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RESEARCH ARTICLE

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Astaxanthin deposition in the cuticle of juvenile American lobster (*Homarus americanus*): implications for phenotypic and genotypic coloration

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Abstract The phenotypic brownish-green color of an American lobster (*Homarus americanus* Milne-Edwards, 1837) is determined by genetic and dietary mechanisms. Diet influences color through the carotenoid astaxanthin. This pigment can appear red or blue depending if it is bound to protein, and the relative amounts of each form influence lobster color. Carotenoprotein formation was examined in sibling American lobsters rendered white by a diet low in astaxanthin, by feeding a diet containing 0, 55, 110, or 220 μg astaxanthin g^{-1} for 110 days beginning on 7 February 2004. The relative levels of red and blue in these lobsters were assessed through the analysis of digital photographs of the lobsters, which were taken monthly. The red/blue ratio was used to assess if free (red) or protein-bound astaxanthin (blue) was the dominant form in the lobsters, or if the two forms occurred in ratios allowing for natural coloration. Naturally colored lobsters developed only in the highest astaxanthin diet group, while lobsters fed the 55-μg diet were blue. Differences were observed among different parts of the body, and were presumed to be a function of cuticle thickness. However, within each diet treatment, some lobsters initially became red, suggesting an accumulation of free astaxanthin, while other lobsters initially became blue, suggesting an accumulation of protein-bound astaxanthin. This variation may be a result of differences in the uptake of astaxanthin, the rate of carotenoid-protein complexing, or in the total amount of protein available for binding. This variability is likely to be the key to understanding the underlying basis of genetically determined color in American lobsters.

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Introduction

The color of American lobsters (*Homarus americanus*) is typically a muddy-brownish-green color. Color variants are rare in the wild and include, in order of decreasing frequency, blue, orange yellow, calico, and even white. The color of a lobster can be independent of or dependent on diet. Dietary-independent color is often referred to as being genetic. While no mechanisms of inheritance have been determined, blue coloration was assumed to be a recessive trait, as blue offspring occur only through the mating of two blue adults (Beal et al. 1998).

Dietary-dependent lobster color is a function of the carotenoid pigment astaxanthin. This pigment occurs in a free, esterified state, or in one of three carotenoid-protein complexes (α- and β-crustacyanin or crustochrin). Astaxanthin is typically red, but undergoes a bathochromic or hypsochromic shift, respectively, when forming the crustacyanins or crustochrin, yielding a purple color in β-crustacyanin, blue in α-crustacyanin, and yellow in crustochrin (Cianci et al. 2002). Whereas the free astaxanthin occurs in the epidermal layer below the cuticle, α- and β-crustacyanin occur in the cuticle (α-crustacyanin being the dominant form), while crustochrin occurs in the epicuticular layer (Buchwald and Jencks 1968; Salares et al. 1977; Mackenthun et al. 1979; Young and Williams 1983; Cianci et al. 2002). Thus, the phenotypic color of an American lobster is a result of differential light scattering throughout these layers of astaxanthin and astaxanthin complexes.

Dietary studies of American lobsters have determined that insufficient astaxanthin causes a lobster to become blue (D'Agostino 1980; Lim et al. 1997). More specifically, 0.1 mg of carotenoid derived from crustacean shell per 100 g of feed was necessary for lobsters to retain their "wild" color (D'Abramo et al. 1983), and purified carotenoids resulted in less deposition of the carotenoid in the lobster's shell than an equal amount of "natural" carotenoids (D'Abramo et al. 1983). Incorporating an understanding of the forms and locations of the different

colors into the observation that total body astaxanthin was correlated with dietary levels of astaxanthin (D'Abramo et al. 1983), it can be postulated that lobsters fed diets lacking astaxanthin became blue, because the process of binding astaxanthin to the proteins continued, thus increasing the ratio of protein-bound to free astaxanthin.

Thus, color in American lobsters can be conceptualized as a ratio of free esterified astaxanthin to that bound by protein. Lobsters with an abundance of carotenoproteins will appear blue, while those deficient in the carotenoproteins, but with a relatively high total body astaxanthin content, will appear red. Wild coloration is a balance of free and protein-bound astaxanthin. This red/blue ratio method was used to assess how American lobsters made white by an astaxanthin-deficient diet added color as they amassed pigment when fed *Artemia* sp. (Tlusty 2005). However, in *Artemia* sp., canthaxanthin, also a red hue, is the dominant carotenoid (D'Abramo et al. 1983). Thus, lobsters had to first convert the canthaxanthin to astaxanthin prior to assimilation in the cuticle. This process increased the length of time free carotenoids were present in the lobsters, and caused them to initially appear red.

