013a; 2013b).

ImageJ (National Institutes of Health, Bethesda, MD, USA) was utilized to measure total length of each fish specimen. In order to reduce the potential short- and long-term stress related color changes (Mazeaud 1969, 1973), fish mass was inferred from length-mass relationships. Total length (TL) measurements were acquired from the necessary color data images, and reduced the handling time and potential stress on the specimens. Fish mass was estimated from the formula $M = a \times L^b$, where mass (M) is in grams, length (L) is total length in cm, the coefficient a = 0.0189, and the exponent b = 3.19 for *Amphiprion* spp. (Kulbicki et al 2005).

The primary color parameter in amphiprioninids that is affected by diet and external parameters is hue (Yasir & Qin 2009a, b, 2010; Ho et al 2013a; Ho et al 2013b). To test the hypothesis of dominance mediated coloration, the differences in hue between different members of the social hierarchy were compared, under the premise that the larger individuals are dominant over successively smaller individuals (Iwata et al 2008). The fish in each of the tanks are designated specimen α, β, and γ in order of decreasing total length. To test the hypothesis of superior coloration (lower hue) of the largest individual (the putative female), the hue value of the largest specimen (α) was subtracted from the average hue value (denoted by avg) of the smaller specimens ([β+γ]/2); this value is referred to as "Δ $Hue_{\alpha\text{-}avg}$" (α - avg = α vs. average). To resolve potential pecking order influence on coloration the difference between the α and β (Δ $Hue_{\alpha\text{-}\beta}$: where α-β = α vs. β), the difference between specimen α and γ (Δ $Hue_{\alpha\text{-}\gamma}$: where α-γ = α vs. γ) were also calculated. The difference values were calculated for time periods of 35–155 DPH, when at least a median of two specimens survived (Figure 2). The difference in hue will be referred to as Δ Hue, and positive Δ Hue values will denote superior coloration of the dominant fish.

Statistical analyses. When applicable, normality or normality of residuals were assessed using a Shapiro–Wilk Test and Normal Q-Q plots, while sphericity with a Mauchly's Test. Data sets that failed to meet normality requirements were rank transformed. Parametric data are presented as mean ± SE and non-parametric as median ± MAD (Median Absolute Deviation). Statistically significant differences were set at $p < 0.05$. All statistical tests were performed on SPSS v 18.0 software (SPSS Inc., Chicago, IL, USA). When applicable, Wilk's λ was used to test for multivariate significance

and Fisher's Least Significant Difference multiple comparisons test was used to compare the effects across factors for each subsequent univariate test.

To test for coloration, the averages of the three fish for each sample were used for statistical analysis; n = 8 for each of the three treatments during each of the seven sampling periods. Although mortality was observed, it had no effect on test power as the tank was the replicable unit and not the individual fish. The effects of dietary exposure time (age), dietary regime (treatment), and their interaction on (1) hue, (2) saturation, and (3) luminosity of *A. ocellaris* were analyzed with a Multivariate Repeated Measures Analysis of Variance (MANOVAR) with time as the within-subjects factor and dietary regime as the between-subjects factor.

To test for survivorship over time, the number of fish surviving in each tank was rank transformed to account for the discreet nature of the data (count); n = 8 for each of the three treatments during each of the seven sampling periods. The effects of dietary exposure time (age), dietary regime (treatment), and their interaction on survivorship was analyzed with a Repeated Measures Analysis of Variance (ANOVAR) with time as the within-subjects factor and dietary regime as the between-subjects factor.

To test for physiological parameters of lengths and mass, the averages of the three fish for each sample were used in the case of length, while the sum of the specimens' masses for each tank were used for statistical analysis; n = 8 for each of the three treatments during each of the seven sampling periods. Although the mass is derived from length measurement, this section seeks to test the total (sum) of each tank, which would be decoupled from the mass-length relationship. The effects of dietary exposure time (age), dietary regime (treatment), and their interaction on (1) average length and (2) total mass of *A. ocellaris* a MANOVAR with time as the within-subjects factor and dietary regime as the between-subjects factor was used.

