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Control of shell colour changes in the lobster, *Panulirus cygnus*

Nicholas M. Wade[1,2], Roy Melville-Smith[3], Bernard M. Degnan[1] and Michael R. Hall[2,*]

[1]School of Integrative Biology, University of Queensland, Brisbane, Queensland, 4072, Australia, [2]Australian Institute of Marine Science, PMB3, Townsville, Queensland, 4810, Australia and [3]Western Australian Marine Research Laboratories, PO Box 20, North Beach, Western Australia, 6020, Australia

*Author for correspondence (e-mail: m.hall@aims.gov.au)

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SUMMARY

The transition from juvenile to adult in the Australian western rock lobster, *Panulirus cygnus* (George), is preceded by a mass migration from inshore nursery reefs to offshore breeding grounds. Associated with this migration is a moult which results in the animals that are due to migrate undergoing characteristic colour change from deep red to pale pink, known as the 'white' phase, which is believed to be triggered by environmental factors. To investigate this phenomenon, the colour change of wild-caught animals was measured over two separate years in response to two important modifiers of crustacean shell colour, dietary carotenoid and background substrate colour. Changes in shell colour during this colour transition period were influenced more greatly by other factors independent of diet or background substrate and no mass colour change was induced during this time. Shell colour measurement and carotenoid quantification confirmed the presence of animals similar to wild-caught 'whites', regardless of the treatment. From these experimental observations we infer that the 'white' phase of the western rock lobster is not triggered by dietary modification or in response to background substrate. We propose that this transition is under the regulation of an ontogenetic program activated at a specific moult, which induces presently unidentified molecular changes linked to shell colour production. This unique colour transition may have evolved to provide protective camouflage during migration, and serves as an excellent model to study the genetic mechanisms underlying crustacean shell colouration. These data also provide insight into the changes in carotenoid levels induced by environmental factors, and the ability to modify crustacean shell colour in aquaculture.

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Key words: carotenoid, crustacean, modification, diet.

INTRODUCTION

Colour change in crustaceans occurs either by quantitative adjustments in chromatophores or by changes in exoskeletal pigment concentration or composition (Kleinholz, 1961; Rao, 2001). In some crustaceans, physiological colour changes are rapid, reversible and often rhythmic, being driven largely by the dispersion or concentration of pigment granules within chromatophores (Rao, 1985). In contrast, morphological changes in the exoskeletal pigments tend to be slow and more permanent. Regardless of the mechanism, carotenoids play a central role in the colouration of many crustacean tissues such as the shell and epithelial tissue, eggs and ovaries, and hepatopancreas (Castillo et al., 1982; Lenel et al., 1978). Crustaceans are unable to synthesise carotenoids and must therefore obtain them from their diet (Lee, 1977). In addition to the type and amount of carotenoids ingested, a number of environmental factors, such as the colour of the background substrate, light intensity, photoperiod and temperature, can also produce physiological colour changes in crustaceans (Rao, 1985). By comparison, mechanisms of morphological colour change and the deposition of colour in the exoskeleton are poorly understood.

Juvenile Australian western rock lobsters, *Panulirus cygnus* (George), within a size range of 65–85 mm carapace length congregate on inshore reefs and over a 3–4 week period undergo a highly synchronised moult that is associated with a colour change from a deep red to pale pink (Chubb and Barker, 2005; Gray, 1992; Melville-Smith et al., 2003). These juvenile lobsters are herein referred to as 'Prewhites' and the pale pink migratory phase referred to as 'Whites'. Male and female White lobsters aggregate in equal numbers on these reefs until an unknown trigger signals their migration in early December each year to deeper waters and their future breeding grounds (Chubb and Barker, 2005; George, 1958). Upon reaching deeper waters, the White lobsters gradually return to their normal deep 'Red' colouration and become sexually mature over the forthcoming months for reproduction in the following year (George, 1958; Gray, 1992; Melville-Smith et al., 2003).

The colour of seafood is the first characteristic noted by the consumer and is directly related to acceptability (Shahidi et al., 1998). Asian markets, in particular the Japanese, have been shown to prefer the deep red colour of the western rock lobster (Monaghan, 1989). Comprising up to approximately 25% of the annual catch (Chubb and Barker, 2005), the marketing of White lobsters results in a significant annual loss of revenue to the fishery. Among all crustaceans, the western rock lobster is the only reported species to experience this distinct mass colour change during migration and maturation. Pale-coloured animals are discovered occasionally in some spiny lobster species, such as the Australian southern rock lobster *Jasus edwardsii* captured in deep water (McGarvey et al., 1999); however, this colour change is thought to be caused by local availability of carotenoids and does not occur in a synchronised manner throughout a large population. In the case of the western rock lobster, the colour of Whites during their migration may provide protective colouration across open stretches of sand (Melville-Smith

et al., 2003). It has therefore been suggested that the initial cause of the White phase in the western rock lobster is due to pre-moult dietary modification or adaptation to substrate colour. Although inducing a small change in shell colour, environmental factors such as dietary carotenoid content, background substrate colour, light intensity, incident light wavelength and photoperiod have been previously shown to be ineffective in rapidly returning White lobsters to their normal Red colouration (Melville-Smith et al., 2003).

In this study, we measured the effect of two environmental variables, diet and background substrate colour, on shell colour during the Prewhite to White phase moult over two separate years using the Commission Internationale de l'Eclairage (CIE) L*a*b* system of colour notation (Commission Internationale de l'Eclairage, 2004). This system measures the absolute colour of a sample on a three-dimensional scale of value, hue and chroma. The value of colour (or lightness represented by L*) has a scale of 0 (pure black) to 100 (pure white). The hue has two components that distinguish opposing colours. The first is a*, which represents the red–green scale, and the other is b*, which represents the blue–yellow scale. Chroma (or saturation) indicates the amount of hues, positive a* towards red, negative a* towards green and positive b* towards yellow, negative b* towards blue. This technology has been adapted successfully for use in assessing the colour changes in White lobsters in response to environmental change (Melville-Smith et al., 2003) and the onset of colouration in juvenile *Homarus americanus* (Tlusty, 2005).