To assess carotenoprotein formation in American lobsters independent of the canthaxanthin/astaxanthin conversion, purified astaxanthin was fed to juvenile lobsters at one of four concentrations, ranging from 0 to 220 μg g^{-1} diet. Digital macrophotography was used to repeatedly sample the color of individual lobsters over a period of 110 days. If the uptake of ingested astaxanthin was lower than the rate of carotenoprotein formation, then lobsters should have initially become blue (no accumulation of free astaxanthin). If uptake of ingested astaxanthin was greater than the rate of carotenoprotein formation, then lobsters should have initially become red (an accumulation of free astaxanthin). A wild coloration will occur only when astaxanthin exists in both free and protein-bound states.

Materials and methods

Twenty-four sibling American lobsters (*Homarus americanus* Milne-Edwards, 1837) used in this study were reared at the New England Aquarium, Boston, Mass., USA. These lobsters were hatched from a wild-colored, 81-mm carapace length female that was caught in October 2002 on the southern coast of Massachusetts. While multiple paternity is possible in American lobsters (Nelson and Hedgecock 1977), the parentage within this sibling group was not investigated, and it is unknown if these lobsters were full or half siblings. Upon arrival at the aquarium, the female was held at 19–23°C for 2 months, then transferred to 18–19°C for 1 month until the embryos hatched and the larvae were released by the female. The larvae were fed live *Artemia* sp. nauplii and frozen adult omega-3-enriched *Artemia* sp. until stage IV at which time they were fed Economac 4 (Aquafauna Bio-Marine, Hawthorn, Calif., proximate analysis available at http://www.aquafauna.com/Profiles-Econo-Mac.htm), a commercially formulated crustacean feed, for 13 months until the initiation of this experiment. This diet was initially fed in its extruded form, but, at 6 months, was bound in gelatin and fed in cubed form. The Economac 4 diet was low in astaxanthin, and lobsters fed this diet were phenotypically white (Tlusty 2005). Beginning at stage IV and throughout the experiment, all lobsters were housed individually in plastic mesh containers, 70.9 cm^2 in surface area and 9 cm deep.

On 7 February 2004, the abdominal and tail segments of these lobsters were photographed, and they were randomly divided into four groups. Each group was assigned a diet with 0, 55, 110, or 220 μg astaxanthin g^{-1} as feed. The 110 μg g^{-1} diet was made by mixing 0.053 g dry powdered astaxanthin (Naturose, Cyanotech, Kailua-Kona, Hawaii) with 7.150 g Economac 4, and binding it with 9 ml gelatin (7 g gelatin dissolved in 90 ml deionized water). The astaxanthin was doubled or halved, with the total dry ingredients remaining at 7.203 g to make the 220 and 55 μg g^{-1} diets, respectively. The lobsters were fed this diet daily for the first 7 days, then every other day until day 30, and then daily again until the termination of this experiment at 110 days. Photographs of the lobsters were taken on days 42, 72, and 110. For ease of discussion, the sampling days were referred to as initial (day 0) and months 1 (42 days), 2 (72 days), or 3 (110 days).

Photograph analysis

Photographs were taken of each lobster's tail, with the macro function of a Nikon Coolpix 5000 digital camera, with spot lighting provided by a Nikon SL1 Macro Cool Light. Under these conditions, the focal length was 2 cm, shutter speeds were 1/15–1/30, and the aperture was F2.6. There was a reference white square in each photograph that was used to adjust for variation in lighting. After the picture of the tail was acquired, the endopodite (END), exopodite (EXO), telson (TEL), and the last-abdominal-segment-medial (LASM) were analyzed for corresponding hues using Photoshop 6.0. Regions of interest (ROI) for each body location were first selected. Pictures were opened in RGB color mode, and the average value for %red and %blue recorded from the histogram plot of the ROI. The picture was then translated into a Commission Internationale de l'Éclairage (CIE) L*a*b color mode, and the CIE-L average value and standard deviation were recorded again utilizing the histogram function. Histograms of the reference square were also scored, and, within each picture, each color parameter was corrected for deviation from pure white. Color hue scores ranged from 0 to 100, with pure white in the RGB scheme being (100, 100, 100) for %red, %green, and %blue, respectively. Black was a score of (0, 0, 0), while a pure color (red, green,

blue) was 100 for that particular hue and 0 for the other two. Lightness (CIE-L) ranged from 0 (black) to 100 (white). Previous work (Tlusty 2005) found the EXO, END, TEL, and LASM of adult, wild-colored American lobsters to have %red scores of 36.3, 33.5, 15.0, and 11.7, respectively. Scores for %blue (13.4, 11.6, 8.7, and 8.2) and CIE-L (31.2, 27.3, 13.7, and 10.3) followed the same trend.