To test for dominance mediated coloration, Δ Hue$_{\alpha\text{-avg}}$, Δ Hue$_{\alpha\text{-}\beta}$, and Δ Hue$_{\alpha\text{-}\gamma}$ values were tested for significant deviation from 0 using a directional Paired t-Test. For Δ Hue$_{\alpha\text{-avg}}$ and Δ Hue$_{\alpha\text{-}\beta}$ variables, sample sizes were n = 6, n = 6, and n = 5 for negative control, switch, and positive control diet regimes, respectively. The Δ Hue$_{\alpha\text{-}\gamma}$ response variable had total samples sizes (all treatments combined) of n = 17, n = 16, n = 11, n = 4, and n = 0 for time periods 35 through 155, respectively. Because of the decreasing within-subjects factor (time) sample sizes for Δ Hue$_{\alpha\text{-}\gamma}$ comparisons, it was excluded from the MANOVAR test to maintain the integrity of the repeated measures sample size of the other two response variables. The effects of dietary exposure time (age), dietary regime (treatment), and their interaction on (1) Δ Hue$_{\alpha\text{-avg}}$ (2) Δ Hue$_{\alpha\text{-}\beta}$ values of *A. ocellaris* were analyzed with a Multivariate Repeated Measures Analysis of Variance (MANOVAR) with time as the within-subjects factor and dietary regime as the between-subjects factor.

Results

Fish coloration. A MANOVAR showed a significant effect of time and dietary regime on the combined coloration parameters, but no significant interaction effect was observed (time × dietary regime; Table 1). Univariate tests showed that hue, saturation, and luminosity were significantly affected by time. Furthermore hue was also significantly affected by dietary regime, as well as the interaction of time and dietary regime (Table 1). However, saturation and luminosity was not affected by dietary regime or the interaction of time and dietary regime. Fish across all treatments exhibited a median hue of ~33° at baseline (35 DPH, Figure 1A). The negative control treatment decreased significantly by ~6° by 65 DPH, and subsequently leveled out to ~25°. The supplemented diet treatments (positive control and switch treatment) decreased significantly by ~17° after 30 days, and leveled out at ~14° (Figure 1A). The switch treatment continued to mirror the positive control up to 215 DPH, even after the switch to the negative control diet at 125 DPH. At 215 DPH, the switched treatment hue was significantly higher than the positive control, but significantly lower than the negative control treatment (Figure 1A). Saturation increased to a median of ~80% from a ~ 60% baseline and plateaued for 90 days; after which it fluctuated by ~10% every 30 days (Figure 1B). Luminosity

dropped from a baseline median of ~41% to ~30% from 65–185 DPH, with slight increase at 215 DPH (Figure 1C).

Table 1

Summary of MANOVAR* results of coloration parameters. Statistically significant p-values are highlighted in bold. Data were rank transformed

Statistical model	*Independent variable*	*Measure*	*F*	*d.f.*	*P*	*Wilk's λ*
Multivariate Test	Within-subject effects					
	Time	Combined	77.92	18, 4	**< 0.001**	0.003
	Time × Diet	Combined	2.799	36, 8	0.064	0.005
	Between-subject effects					
	Diet	Combined	15.42	6, 38	**< 0.001**	0.085
Univariate Tests	Within-subject effects					
	Time	Hue	70.77	6, 126	**< 0.001**	
		Saturation	58.39	6, 126	**< 0.001**	
		Luminosity	40.10	6, 126	**< 0.001**	
	Time × Diet	Hue	6.980	12, 126	**< 0.001**	
		Saturation	0.929	12, 126	0.520	
		Luminosity	0.806	12, 126	0.644	
	Between-subject effects					
	Diet	Hue	55.64	2, 21	**< 0.001**	
		Saturation	0.782	2, 21	0.481	
		Luminosity	0.759	2, 21	0.470	

*Type I Sum of Squares Repeated Measures Multivariate Analysis of Variance; Time = Time exposed to diet(s), and Diet = dietary regime.

Survivorship, length, and mass. An ANOVAR revealed a significant effect of time on survivorship ($F_{(6,126)}$= 57.502, p < 0.001). However, no significant effect of diet ($F_{(2,21)}$= 0.963, p = 0.408) or the interaction of diet and time was detected ($F_{(12,126)}$= 0.761, p = 0.689). Survivorship decreased significantly with time (Figure 2). From the stocked three specimens, a median of two fish was left at 95 DPH, and one by 185 DPH.