MATERIALS AND METHODS

In October of both 2002 and 2003, 64 sub-adult Prewhite western rock lobsters selected to be within the size class expected to enter the White phase upon their next moult (approximately 65–85 mm carapace length) were captured from shallow reef waters by professional fishers in Western Australia. Despite being the only method available, size-class selection of animals does not ensure that each animal is indeed ready to undergo their White phase transition, and hence some animals were expected not to change colour and remain Red. Captured lobsters were held in aquaria for less than 48 h without feeding, and then shipped live to the Australian Institute of Marine Science (AIMS), Queensland.

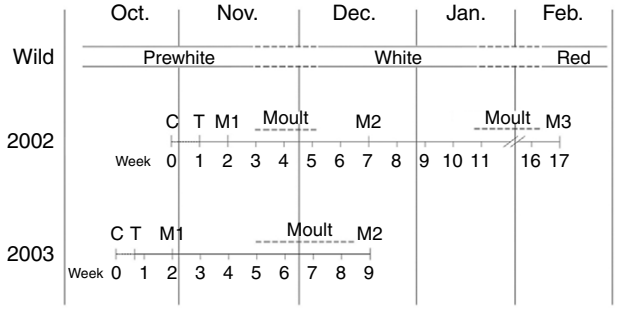

Fig. 1. Experimental design to test environmental effectors of the White phase. Two experiments were run over successive years. Experiment 1 encompassed the entire Prewhite–White–Red transition beginning with a moult in November/December and ending after a second moult in January/February. The second experiment acclimated Prewhite lobsters under experimental conditions for a longer period of time before their initial moult in November/December, after which the experiment was terminated. C, capture date; T, tagging date; M1, measurement 1; M2, measurement 2; M3, measurement 3.

Individual lobsters were sedated by cooling in 12°C seawater before intra-muscular insertion of a Hallprint T-bar anchor tag in the mid-ventral abdomen along with a separate moult indicator glued to the lateral carapace. Sixteen tagged lobsters were allocated randomly to each of four treatments: high dietary carotenoid supplementation while on either light or dark substrate, and the corresponding low carotenoid diet on light or dark substrate. Two experiments were conducted of varying duration (Fig. 1), encompassing the period during which Whites would appear naturally in the wild. The 2002 experiment consisted of 7 days acclimation before initial measurement, followed by 37 days under experimental treatment conditions before the second colour measurement, and a further 57 days before final colour measurement. In the 2003 experiment, a slightly extended 11 days of acclimation before initial measurement preceded 48 days under experimental conditions until the second shell colour measurement. For each experiment, all animals recorded a moult between measurements as assessed by moult indicators attached to the carapace.

Animals were maintained in two 10 000 l, 4 m diameter circular holding tanks at 22–25°C. Water quality was maintained by continuous recirculation through a foam fractionation tower and daily 50% water exchanges. Each tank was divided in half by plastic netting and painted either black or white to simulate dark or light substrates. A squid-based artificial diet supplemented with vitamin and mineral mixes, cholesterol and binders was prepared to control carotenoid intake (Table 1). Gelatine was introduced to bind the diet mixture, and prevented dispersion of feed components in the seawater and thereby maximised the carotenoid intake. Lobsters were fed approximately 30 g of the diet per animal per day prepared either with or without an astaxanthin supplement in the form of Carophyll Pink (Hoffman-La Roche, Basel, Switzerland).

Two points on the shell were chosen to measure the colour of all lobsters: the mid-dorsal carapace and the dorsal surface of the first abdominal segment. Lobster shell L*a*b* colour was measured using a MiniXE Colorimeter (HunterLab, Reston, VA, USA) over a 1 cm diameter circle standardised under D65 illumination that most accurately replicates natural sunlight. Fourteen deep-water Red and 13 migratory White western rock lobsters were captured by

Table 1. Composition of lobster diet

Ingredient	Content
Squid (minced)	53.7%
Mineral mix[a]	1.2%
Vitamin mix[b]	1.2%
Cholesterol	0.6%
Shrimp activa	0.6%
Binders: guar gum	1.4%
Manugel GMB[c]	2.1%
tetrasodium pyrophosphate	2.1%
gelatine	7.1%
Water	30.0%
± Carophyll Pink[d]	0.15%

[a]Mineral mix (Rabar Ltd, Beaudesert, Australia): di-calcium phosphate 61.9%, magnesium sulphate 30.2%, sodium phosphate monobasic 7.9%.
[b]Vitamin mix (Rabar Ltd, Beaudesert, Australia): prebase 36.6%, vitamin C 31.6%, choline chloride 15.8%, inositol 6.32%, Endox 2.5%, Roxophyll 112 2.02%, vitamin D3 1.52%, Calpan 1.06%, vitamin E 0.64%, niacin 0.64%, Rovimix H-2 0.32%, vitamin K 0.28%, pyridoxine HCl 0.18%, vitamin B2 0.16%, para-aminobenzoic acid 0.16%, vitamin B12 0.12%, thiamine HCl 0.08%, folic acid 0.02%.
[c]Janbak Industries Pty Ltd, Tingalpa, Australia.
[d]F. Hoffman-La Roche Ltd, Basel, Switzerland.

professional fishers during the months of December and January in 2003 and 2004. The average shell colour values of these wild-caught animals were used as a comparison to assess colour change in tank-reared experimental animals. The change in the dependent variable L* across a moult (either between M1 and M2, or between M2 and M3) was analysed by linear regression analysis using the least-squares method to identify statistical significance with respect to the two independent variables high carotenoid diet and dark background tank colour, and an interaction between these two variables. The intercept of the regression can be interpreted as a combination of all other factors that are assumed to affect every individual uniformly. Where comparison between individual measurements was required, statistical significance was assessed by single factor analysis of variance (ANOVA), followed by Fisher's test allowing 5% error.