Statistical analyses

The premise of pigment addition in American lobsters was that overall color could be parsimoniously identified by the %red/%blue ratio (R/B) of the sample. Therefore, the R/B ratio was calculated for each sample, and dietary and body location differences were analyzed for statistically significant differences using a three-way repeated-measures ANOVA. However, significant three-way interactions precluded interpretation of a full-effects model, and simple effects were assessed. Thus, two-way repeated-measures ANOVAs with one-factor (body location) repetition were utilized separately for images captured in months 1, 2, and 3 of the experiment. If the parametric data failed either a normality or equal variance test, the data were ranked and then similarly analyzed. Power of the test was calculated with $\alpha = 0.05$, and is reported only when < 0.80. Statistical differences between all paired comparisons were examined with the Holm–Sidak method (SigmaStat 3.0).

To discretely classify color, photographs of lobsters were assessed if the LASM location was colorless or white, predominantly blue, predominantly red, purple (equal blue and red), or a natural wild color. This method did not distinguish between color intensity for red and blue lobsters. Thus, lobsters determined to be blue or red could range between a pale faded hue and a very bright intense hue of each respective color. While it was possible that purple may have been a less intense wild hue, it was recorded as a separate category. The discrete color scores of photographs of lobsters at 2 months were compared to their R/B ratios, as at this point, all of the diet groups containing astaxanthin had lobsters in at least three discrete color categories. These data were analyzed for significance using a one-way ANOVA, with multiple comparisons examined via the Holm–Sidak method.

The endpoint for color addition in American lobsters was a wild color. In the R–B color scheme, lobsters moved from a whitish coloration within the scale of 100 being pure white to lower values as the lobsters became wild colored. Thus, the change in color as a white lobster added pigment to its carapace could be envisioned as the two-dimensional distance between a lobster's initial and sampled coloration. Samples at discrete time intervals could then be assessed for how close they were to attaining the final (wild) coloration. In a two-dimensional space (e.g. red–blue), the percent final color (%FC) was calculated as the distance a sample point was from the initial point as a percent of the total distance between initial and adult coloration:

$$\%\mathrm{FC} = \left(\sqrt{(R_i - R_s)^2 + (B_i - B_s)^2} \Big/ \sqrt{(R_f - R_i)^2 + (B_f - B_i)^2} \right) * 100 \qquad (1)$$

where R and B indicated %red and %blue color scores, respectively, i represented the initial color score, s was the sample color score, and f was the color score of an adult wild color lobster. %FC could be expanded to three dimensions (e.g. red–green–blue) by adding a third color term in both the numerator and the denominator. However, in this study, adding a third color term did not significantly change the patterns of statistical significance from those determined with a two-dimensional (red–blue) analysis. Hence, the data for the three-dimensional distance measures are not presented here. The %FC score will range from 0 (same color as the initial sample) to 100% (adult coloration). The %FC values were analyzed using a two-way repeated-measures ANOVA with one-factor (body location) repetition as described previously. Finally, the patchiness of dark coloration was measured by comparing the coefficient of variation (CV) of CIE-L of the LASM. Statistical significance across diet treatment and months was examined using a two-way repeated-measures ANOVA with one-factor (month) repetition, and multiple comparisons were examined via the Holm–Sidak method. Lobsters that did not survive for the duration of the experiment were excluded from this final analysis.

Results

Red/Blue ratio

The *Homarus americanus* survived well on these prepared diets, and, during the course of this experiment, only one lobster fed the 0 μg g^{-1} diet died on day 83.