A MANOVAR showed a significant effect of time on the combined physiological parameters of length and mass. However, no significant effect of dietary regime or the interaction time and dietary regime (time × dietary regime) was observed (Table 2). Univariate tests showed that length and mass were significantly affected by time, but not by dietary regime. Furthermore a significant interaction effect between time and dietary regime was detected for length, but not for mass (Table 2). Median length increased consistently over the entire experimental period from ~19 mm to 36 mm (Figure 3 left). Total mass (sum of replicate mass) also increased consistently over time from ~0.46 g at 35 DPH to ~1.2 g at 155 DPH, when total mass plateaued (Figure 3 right).

Dominance mediated coloration. Paired t-tests showed that all Δ Hue values at 35 and 155 DPH did not differ significantly from zero (paired t-tests: *d.f.* = 16, p-value ranges = 0.095 – 0.433; Figure 4). However, Δ Hue values from 65–125 DPH did differ significantly from zero (paired t-tests: *d.f.* = 16, p-value ranges = 0.05 to <0.001; *d.f.* = 15, p < 0.001; *d.f.* = 10, p < 0.001; *d.f.* = 3, p = 0.002). A MANOVAR showed a significant effect of time on the combined dominance mediated coloration parameters. However, no significant effect of dietary regime or the interaction time and dietary regime (time × dietary regime) was observed (Table 3). Univariate tests showed that both $\Delta\ Hue_{\alpha\text{-}avg}$ and $\Delta\ Hue_{\alpha\text{-}\beta}$ were significantly affected by time, but not by dietary regime or the interaction between time and dietary regime (Table 3).

Between 35 and 65 DPH $\Delta\ Hue_{\alpha\text{-}avg}$ values increased significantly by ~7°, and decreased gradually to mean $\Delta\ Hue_{\alpha\text{-}avg} \approx 1°$ by 65 DPH (Figure 4). The same pattern was observed for both $\Delta\ Hue_{\alpha\text{-}\beta}$ (Figure 4B) and $\Delta\ Hue_{\alpha\text{-}\gamma}$ (Figure 4C). Although values

across different Δ Hue parings were not statistically compared, Δ $Hue_{\alpha-\beta}$ values were ~2° lower than Δ $Hue_{\alpha-avg}$ and Δ $Hue_{\alpha-\gamma}$ values were ~2° higher than Δ $Hue_{\alpha-avg}$.