A conservative approach was employed when assigning colorimeter data to lobsters that had lost their individual identification tags during moulting. In the first experiment this occurred for three and five individuals from the light and dark treatments, respectively, from the low carotenoid diet (LL and DL treatments), and for four individuals in each of the high carotenoid tanks (LH and DH treatments). At any one measurement, unidentified lobsters were ranked in order of their L* value from highest to lowest. These values were then allocated in rank order to the unidentified set of previous measurements that had also been ranked from highest to lowest. That is, the highest unknown L* value for the second measurement was linked to the highest unknown L* value from the first measurement and so on for each unidentified individual. The second experiment did not suffer any loss of ID tags and every individual could be tracked throughout.

Shell, hypodermis and muscle were sampled from eight lobsters in the 2003 experiment, representing the extremes of measured L*a*b* colour values from each of the four treatments. These animals were used to further compare the Prewhite and White individuals from the tank experiment with their Red and White wild-caught counterparts and biochemically investigate whether these animals could be truly considered Whites. Animals were sedated by cooling in 4°C seawater for 30 min followed by cerebral ganglion bisection and cutting of the ventral nerve cord. Carotenoid quantification was performed using reverse-phase high pressure liquid chromatography (RP-HPLC) from freeze-dried tissue or ground shell that was extracted three times in acetone, evaporated under nitrogen and the absorbance (A_{470}) measured in hexane. Carotenoid content in abdominal muscle tissue was used as a control and always found to be zero. RP-HPLC analysis followed previously published procedures on this species (Wade et al., 2005). Where possible, data were converted to carotenoid content (micrograms of carotenoid per gram of dry weight, μg g^{-1}) using a standard curve generated from an astaxanthin standard (A9335, Sigma, St Louis, MO, USA) or the following formula: total carotenoid content=($A_{470}\times$10 000)/E/dry weight [where E=2500, the standard absorbance of a 1% mixed carotenoid solution (weight/volume) in a 1 cm cuvette at 474 nm in hexane; and dry weight of the tissue is in g). Relative quantification of RP-HPLC peaks (peak area per gram of dry weight tissue) was calculated using a peak width of 1 min and 2% peak sensitivity.

RESULTS

The characteristic colour change from Red to White of the western rock lobster has been defined in absolute terms using the CIE L*a*b* system of colour notation. Average L*a*b* values are shown in Table S1 (see supplementary material). Represented graphically in three dimensions (Fig. 2A), there was a clear distinction between Red (N=14) and White (N=13) individuals. Lightness (L*) showed the greatest separation between Reds and Whites and thus produced the greatest statistical significance between the sample groups ($F_{1,25}$=180.51; P=6.15×10^{-13}). The next most significant variable was b* ($F_{1,25}$=91.61; P=7.7×10^{-10}) and finally a* ($F_{1,25}$=12.88; P=0.0014). Total carotenoid content of the shell and hypodermal tissue was shown to be lower in Reds (N=3) compared with Whites (N=3), with the average values shown in Table S1 (see supplementary material). Furthermore, the total carotenoid content of the shell correlated with each of the tristimulus colour values and hence the observed colour of the shell (Fig. 2B). Although total sample numbers were low, linear regression analysis showed significant correlations with a* (R^2=0.9138), b*

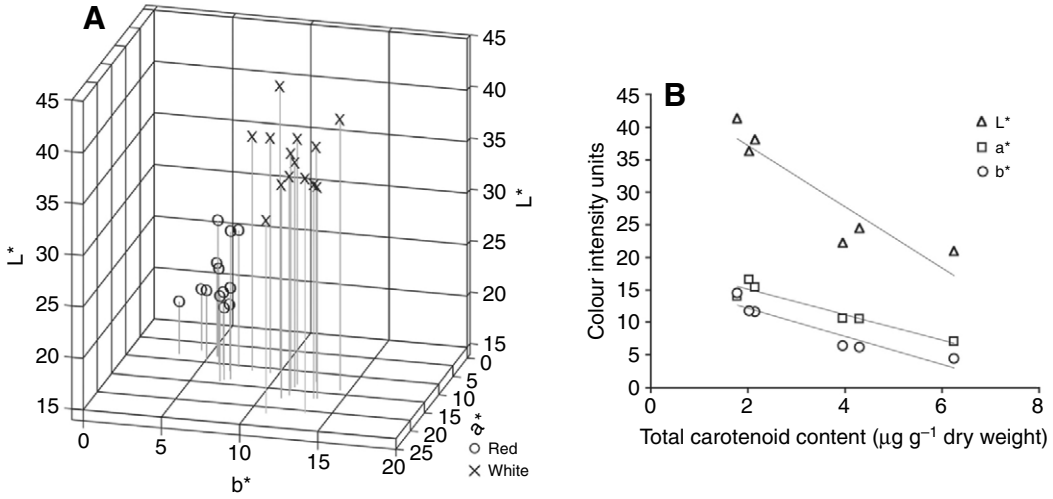

Fig. 2. L*a*b* colour measurement of Reds and Whites and correlation with shell astaxanthin content. (A) Three-dimensional representation of absolute shell colour of 14 Red (open circles) and 13 White (crosses) lobsters collected from the wild and measured on the CIE L*a*b* scale (see Materials and methods) showing a clear distinction between the two groups. (B) The amount of carotenoid extracted from these shells correlated with the measured tristimulus values L*a*b*. The amount of total carotenoid is expressed in micrograms per gram of dry weight as measured by absorbance at 470 nm in hexane.

Table 2. Important factors driving the change in L*

2002 Experiment	M2
Diet	P=0.28
Background	P=0.63
Interaction	P=0.91
Starting L*	P=0.0007

M2, measurement 2; L*, lightness according to the CIE L*a*b* scale (see Materials and methods).