For all three sampling dates after white American lobsters were switched to diets containing astaxanthin, significant two-way interactions in the R/B ratio existed between diet treatment and body locations (two-way repeated-measures ANOVA on ranked data, month 1, $F_{9,60} = 6.30$, $P < 0.001$; month 2, $F_{9,60} = 8.41$, $P < 0.001$; month 3, $F_{9,57} = 6.59$, $P < 0.001$). An analysis of simple effects demonstrated that in the first month's sample, there were no significant diet differences in the R/B ratio within each body location (Holm–Sidak multiple comparison method, $t < 1.56$, $P > 0.05$ for all comparisons, Table 1). However, within each diet, the R/B ratio of the exopodite was significantly larger than that of the telson (Holm–Sidak multiple comparison method, $t > 2.77$,

$P < 0.05$ for all comparisons) and was equivalent to that of the endopodite (Holm–Sidak multiple comparison method, $t < 1.23$, $P > 0.05$ for all comparisons in diets containing astaxanthin). After the second month, significant diet treatment differences existed within body locations, with the exception of the LASM in which all diet treatments had an equivalent R/B ratio (Holm–Sidak multiple comparison method, $t < 2.75$, $P > 0.05$ for all comparisons, Table 1). In the three other body locations, the 220 and 110 μg diets were always in a group having the largest R/B ratio, whereas the 0 and 55 μg g^{-1} diets were always in a group with the smallest R/B ratio. Within each diet treatment, the R/B ratio of the exopodite was always significantly greater than that of the LASM (Holm–Sidak multiple comparison method, $t > 3.68$, $P < 0.05$ for all comparisons), while the exopodite and the endopodite were statistically similar to each other (Holm–Sidak multiple comparison method, $t < 1.81$, $P > 0.05$ for all comparisons). Colors in the third month followed the trends of the second month. Within body locations, the lobsters fed the 220 μg g^{-1} diet always had the highest R/B ratio (endopodite and telson, Holm–Sidak multiple comparison method, $t > 2.80$, $P < 0.05$ for all comparisons) or were within the group of treatments (exopodite and LASM) that had the largest R/B ratios. The 55 μg g^{-1} diet group was ranked in the group with the lowest R/B ratio for all four body locations. The analysis of simple effects demonstrated that, within all diets, there were no statistical differences between the exopodite and the endopodite (Holm–Sidak multiple comparison method, $t < 0.82$, $P > 0.05$ for all comparisons) or between the telson and the LASM (Holm–Sidak multiple comparison method, $t > 2.12$, $P > 0.05$ for all comparisons, Table 1).

The discrete coloration of lobsters changed throughout the course of the experiment (Fig. 1). Wild-colored lobsters appeared in the first month in the 110 and 220 μg g^{-1} diet treatments. At 2 months, there was one wild-colored lobster in the 55 μg diet treatment, but this lobster did not maintain its coloration, and faded to purple by the third month. In the third month, only the 220 μg g^{-1} diet treatment had wild-colored lobsters. Red was the least common color category, with six, three, and two lobsters, respectively, per month in this category. The second month's sample was unique in that within each of the diet treatments containing astaxanthin, the lobsters were categorized into at least three discrete color categories. In this month, the R/B ratios of the LASM for the five discrete colors were significantly different (one-way ANOVA, $F_{4,19} = 8.71$, $P < 0.001$). Lobsters determined to have a blue color had the lowest R/B ratio, and the only group with an average $R/B < 1.0$ ($0.99 + 0.11$, mean + 1 SD). The value for a discrete blue color was significantly lower than for all colors (Holm–Sidak multiple comparison method, $t > 4.02$, $P < 0.05$), except purple ($t = 2.57$, $P > 0.05$). The values for all other colors were not significantly different from one another (Holm–Sidak multiple comparison method, $t < 2.57$, $P > 0.05$).

The color of the LASM generally matched that of the END and EXO. In the three sample months, 12, 13, and 10 of the 18 lobsters fed diets containing astaxanthin had END and EXO colors that were similar to that of the LASM. When the colors were different, END and EXO were red, while the LASM was blue, purple, or wild colored. The only time END and EXO were blue was when the LASM was also blue.

Red–Blue distance

The %FC in a two-dimensional red–blue space did not demonstrate any significant two-way diet treatment–body location interactions within each measurement period (two-way repeated-measures ANOVA on ranked data, month 1, $F_{9,60} = 0.64$, $P > 0.75$; month 2,

Table 1 *Homarus americanus*. Significance of simple-effects analysis of red/blue ratios for four body locations of American lobsters fed diets differing in the amount (μg g^{-1}) of astaxanthin. Patterns of significance for diet treatments (*rows*) indicated by *letters* and for body locations (*columns*) by *numbers*, with identical notation indicating statistical similarity (two-way repeated-measures ANOVA on ranked data with Holm–Sidak multiple comparisons with $P < 0.05$). In the first month, there were no significant differences between diet treatments within body locations, thus letters denoting statistical similarity are omitted