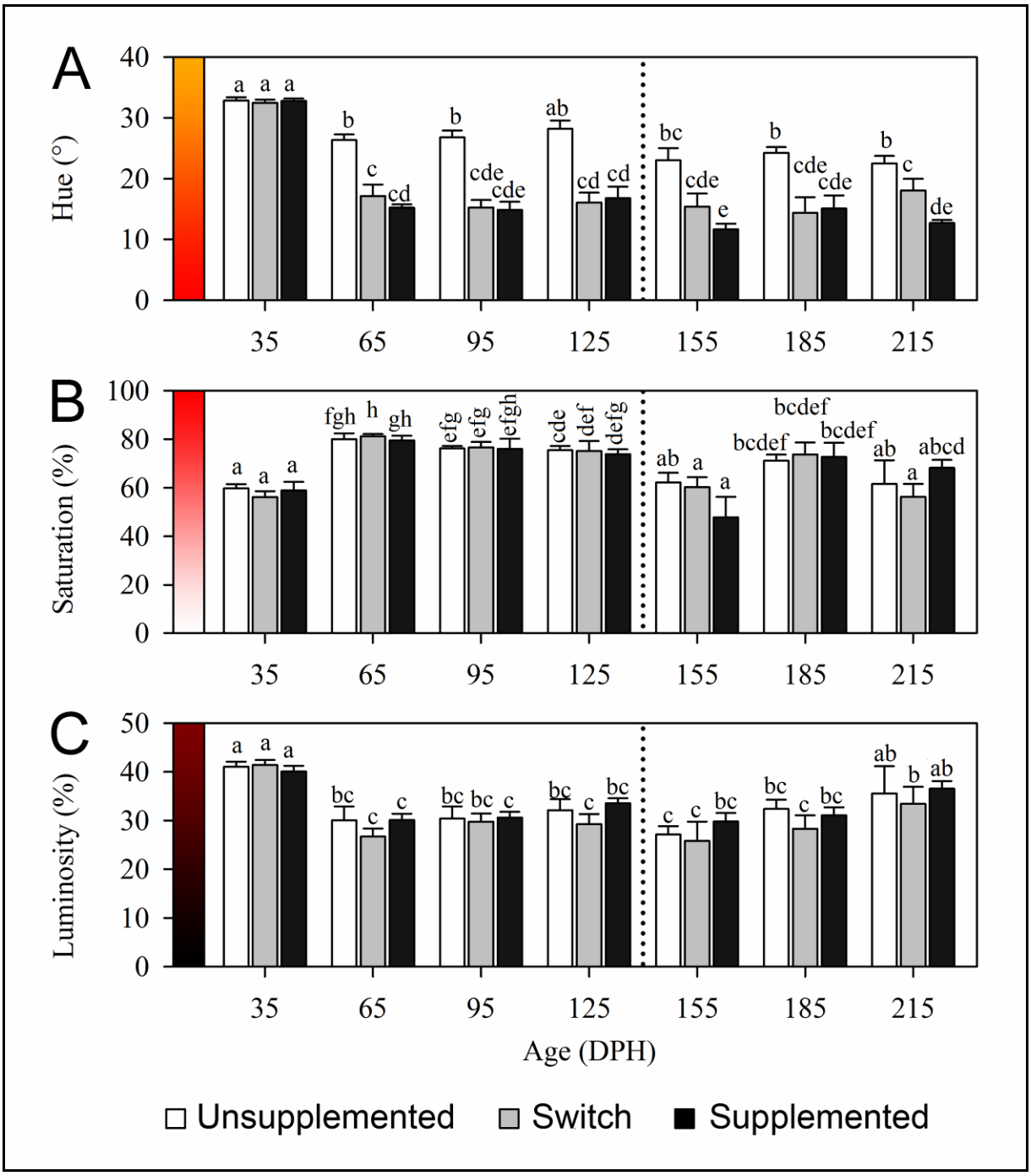

Figure 1. Median ± MAD (Median Absolute Deviation) *Amphiprion ocellaris* skin color response variables – measured as (A) hue, (B) saturation, and (C) luminosity – to dietary exposure time and dietary regime. Different lower case letters represent significantly different groups across all means of diet regime and time within each pane. DPH = days post hatch and for saturation. Representative color gradients are given on each *y*-axis, and saturation and luminosity gradients are given with a hue = 0°. Unsupplemented (19.3 mg kg^{-1} total carotenoids) and supplemented (75.5 mg kg^{-1} total carotenoids) diets served are negative and positive controls, respectively. The switch diet regime refers to switch from supplemented diet (35–125 DPH) to unsupplemented diet (125–215 DPH); timing of the switch is highlighted by the dotted line.

Table 2

Summary of MANOVAR* results of length and mass. Statistically significant P-values are highlighted in bold. Data were rank transformed

Statistical model	*Independent variable*	*Measure*	*F*	*d.f.*	*P*	*Wilk's λ*
Multivariate Test	Within-subject effects					
	Time	Combined	54.38	12, 10	**< 0.001**	0.150
	Time × Diet	Combined	0.777	24, 20	0.724	0.268
	Between-subject effects					
	Diet	Combined	1.710	4, 40	0.167	0.729
Univariate Tests	Within-subject effects					
	Time	Length	179.4	6, 126	**< 0.001**	
		Mass	34.75	6, 126	**< 0.001**	
	Time × Diet	Length	3.554	12, 126	**< 0.001**	
		Mass	2.789	12, 126	**0.002**	
	Between-subject effects					
	Diet	Length	1.748	2, 21	0.198	
		Mass	2.081	2, 21	0.150	

*Type I Sum of Squares Repeated Measures Multivariate Analysis of Variance; Time = Time exposed to diet(s), and Diet = dietary regime.