Table 3. Multiple regression analysis of the change in lobster shell colour

	M1 to M2		M2 to M3	
2002 Experiment				
High diet	–2.00	P=0.28	–1.72	P=0.33
Dark background	–0.89	P=0.63	–8.60	$P=1.89\times10^{-5}$
Interaction	–0.36	P=0.89	–0.80	P=0.73
Intercept	4.25	$P=1.5\times10^{-3}$	5.76	$P=2.8\times10^{-5}$
2003 Experiment				
High diet	–2.03	P=0.14	–	–
Dark background	–7.16	$P=2.66\times10^{-6}$	–	–
Interaction	4.26	P=0.0295	–	–
Intercept	11.33	$P=8.23\times10^{-17}$	–	–

Regression coefficient (β) and P values are shown for measurements 1–3 (M1–3) of lobster shell colour according to the CIE L*a*b* scale (see Materials and methods).

(R^2=0.8877) and L* (R^2=0.8428). A similar correlation was observed with the total carotenoid content in the underlying hypodermal tissue (data not shown). Combined, these data demonstrated that L* was the most effective measure for distinguishing Red and White individuals and it was hence used for analysis in further experiments. Despite the fact that a* showed an excellent correlation with external shell colour, the lack of overall variation in a* between Reds and Whites meant this value alone poorly distinguished between the two groups.

Initially, it was necessary to determine the most important factor driving the change in L*, and hence colour. Using the data from the 2002 experiment in a linear regression, we established which of the factors [diet, background colour, interaction of diet and background or colour at first measurement (M1)] was the most important for determining the colour at the second measurement (M2). M2, which was performed within the characteristic moult and colour change specifically under investigation, was shown to be most dependent upon the starting L* value at M1 (P=0.0007; Table 2). Similar observations were evident for the 2003 experiment (data not shown). This indicates that the most important factor affecting the colour at M2 is the starting colour at M1. Due to this and the fact that our primary focus was to investigate which factors drive the change in colour over this time (not just colour itself), we removed the observed effect of M1 on M2 and modelled the difference between individuals between M1 and M2 (and also M2 and M3 for the 2002 experiment).

Thus, in our regression model framework, individual colour changes were set as dependent variables, while high carotenoid diet, dark background colour and the interaction between diet and background were the explanatory variables. The regression coefficients and P values describing the association with the measured colour change from M1 to M2 or M2 to M3 in the 2002 experiment, and from only M1 to M2 for the 2003 experiment are shown in Table 3. The intercept of the regression can be interpreted as a combination of all other factors that are assumed to affect every individual uniformly. We therefore herein refer to all the regression intercepts unrelated to diet or background colour as 'other factors'. For the moult that encompassed the transition from Prewhite to White (M1 to M2), the only significant regression coefficient for the 2002 experiment was 'other factors' (P=0.0015). This coefficient had a strong positive effect (β=4.25) on the individual colour change of all the animals in the experiment; that is, on average the L* value for all animals regardless of treatment group was increased and hence the animals appeared lighter in colour. Within treatment groups, there was generally a small increase in the average L* value for the group as a whole, combined with a large increase in the range of L* values for individuals within each group at M2 compared with M1 (Fig. 3A). This indicated that some animals had undergone a strong colour change (similar to a White phase transition), while others had remained in their Prewhite stage. Interestingly, none of the other variables showed any significant relationship with the observed shell colour changes during this time. These data suggest that there are other strong factors, in addition to and independent of diet or background colour, that are responsible for the colour change observed in these animals. This first experiment was extended to included a second moult between M2 and M3, to examine the longer-term effects of each of these variables and to complete the transition from White back to Red, as observed in the wild. During this period, a similarly strong effect of 'other factors' on the colour change was reflected in its significant positive regression coefficient (β=5.76; $P=2.8\times10^{-5}$). However, in this instance, the change in colour was also significantly affected by a more strongly negative regression coefficient for background colour (β=–8.76; $P=1.89\times10^{-5}$). These relationships indicate that the strongest and most significant influence on the change in shell colour during this time was the background colour of the tank. As the regression used dark background as the explanatory variable, this negative value indicated an increase in L* value and a change in animal colour towards the lighter coloured background. This caused a more pronounced shift in the average L* value for the group as a whole by the third measurement (M3), which was visible in the light background treatments (Fig. 3A, light low and light high).

The 2003 experiment encompassed only the moult from Prewhite to White (M1 to M2) and, similar to the previous year, the most significant regression coefficient was 'other factors' ($P=8.23\times10^{-17}$), which exerted an even stronger positive effect on the L* value (β=11.33). At the same time, however, the extended period of exposure to treatment conditions (from 3 weeks in 2002 to 5 weeks in 2003) resulted in an enhanced impact of the background substrate colour (β=–7.16; $P=2.66\times10^{-7}$). We observed an increase in the average L* values in each of the treatments (Fig. 3B), an effect that was accentuated by the light-coloured background (Fig. 3B, light low and light high). That is, a light background further increased the L* value and hence further lightened shell colour. Although significant, the effect of background substrate was not as strong as that of 'other factors' and these two effects could be distinguished using our regression analysis model. Similar to the 2002 experiment, the measurements at M2 showed a general increase in the range of L* values (Fig. 3B), representative of the appearance of some markedly paler animals alongside ones that remained dark.