Diet	Exopodite	Endopodite	LASM	Telson
Month 1				
0	$1.12 + 0.06^{a}$	$1.09 + 0.05^{a}$	$1.26 + 0.08^{b}$	$1.17 + 0.05^{b}$
55	$1.16 + 0.15^{a}$	$1.13 + 0.16^{a,b}$	$1.14 + 0.19^{a,b}$	$1.07 + 0.12^{b}$
110	$1.43 + 0.38^{a}$	$1.38 + 0.35^{a}$	$1.20 + 0.26^{b}$	$1.17 + 0.21^{b}$
220	$1.51 + 0.35^{a}$	$1.49 + 0.37^{a,b}$	$1.31 + 0.36^{b}$	$1.27 + 0.24^{b}$
Month 2				
0	$1.16 + 0.07^{a}{}_{3}$	$1.15 + 0.07^{a}{}_{3}$	$1.30 + 0.05^{a,b}{}_{1}$	$1.16 + 0.05^{b}{}_{1,2}$
55	$1.32 + 0.20^{a}{}_{2,3}$	$1.22 + 0.13^{a,b}{}_{2,3}$	$1.13 + 0.09b,^{c}{}_{1}$	$1.06 + 0.07^{bc}{}_{2}$
110	$1.47 + 0.18^{a}{}_{1,2}$	$1.38 + 0.14^{a}{}_{1,2}$	$1.21 + 0.07^{b}{}_{1}$	$1.18 + 0.09^{b}{}_{1,2}$
220	$1.83 + 0.33^{a}{}_{1}$	$1.80 + 0.32^{a}{}_{1}$	$1.27 + 0.22^{b}{}_{1}$	$1.34 + 0.19^{b}{}_{1}$
Month 3				
0	$0.90 + 0.01^{a,b}{}_{3}$	$0.89 + 0.01^{a}{}_{3}$	$0.93 + 0.06^{a,b}{}_{2}$	$1.03 + 0.02^{b}{}_{1,2}$
55	$1.15 + 0.26^{a}{}_{2,3}$	$1.12 + 0.25^{a}{}_{3}$	$0.85 + 0.21^{b}{}_{2}$	$0.94 + 0.19^{b}{}_{2}$
110	$1.51 + 0.29^{a}{}_{1,2}$	$1.58 + 0.40^{a}{}_{2}$	$1.25 + 0.38^{b}{}_{2}$	$1.21 + 0.29^{b}{}_{1}$
220	$2.39 + 0.55^{a}{}_{1}$	$2.77 + 0.44^{a}{}_{1}$	$1.69 + 0.36^{b}{}_{1}$	$1.63 + 0.24^{b}{}_{1}$