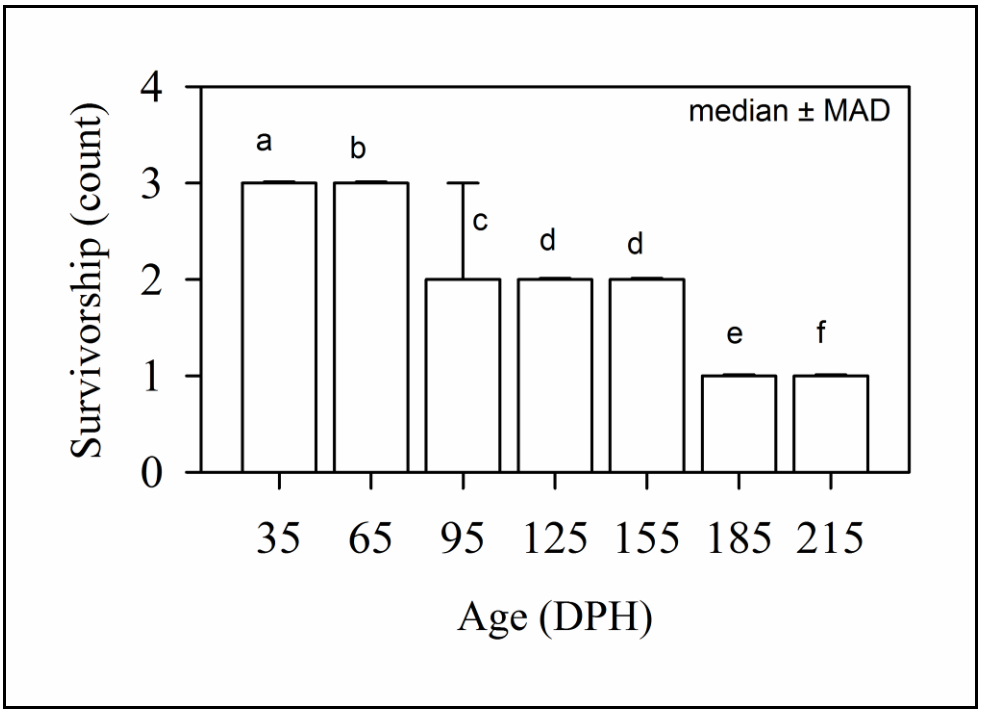

Figure 2. Median ± MAD (Median Absolute Deviation) *Amphiprion ocellaris* survivorship (count) across time. Sample sizes are n = 24 at each time period. Different lowercase letters represent significantly different groups across the within subjects factor (time) irrespective of treatment. DPH = days post hatch.

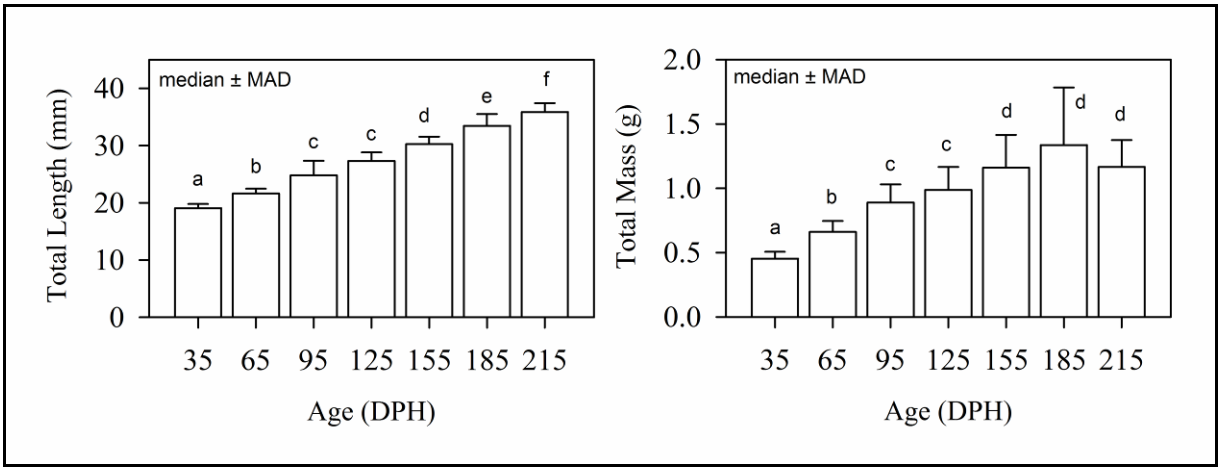

Figure 3. Median ± MAD (Median Absolute Deviation) *Amphiprion ocellaris* total length average (average of all specimens within one replicate; left pane), and Median ± MAD total fish mass (sum in individual specimens masses within each replicate; right pane) across time. Sample sizes are n = 24 at each time period. Different lowercase letters represent significantly different groups across the within subjects factor (time) irrespective of treatment. DPH = days post hatch; masses derived from mass = $a \times L^b$, where a and b terms for *Amphiprion* spp. was used (Kulbicki et al 2005).