Regardless of the treatment and in the two experiments over successive years, some individuals underwent a strong colour change resembling that of wild-caught Reds and Whites. Although not every animal underwent this change, two individuals that

represented the extremes of L* value within each treatment (and hence the darkest and the lightest individual from each treatment) were selected based on their L* value. Selected individuals from each of the four treatments in both the 2002 and 2003 experiments showed a striking similarity to wild-caught Whites in the colour of their shell (see Fig. S1 in supplementary material) and complementary tristimulus values (see Table S1 in supplementary material). These data suggest that White phase individuals had appeared in each of the tanks, regardless of the treatment. The statistical significance of the difference in L* value between individuals within a tank or the change in L* value of an individual between M1 and M2 could not be assessed due to the low number of repeated measurements. Therefore, to explore more thoroughly whether this colour change in tank-reared individuals corresponded to the White phase, we investigated the carotenoid levels in the shell and hypodermis of these individuals by RP-HPLC, in comparison with previous data on wild-caught Reds and Whites (Wade et al., 2005). To simplify the analysis, carotenoid peaks for each animal were grouped into astaxanthin-like compounds (peaks 1–4), unknown carotenoids (peaks 5–10) and astaxanthin esters (peaks 11–15). The shells of tank-reared Whites contained, in general, a lower amount of astaxanthin-like compounds than those of Reds from the same treatment group (Fig. 4A). Furthermore, the hypodermis of experimental Whites contained less of each of the carotenoid groups than that of Reds under similar conditions. Similar to wild-caught Reds and Whites, there was a lack of esterified astaxanthin (peaks 11–15) in tank-reared Whites in comparison to tank-reared Reds.

One trend that could be observed was that hypodermal carotenoid levels best reflected the artificial treatment conditions, in that animals fed the low carotenoid diet contained less total hypodermal carotenoid compared with high carotenoid diet animals (Fig. 4B). However, these measurements were recorded from only four individuals, comprising two Reds and two Whites within the high and low carotenoid diet treatments and thus were not statistically significant. Carotenoid levels from the hypodermis of the Red and White individuals selected from the dark substrate and high carotenoid diet (Fig. 4A, DH-R and DH-W) displayed carotenoid profiles most similar to those of wild-caught Reds and Whites. Interestingly, however, none of the environmental treatments was observed to maintain or elevate the level of carotenoids in these animals above that of their wild-caught counterparts. Experimental Reds on a high carotenoid diet contained markedly elevated levels of one unidentified RP-HPLC peak 8 compared with wild-caught animals (see Fig. S2 in supplementary material); however, this increase was not accompanied by an increase in shell carotenoid levels. All muscle tissue tested throughout as a control did not contain detectable levels of carotenoids. As a positive control, the high carotenoid artificial diet was confirmed to contain elevated levels of astaxanthin, approximately 71.5 μg of carotenoid per gram of dry weight compared with 6.3 μg for the low carotenoid diet (Fig. 4C). Carotenoids extracted from the high carotenoid diet had a peak absorbance at 470–474 nm in hexane, and RP-HPLC analysis showed a single carotenoid peak that co-eluted with an astaxanthin standard (data not shown).

DISCUSSION

Inshore juvenile Australian western rock lobsters undergo a highly synchronised moult and colour change from a deep red (Red) to pale pink (White) prior to migrating to deeper waters where they attain sexual maturity and return to their dark red colour (Chubb and Barker, 2005; Gray, 1992; Melville-Smith et al., 2003). Here we sought to prevent or induce the onset of the White phase by modifying diet and background substrate colour in Prewhites and

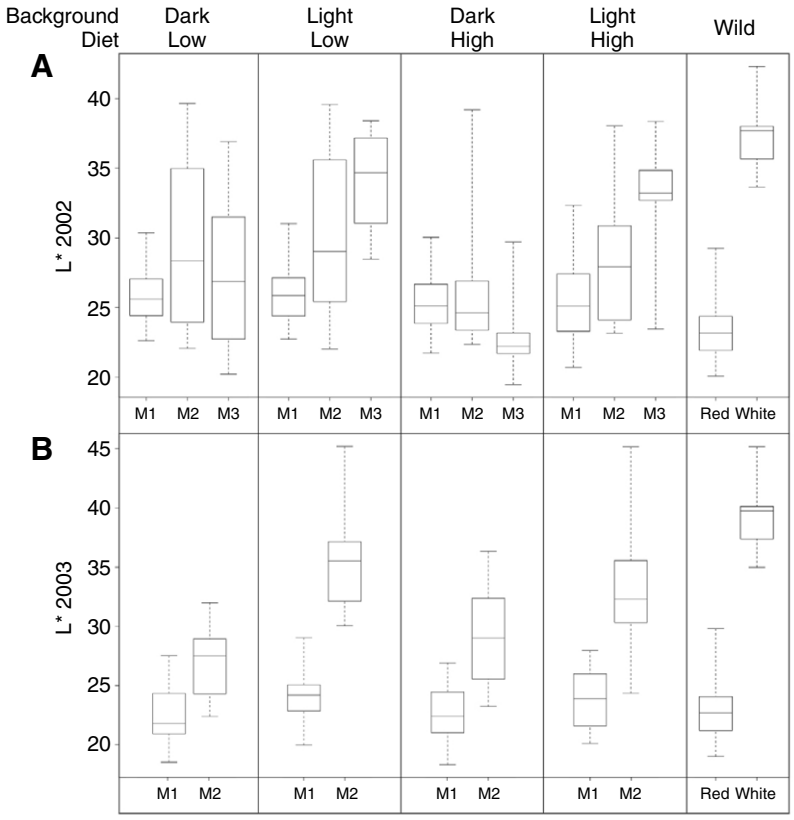

Fig. 3. Changes in L* values of individual lobsters under treatment conditions. (A) Box and whisker plot of colorimeter data from tank-reared individuals under treatment conditions over approximately 4 months of the 2002 experiment that encompassed two moults and the complete period from Prewhite (M1) to White (M2) to Red (M3). Boxes indicate the range of the first and third quartiles, the line across the box shows the median of the data and the whiskers represent the range of the data from each treatment group. (B) A similar box plot of data from the 2003 experiment that covered only the transition between Prewhite to White and included an extended acclimation period after measurement and before moulting. In both experiments, linear regression analysis demonstrated that the most important effector of shell colour between the moult specifically under investigation (between M1 and M2) was 'other factors' unrelated to diet or background colour. This was followed by a significant influence of background colour over a longer time under treatment conditions and a shift in average L* values at M3 in 2002. An extended range of L* values could be observed at M2 in both experiments and suggested that Whites had appeared in each treatment, irrespective of the influence of the variables diet and background colour.