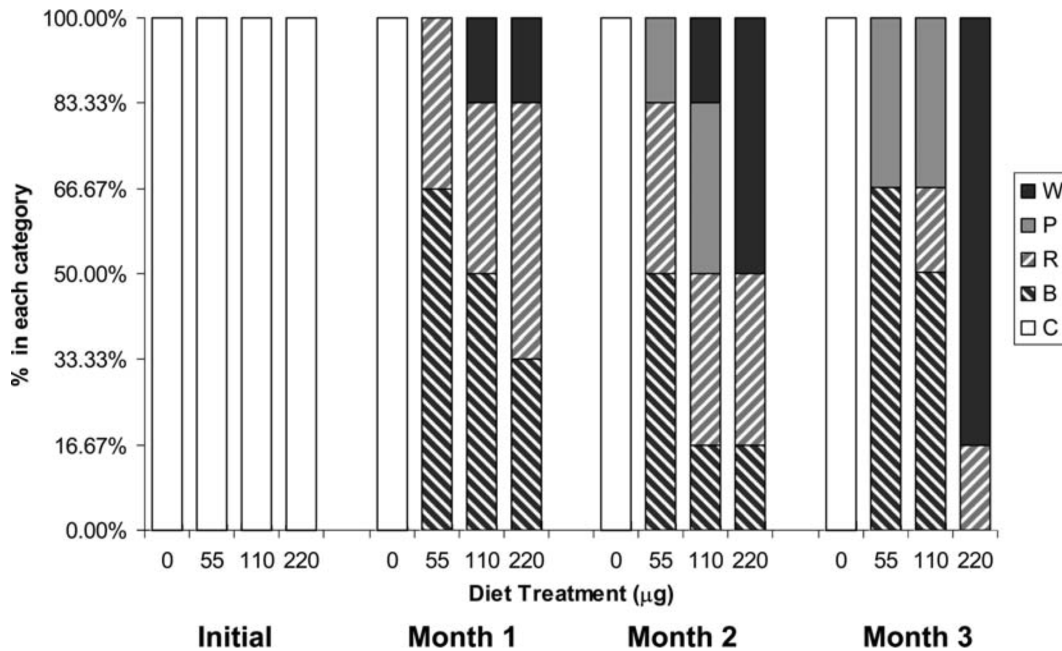

Fig. 1 *Homarus americanus*. Discrete colors of last abdominal segment medial of American lobsters fed a diet of 0, 55, 110, or 220 µg astaxanthin g^{-1} for month 1 (42 days), month 2 (72 days), and month 3 (110 days). Colors were classified as predominantly wild (*W*), purple (*P*), red (*R*), blue (*B*), or white (*C*). Color scheme assessed color relative to other samples within a month, and did not account for color intensity

$F_{9,60} = 0.68$, $P > 0.70$; month 3, $F_{9,57} = 0.93$, $P > 0.50$). Thus, main treatment effects were subsequently analyzed.

After 1 month of being fed a diet containing astaxanthin, the %FC in a two-dimensional red–blue space was significantly different for both diet treatment and body location (two-way repeated-measures ANOVA on ranked data, $F_{3,20} = 3.33$, $P < 0.05$, power = 0.495 and $F_{3,60} = 14.08$, $P < 0.001$, respectively, Table 1). Because of the low power associated with the diet treatment, there were no significant differences between paired treatment comparisons. Comparing body locations, EXO (35.1 ± 5.7, mean ± 95% CI) and END (35.3 ± 5.0) were statistically similar to one another (Holm–Sidak multiple comparison method, $t = 0.14$, $P > 0.05$), and greater than LASM (26.2 ± 5.3) and TEL (22.1 ± 4.6), which were statistically similar to one another (Holm–Sidak multiple comparison method, $t = 0.12$, $P > 0.05$). Two months into the study, lobsters fed the 220 µg g^{-1} diet had a significantly larger %FC than the other three diets (two-way repeated-measures ANOVA on ranked data, $F_{3,20} = 3.33$, $P < 0.05$, power = 0.99, Holm–Sidak multiple comparison method, $t > 3.29$, $P < 0.05$, Table 1; Fig. 2). The body locations (two-way repeated-measures ANOVA on ranked data,

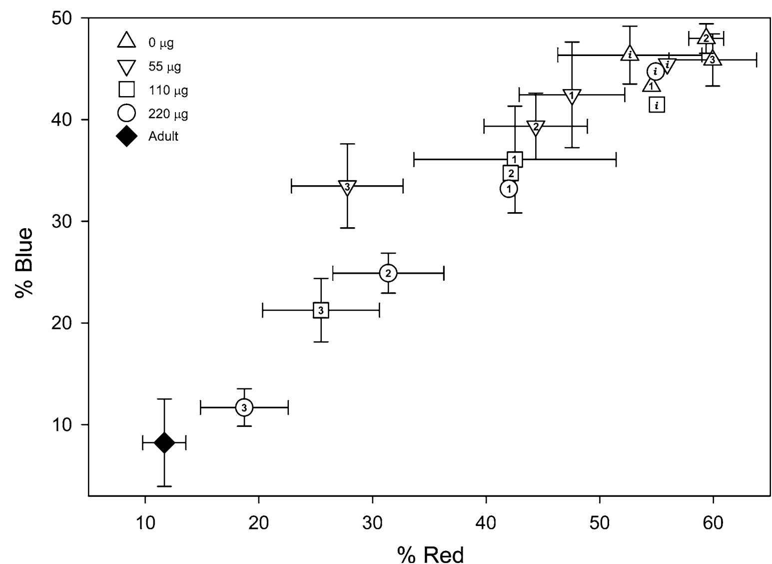

Fig. 2 *Homarus americanus*. Plot of %red compared to %blue from RGB analysis of last abdominal segment medial for American lobsters fed 0, 55, 110, or 220 µg astaxanthin g^{-1} diet. *Numbers within symbols* refer to monthly sample periods (*i* initial measurement). *Error bars*: 95% confidence intervals; some intervals omitted to improve clarity of presentation