Table 3

Summary of MANOVAR* results of dominance mediated coloration parameters. Statistically significant p-values are highlighted in bold. Results are for sphericity assumed, with the exception of Δ $Hue_{\alpha-\beta}^{\dagger}$ (denoted with †) where the Huynh-Feldt correction was applied

Statistical model	*Independent variable*	*Measure*	*F*	*d.f.*	*P*	*Wilk's λ*
Multivariate Test	Within-subject effects					
	Time	Combined	9.230	8, 7	**0.004**	0.087
	Time × Diet	Combined	1.094	16, 14	0.437	0.179
	Between-subject effects					
	Diet	Combined	1.012	4, 66	0.419	0.749
Univariate Tests	Within-subject effects					
	Time	$\Delta Hue_{\alpha-x}$	11.32	4, 56	**< 0.001**	
		$\Delta Hue_{\alpha-\beta}^{\dagger}$	58.39	4, 56.0	**0.003**	
	Time × Diet	$\Delta Hue_{\alpha-x}$	1.371	8, 56	0.229	
		$\Delta Hue_{\alpha-\beta}^{\dagger}$	0.929	8, 56.0	0.322	
	Between-subject effects					
	Diet	$\Delta Hue_{\alpha-x}$	0.258	2, 14	0.776	
		$\Delta Hue_{\alpha-\beta}^{\dagger}$	0.644	2, 14	0.540	

*Type I Sum of Squares Repeated Measures Multivariate Analysis of Variance; Time = Time exposed to diet(s), Diet = dietary regime, Δ Hueα-avg = Difference in Hue between largest specimen and average hue of remaining smaller specimens. Δ Hueα-β = difference in hue between largest specimen and the second largest specimen.