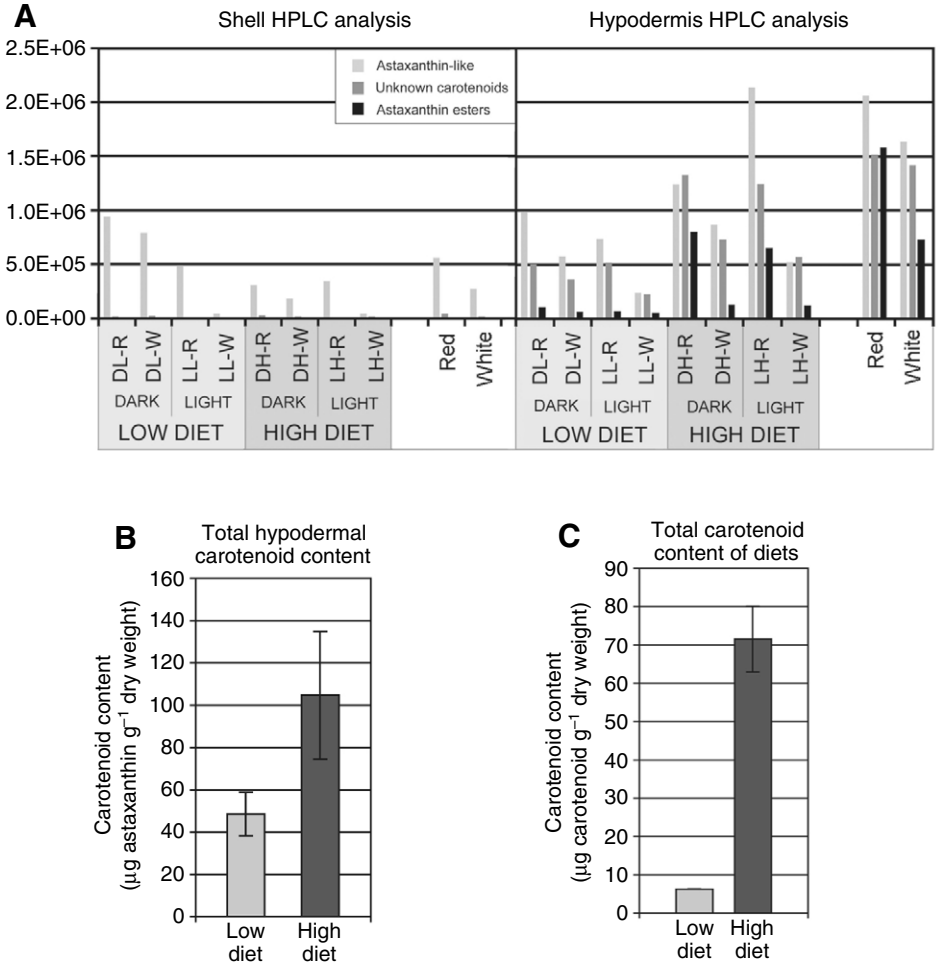

Fig. 4. Shell and hypodermis responses of selected tank-reared lobsters. Two animals were selected from each treatment group in the 2003 experiment based on their L* value at M2 and these animals represented the darkest and lightest animal in each treatment. To further investigate the appearance of Whites under experimental conditions, total carotenoids were extracted from the shell and hypodermis of these selected animals, followed by separation on RP-HPLC. (A) Quantitative peak data from the shell and hypodermis of selected lobsters were grouped for further analysis according to previous studies (Wade et al., 2005) into astaxanthin-like carotenoids, unknown carotenoids and esterified astaxanthin. The selected Red individual (R) from each treatment tank was paired with the corresponding White individual (W) from the same treatment tank. In conjunction with colorimeter measurement, comparison of RP-HPLC data from tank-reared lobsters with wild-caught Reds and Whites suggested the White phase had been reproduced successfully under controlled conditions and that White individuals were present regardless of the treatment tank. DL, dark tank, low carotenoid diet; LL, light tank, low carotenoid diet; DH, dark tank, high carotenoid diet; LH, light tank high carotenoid diet. (B) Tank-reared lobsters fed a high carotenoid diet for 5 weeks showed a concurrent increase in hypodermal total carotenoid levels compared with lobsters fed the low carotenoid diet (N=4). Units are expressed as micrograms of carotenoid per gram of dry weight tissue calculated from an astaxanthin standard curve. Error bars (s.e.m.) are included on graphs. (C) Quantitative RP-HPLC peak data of diet preparations showed an approximate 10-fold enrichment of astaxanthin in the high diet (N=8). Units are expressed as micrograms of carotenoid per gram of dry weight of diet.

investigate whether these environmental factors are sufficient to produce a White phase transition in tank-reared western rock lobsters.

The linear regression model used to analyse the data presented here incorporated individual shell colour changes from M1 to M2 or M2 to M3. It further defined the influence of various factors affecting individual colour change over time and was able to distinguish between and separate factors that may be working in combination (with either net additive or net subtractive effects on shell colour). In both years we observed that the strongest and most significant effector of shell colour change of each individual across the White phase moult (from M1 to M2) was ‘other factors’. This effect was positive, thereby indicating that there was a significant lightening of shell colour across this moult. No relationship was observed between background colour or diet in the M1 to M2 period of the 2002 experiment, although as expected, a relationship developed after a further 4 months of treatment conditions between shell colour change and background that rivalled the effect of ‘other factors’. During the M1 to M2 period of the 2003 experiment, background colour exerted some influence on shell colour change and this could be distinguished from the influence of ‘other factors’. However, the strength of the effect of background was far less than the influence of ‘other factors’ and was only evident after an extra 2 weeks under treatment conditions between the two measurements and a longer acclimation period before M1.