$F_{3,60} = 9.16$, $P < 0.001$, power = 0.99, Table 1) still demonstrated a similar pattern of significance as in the first month, with the additional observation that END and LASM were statistically equivalent (Holm–Sidak multiple comparison method, $t = 1.17$, $P > 0.05$). At the final measurement, all four diet treatments were significantly different from one another (Fig. 2, Holm–Sidak multiple comparison method, $t > 3.75$, $P < 0.05$), with the 220 μg g^{-1} diet being 85.6 ± 5.2% of the final color of adults. The 110 and 55 μg g^{-1} diets were 63.5 ± 4.6% and 52.0 ± 3.6%, respectively, while the 0 μg g^{-1} diet was only 13.5 ± 4.3% of the final color of adults. At this time, END, LASM, and EXO were all statistically equivalent (two-way repeated-measures ANOVA on ranked data, $F_{3,57} = 5.86$, $P < 0.001$, power = 0.89, Holm–Sidak multiple comparison method, $t < 1.08$, $P > 0.05$), and were greater than TEL (Holm–Sidak multiple comparison method, $t > 2.61$, $P < 0.05$, Table 1).

Coefficient of variation of CIE-L

One final color change that was noted was the appearance of spots on the abdominal segments of the lobsters. An analysis of the coefficient of variation of CIE-L in the LASM across diet treatments and months indicated a significant interaction term (two-way repeated-measures ANOVA on ranked data, $F_{9,56} = 10.11$, $P < 0.001$, power = 1.00). Initially, there were no differences among diet treatments (Holm–Sidak multiple comparison method, $t < 0.85$, $P > 0.05$). However, after the first month of being fed the experimental diets, the 220 and 110 μg g^{-1} diets were statistically similar (Holm–Sidak multiple comparison method, $t = 0.44$, $P > 0.05$) and significantly larger (Holm–Sidak multiple comparison method, $t > 4.26$, $P < 0.05$) than the 55 and 0 μg g^{-1} diets, which were statistically equivalent (Holm–Sidak multiple comparison method, $t = 2.22$, $P > 0.05$). By month 2, the coefficients of variation of the CIE-Ls of all lobsters fed diets containing astaxanthin were significantly greater than those of lobsters fed 0 μg of astaxanthin (Holm–Sidak multiple comparison method, $t > 5.53$, $P < 0.05$). The coefficients of variation of the CIE-Ls of lobsters fed the 220 μg g^{-1} diet were also significantly larger than those fed the diet with 55 μg g^{-1} of astaxanthin (Holm–Sidak multiple comparison method, $t = 2.97$, $P < 0.05$). By the termination of the experiment, lobsters fed 220 and 110 μg g^{-1} astaxanthin in their diet exhibited similar coefficients of variation for the CIE-L parameter (Holm–Sidak multiple comparison method, $t = 0.17$, $P > 0.05$), and both were significantly larger than the coefficients of variation of the CIE-Ls of lobsters fed the other two diets (Holm–Sidak multiple comparison method, $t > 2.92$, $P < 0.05$). Lobsters fed the 220 μg g^{-1} diet had a coefficient of variation of 23.8 ± 4.2%, whereas those fed the 0 μg g^{-1} diet had a CIE-L coefficient of variation of 2.2 ± 0.6%.

Discussion

Homarus americanus rendered white by a diet low in astaxanthin, then fed a diet containing 220 μg astaxanthin g^{-1}, added color before those in the other diet treatments and were more likely to initially become red. These lobsters had a final color more similar to adults then those fed diets containing less astaxanthin. Lobsters on high-astaxanthin diets also exhibited the largest coefficient of variation in CIE-L on their last abdominal segment, indicating uneven coloring over the ROI. This uneven coloring was a result of pronounced, dark spots compared with a more uniform coloring of lobsters fed the other diets. In contrast, lobsters fed the 55 μg g^{-1} diet were more likely to be blue within the first month of this experiment. Although the exact location and form of astaxanthin was not measured within this study, from previous work on astaxanthin deposition in *Homarus* sp. (Buchwald and Jencks 1968; Salares et al. 1977; Mackenthun et al. 1979; D'Abramo et al. 1983, Young and Williams 1983; Cianci et al. 2002), the following mechanism can be inferred. The shift to red in lobsters fed 220 μg g^{-1} astaxanthin is indicative of free astaxanthin accumulating faster than the carotenoid could be transported and bound to protein in the cuticle. In contrast, the blue color when lobsters were fed a low level of astaxanthin (D'Agostino 1980; D'Abramo et al. 1983; Lim et al. 1997) indicated that the carotenoid did not accumulate as free astaxanthin in the epidermal layer, but resided in the cuticle in a protein-bound form causing the bathochromic shift to blue. Furthermore, the appearance of dark spots on the last abdominal segment indicated that the bathochromatically shifted astaxanthin-protein complexes were not evenly deposited in the cuticle, but instead were initially deposited in discrete areas.