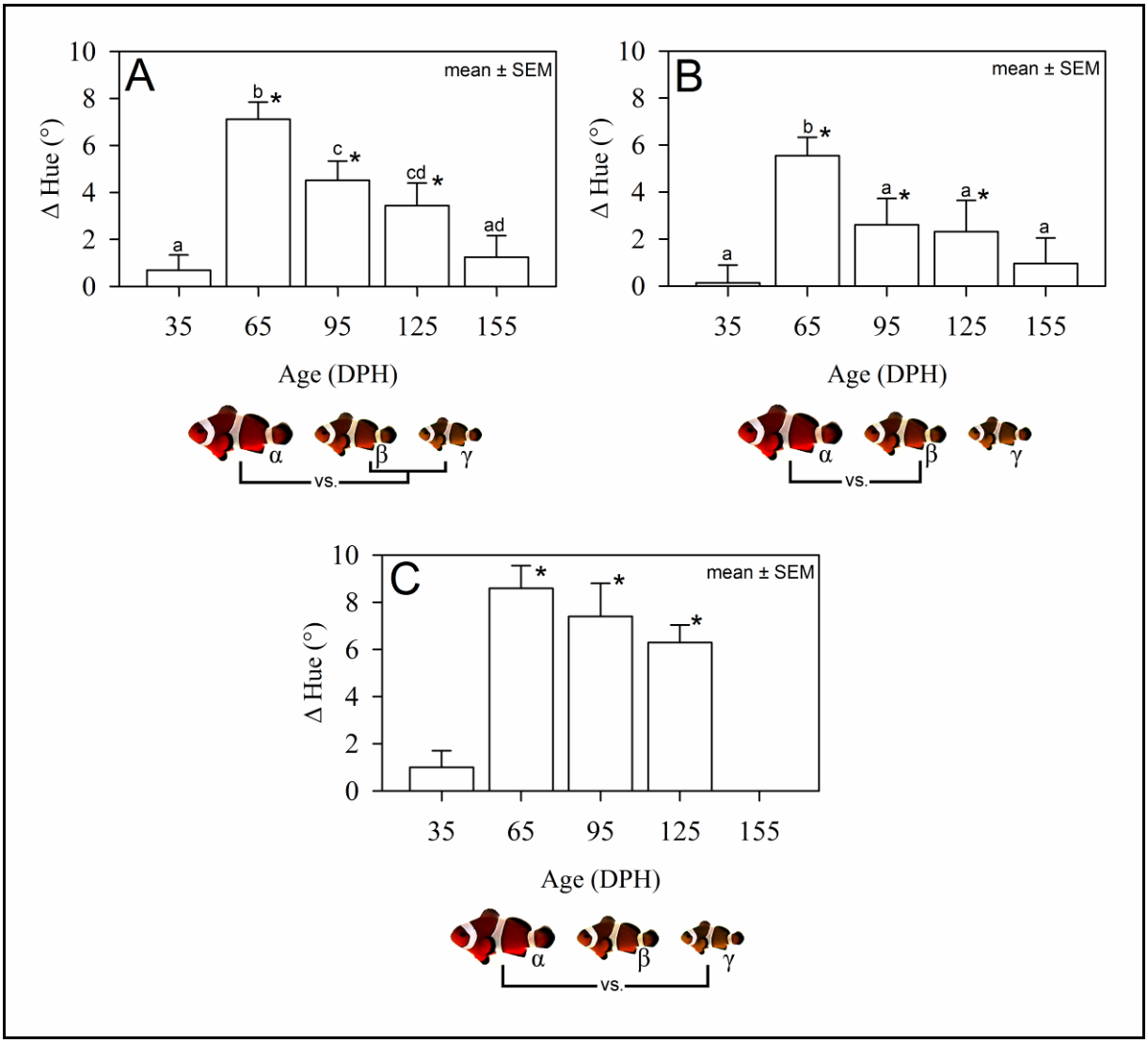

Figure 4. Difference in hue (Δ Hue) in degrees (°) between dominant (largest) *Amphiprion ocellaris* and tanks mates over successive sampling periods. A) Mean ± SEM Δ $Hue_{α\text{-}avg}$ values: between dominant (largest, α) fish and average ([β+γ]/2) hue of remaining smaller specimens. B) Mean ± SEM Δ $Hue_{α\text{-}β}$ values: between dominant (largest, α) fish and the second largest (β) specimen. C) Mean ± SEM Δ $Hue_{α\text{-}γ}$ values: between dominant (largest, α) fish and the third largest (γ) specimen. The comparisons (A-C) are highlighted by the graphic below each graph with representative colors. Sample sizes are n = 17 for A and B, while for C n = 17, n = 16, n = 11, n = 4, and n = 0 for time periods 35 through 155, respectively. Within each pane, different lowercase letters represent significantly different groups across the within subjects factor (time) irrespective of treatment (LSD post hoc) and asterisks (*) denotes significant deviation from 0 (paired directional t-test). Note that pane C lacks post hoc because data from α vs. γ comparisons were excluded from the MANOVAR due to decreasing within-subjects factor (time) sample sizes.

Discussion. To date the focus on *A. ocellaris* color research has been geared towards achieving appropriate coloration under culture conditions (Tanaka et al 1992; Hoff 1996; Yasir & Qin 2009a, b, 2010; Ho et al 2013a), and coloration post-production received very little attention. The unsupplemented and supplemented diets in this study reproduced coloration parameters to a high degree of similarity to previously conducted work following similar protocols, but outperformed the fish from previous work in terms of hue (Ho et al 2013a). Fish from this study started with higher baseline values (hue of ~33° vs. ~28°), and achieved lower hues (~17° vs. ~19°) compared to Ho et al (2013a) at equal dietary exposure time (90d, ~125 DPH) and slightly lower total dietary carotenoid concentration (75 mg kg^{-1} vs. 82 mg kg^{-1}). The parameters of saturation and luminosity followed highly similar trends to those of Ho et al (2013a) as well, with the

exception of increased variability after 120 DPH (termination of Ho et al 2013a). The extension of this experiment to 180 days - in contrast to 90 days (Ho et al 2013a) and 35 days (Yasir & Qin 2010) - shows that under a proper pigment supplementation regime the major coloration parameter of hue stabilizes after 60 days and that saturation and luminosity parameters are more variable and appear to contribute less to the overall appearance. Although, this study achieved superior coloration with lower dietary carotenoid concentration than Ho et al (2013a), the concentration recommended therein (80–160 mg kg^{-1} esterified astaxanthin in wet diet) still holds in order to account for the potential variably in pigment assimilation and expression across different stocks of fish.