The lack of a strong relationship between the change in lobster shell colour and the environmental treatment across the Prewhite to White moult (M1 to M2) suggests that neither of the variables tested is sufficient to induce the White phase colour transition. In further support of this, each of the experimental conditions contained

individuals that resembled wild-caught Reds and Whites visually (see Fig. S1 in supplementary material), by shell colour measurement (see Table S1 supplementary material) and by RP-HPLC carotenoid analysis (Fig. 4A). Similar results were also obtained over successive years. Not every individual underwent this colour change, which may potentially be explained by the notion that the Prewhite animals collected for tank experiments were a random selection of wild-caught animals of a size class expected to turn White at their next moult. However, as there is currently no reliable method of collecting animals that are the same age, this was an accepted condition of this experiment and hence it was not anticipated that every animal would enter their White phase during this experiment. Based on previous research, exposure to a dark background alone or in conjunction with a high carotenoid diet was expected to promote a strong darkening of shell colour (Melville-Smith et al., 2003; Pan et al., 2001; Rao, 1985; Sommer et al., 1991). However, in this experiment, some animals under this treatment became markedly lighter in colour (DH-W), replicating the natural Prewhite to White transition, rather than undergoing the expected change to Red in response to these environmental variables. We therefore conclude that, for the first time under controlled conditions, we have successfully replicated the White phase of the western rock lobster. Furthermore, we have shown that neither dietary carotenoid intake nor background substrate colour, either in combination or independently, is sufficient to prevent or induce the White phase change in experimental animals. It is therefore unlikely that dietary carotenoid or background colour is solely responsible for the onset of the White phase in the western rock lobster, thereby implying another mechanism is responsible for the rapid shell colour transition. More generally, these results clearly indicate that there are other factors present that are able to cause large changes in crustacean shell colour, and a greater understanding of these mechanisms may provide a more effective means of rapidly modifying crustacean shell colour.

These ‘other factors’, identified here as the most significant effector of shell colour change, may be a combination of genetics and other unknown environmental factors. Given these animals were under controlled conditions of light, temperature, substrate and diet, we propose that this sharp individual colour change is induced by a presently unidentified molecular change rather than as a direct physiological response to environmental change. We further suggest this observed change in the western rock lobster from Prewhite to White is underpinned by morphological changes in the quantity of colour in the exoskeleton in conjunction with a specific moult. This distinct change in colour may require the down-regulation of genes involved in shell colouration, perhaps linked to decreased carotenoid levels in the underlying hypodermis, during the moult from Prewhite to White. Whether the primary trigger for the colour change in this species is internally linked to the onset of sexual maturity, or is perhaps initially triggered by other as yet unidentified environmental cues, requires further detailed investigation. Nonetheless, a direct comparison of Red and White lobsters would provide an excellent model system to identify the underlying molecular processes involved in morphological colour changes in crustaceans, in addition to how the metabolism and incorporation of astaxanthin affects shell colour. A greater understanding of these mechanisms and the molecular control of the genes involved in crustacean shell colour formation may give rise to a method to rapidly and significantly modify crustacean shell colour, similar to the colour intensity change observed in this species.

As may be expected in such an experiment, a number of physiological colour adaptations were observed in experimental animals in their respective groups. Over a number of months, background tank colour produced a significant colour change in the treatment group as a whole, causing a shift in the average L* value of each treatment. However, this change was not of comparable intensity to the Prewhite to White transition, did not occur over a single moult and did not prevent Whites from appearing in each treatment. This response was consistent with that observed in previous research on this species, which showed that although background colour was a significant effector of lobster shell colour, it was unable to return White western rock lobsters rapidly to their normal Red colouration (Melville-Smith et al., 2003). This background response may represent a simple adaptive physiological colour change that has been observed in many crustacean species and is elicited through pigmentory effector hormones (Rao, 1985; Rao, 2001). Here we show this physiological effect is independent of other factors involved in modifying crustacean shell colour. Our data suggest that Prewhite individuals would need to aggregate on pale-coloured sandy substrates, perhaps in conjunction with dietary modification, for at least 1–2 months prior to moulting in order to elicit a significant colour change, something that is not observed in the wild.

Dietary carotenoids are known to be essential for shell colour formation in crustaceans (Castillo and Negresadargues, 1995; Lorenz, 1998; Petit et al., 1998; Yamada et al., 1990), with the absence of dietary carotenoids preventing colour formation altogether in clawed lobsters (Tlusty, 2005). The results of the present study also suggest that dietary carotenoid supplementation can elevate hypodermal carotenoid levels, in particular the esterified astaxanthin derivatives. However, even in combination with dark background colour, we showed that individuals were not able to redeploy these carotenoids effectively to the shell and hence were unable to modify their shell colour significantly. Further strong conclusions cannot be drawn from individual data due to low experimental replication. However, of particular interest is RP-HPLC peak 8, which is the predominant carotenoid species present in the hypodermis of animals with a carotenoid-supplemented diet, regardless of background colour. This unidentified carotenoid from a previous study was not differentially accumulated in wild-caught Reds and Whites (Wade et al., 2005), suggesting a potential role in carotenoid storage or intermediary metabolism rather than regulation of shell colour. Hypodermal tissue has been shown to store excess carotenoids (Castillo and Lenel, 1978; Dall et al., 1995; Okada et al., 1994; Sagi et al., 1995), further supporting the involvement of specific carotenoid intermediates or modified astaxanthin esters in the transport, storage and incorporation of astaxanthin into the exoskeleton. The understanding of these metabolic processes and their regulation requires further investigation to best optimise the use of carotenoids during crustacean aquaculture.