This diet study determined that different body locations of juvenile American lobsters changed color at different rates. In the second and third months, both the R/B ratio data and the discrete color data indicated that the uropods (the endo- and exopodite) were redder than the last abdominal segment. The combination of a red last abdominal segment and blue uropods was not observed in the lobsters used in this experiment. Furthermore, the dark spots observed in the last abdominal segment were not observed in the uropods. From these observations, the presence of color as it relates to the structure of the shell can be inferred. The thinner structure of the uropod cuticle will not allow for significant accumulation of carotenoproteins. Thus, any free astaxanthin in the epidermis of the uropods will result in a red color. A blue-colored uropod will occur only when total body astaxanthin is also low and there is no free astaxanthin underlying the small amount of carotenoprotein in the cuticle. The dark spots did not appear in the uropods because the thin cuticle did not allow for an accumulation of the carotenoprotein. Thus, a lobster's phenotypic color appears to be somewhat

influenced by the thickness of the cuticle, where cuticle thickness is positively associated with the amount of blue carotenoprotein.

This result, that different body locations changed color at different rates, was in contrast to a prior study in which five different body locations (the dorsal claw, as well as END, EXO, TEL, and LASM) of juvenile lobsters fed *Artemia* sp. were observed to change color similarly (Tlusty 2005). However, this difference further implicates the importance of free carotenoids in the epidermis in determining lobster color. As mentioned previously, canthaxanthin is the dominant carotenoid form in *Artemia* sp. (D'Abramo et al. 1983), and lobsters have to convert this to astaxanthin prior to assimilation into the cuticle. The increased processing time increased the length of time carotenoids were present in the epidermis, and thus all body locations in these lobsters first appeared red. By utilizing purified astaxanthin in the present experiment, the processing time was eliminated with the result that astaxanthin in the last abdominal segment medial was not amassing in a free state, but would initially appear in its protein-bound form in the cuticle.

Among the sibling lobsters fed the 100 $\mu g\ g^{-1}$ astaxanthin diet, variation in the rate of carotenoprotein formation was evident, as some individuals first became redder, while other individuals first became bluer. This variation may occur because lobsters differ in either their ability to take up carotenoids in the digestive tract, or in their ability to form carotenoproteins. The carotenoprotein α-crustacyanin is a large molecule comprised of 16 protein subunits, each $\sim$20 kDa, bound to 16 astaxanthin molecules (Cianci et al. 2002). Any factor leading to slower synthesis of this molecule will result in a redder lobster when first fed a diet containing carotenoids. Finally, there may be variation in the total amount of protein available for binding to astaxanthin. If a lobster had less protein in its cuticle to bind to astaxanthin, then it would be less able to assemble carotenoprotein molecules, resulting in a relatively redder lobster. A difference in the amount of carotenoprotein could also be a function of the thickness of the cuticle. If cuticle thickness affects a lobster's color, then variation in cuticle thickness among lobsters should correlate to variation in initial color, where lobsters with thick cuticles are bluer, while those with thin cuticles are redder. Unfortunately, these different mechanisms could not be differentiated within our study.

The present study demonstrating variation in color addition by lobsters made white by an astaxanthin-deficient diet provided a presumptive causal mechanism for the genetic control of lobster color. It has been acknowledged that in addition to the dietary color changes utilized here, phenotypic color in American lobsters can also be genetically determined (see "Introduction"), although the genetic mechanism controlling color has never been determined. Since American lobsters bioaccumulate pigments, it is unlikely that genetic control of coloration is a simple case of having alleles that directly determine phenotypic coloration. The differences among sibling lobsters in our study demonstrated variability in the deposition rate or total amount of carotenoproteins in the cuticle. Each of the steps in astaxanthin metabolism, rate of astaxanthin uptake, rate of carotenoprotein formation, or total amount of carotenoprotein formed could be genetically controlled. Although the unknown paternity of these sibling lobsters prevents the full exclusion of any genetic explanation for variation in carotenoprotein formation, in order to disentangle genetic and dietary effects on lobster color, further assessments of diet-independent lobster color will have to assess genetic influences on astaxanthin uptake and rate and amount of carotenoprotein formation.

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