The lack of diet effect on saturation and luminosity, in addition to the lack of changes in median values of these parameters after the diet transition in the switch treatment suggests that the hue parameter by itself is sufficient to evaluate dietary regime effects on coloration. Hue in the experimental switch diet regime mirrored the positive control diet very closely up to 215 DPH. The fish were capable of retaining their high carotenoid diet coloration for 60 days. Even after 90 days, coloration change - although statistically significant - was not drastic. This transitionary hue value (~18°) at 215 DPH can also be achieved with a lower dietary astaxanthin concentration (Ho et al 2013a), and is equivalent to wild type coloration on a qualitative scale (Ho et al 2013a). Thus, after 90 days of substantial dietary carotenoid deprivation, these fish would still appear attractive to the consumer and still be suitable for market. At least on the short term, while the specimens may be at retail outlets, optimum hue can be readily sustained. However, the long term ability - many months to years when a specimen is taken to a consumer aquarium - to maintain coloration after dietary carotenoid deprivation is difficult to predict. However, supplemental feeding at the retail outlet or the consumer's aquarium with diets containing esterified astaxanthin or other carotenoids in concentration lower than the commercial recommendation of 80–160 mg kg^{-1} may prolong or curb the loss of hue and maintain a coloration that would be pleasing to the consumer over a much longer period.

The consistent decline in survivorship over time is not surprising considering the sequential protandric hermaphroditism and social structure of *Amphiprion* spp. (Moyer & Nakazono 1978; Iwata et al 2008; Iwata & Manbo 2013). The consistent aggression from the dominant (putative) female and successive pecking order of the (putative) male and the ambisexual individual leads to a functional outcast of the smallest ambisexual in terms of a social structure of three individuals when bound by limited space (Iwata & Manbo 2013). The smallest (least dominant) individual bears the brunt of the aggression and exhibited the slowest growth rates (data for this study not shown, Iwata et al 2008), and it is rational that the smallest individual was the first to succumb (Figure 2). Aside from the mediating growth (Iwata et al 2008), the social hierarchy is reflected in the individuals' coloration. The Δ hue values varied over time with no effect of diet regime, illustrating that the difference in hue is equivalent across diets (unsupplemented or supplemented with carotenoids) and suggests that the difference is a result of a proportional lack of dietary carotenoids across the hierarchy. Although ample food was provided (in slight excess), the dominance aggression likely resulted in lack of feeding due to *e.g.*, stress. Thus observed hierarchy induced differential growth rates (Iwata et al 2008) and is likely the product of restriction of food ingestion, which is consistent with other species (cichlids) exhibiting size based dominance hierarchies (Koebele 1985). Furthermore, if the hierarchical status dictates food consumption, it is rational that hue (Δ Hue values) would sort out according to the hierarchy. Ho et al (2013b) suggested that skin coloration might be an avenue for sexual selection in amphiprioninids. Given that hue is heavily influenced by total carotenoid concentration in the skin (Ho et al 2013a), that coloration is further mediated by pigment granule size (Ho et al 2013b), that growth rate is influenced by social structure (Iwata et al 2008), and with the findings from this study where hue is influenced by social structure (via food access) it appears more likely that color based sexual selection may be present in amphiprioninids, as it is in other vertebrates (Kodric-Brown 1985; Maan et al 2006) and merits further investigation. Furthermore, the social structure mediated growth and color differences provide a stimulating avenue for further research of the role of the orange coloration on

amphiprioninid social structure and biology regarding colony formations around anemones, mate quality, access to resources, carrying capacity of an anemone, and the capacity of submissive individuals to escape or tolerate aggression.

The social structure mediated coloration may appear to be problematic in terms of color development for commercial production, but when amphiprioninids are housed in high density the social structure observed in trios or small groups disintegrates (personal observation, personal communication, Proaquatix, Inc.). Specimens that do grow faster may still exhibit aggression towards others, but this aggression is diffused among many specimens during grow-out. Furthermore, the difference in hue grew smaller with time and is likely product of increased size of the β individual. In the home aquaria anemonefishes are rarely kept in large groups, but are housed singly or in pairs. Although these specimens are usually acquired at a very young age (months), the likelihood of marked color difference between two individuals is thus likely to be minor.

Conclusions. The goal of this study was to investigate the influence of dietary carotenoid deprivation on coloration and socially mediated coloration in *A. ocellaris*. Because coloration is essential to the marketability of ornamental fishes and customer satisfaction, it is crucial to understand the ability of fish to retain the coloration that was achieved under culture. The deprivation of dietary carotenoids significantly influenced the coloration of the specimens. However, the change was not drastic, and occurred months after a fish would have been sold. Furthermore, appropriate home diets may further curb the loss of coloration. At younger ages, hue was highly subject to social structure, but became less so with fish age. Additionally, commercial culture conditions and hobbyist consumer aquarium conditions are conducive to minimizing the effects of the social structure on hue.

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