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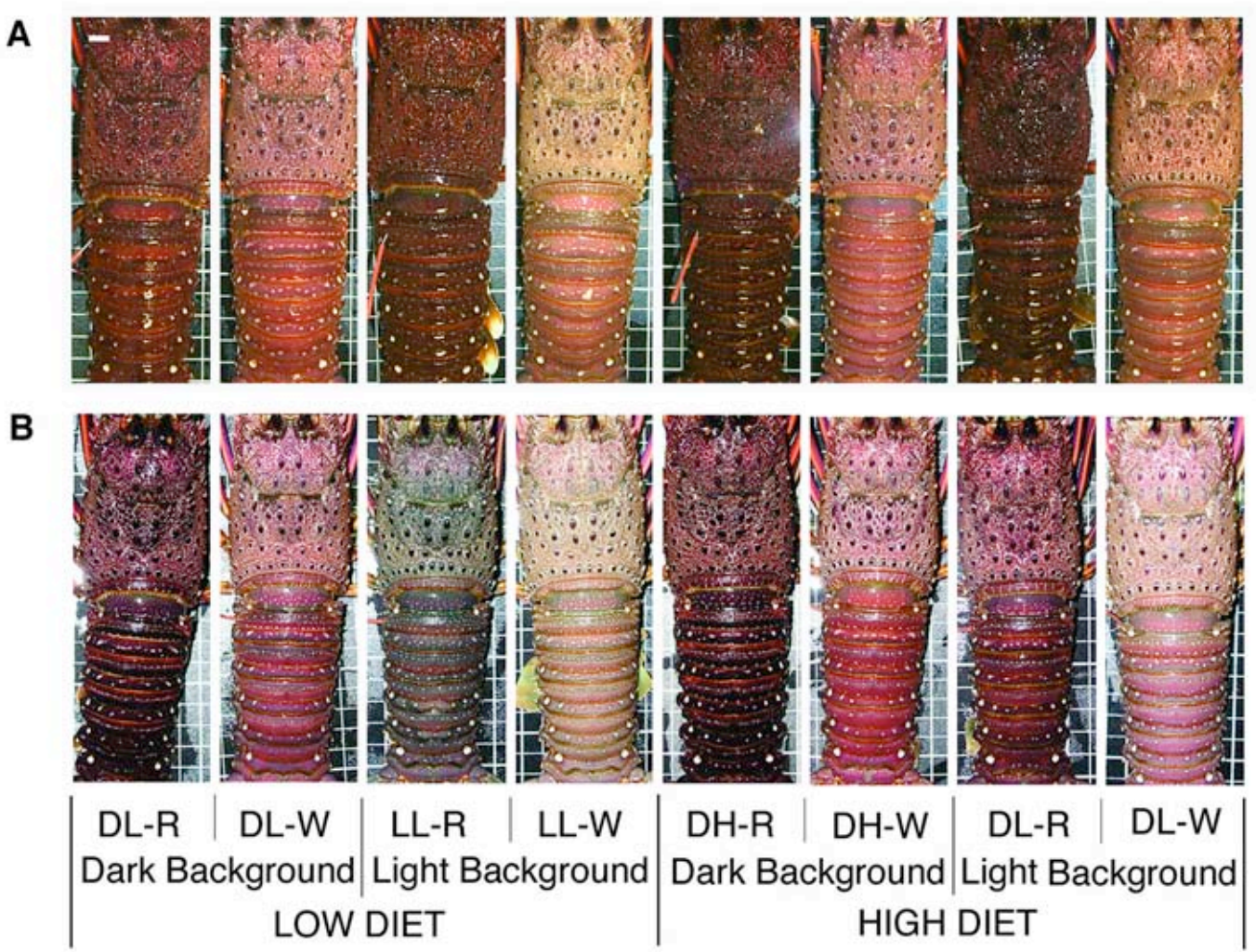
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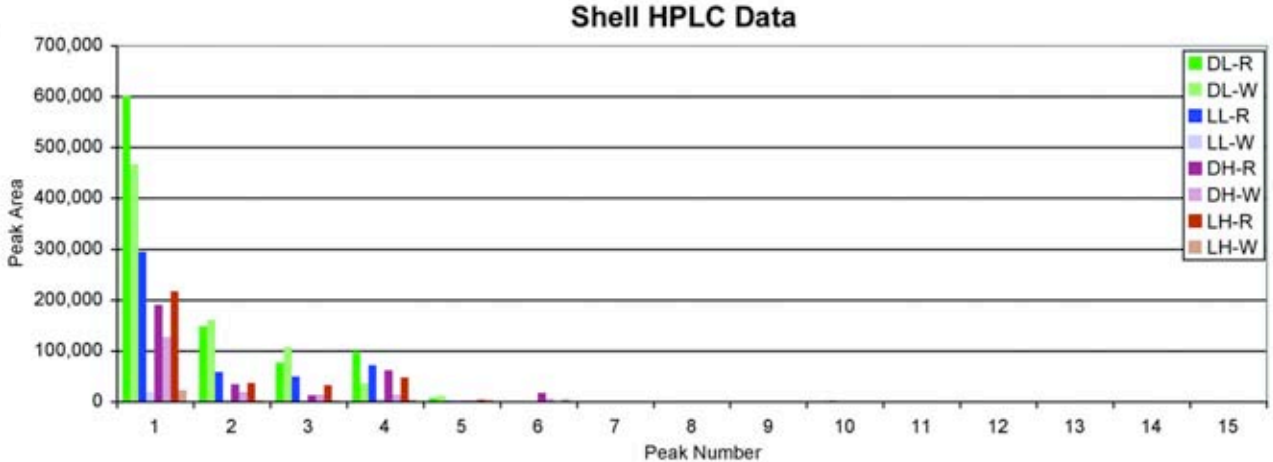
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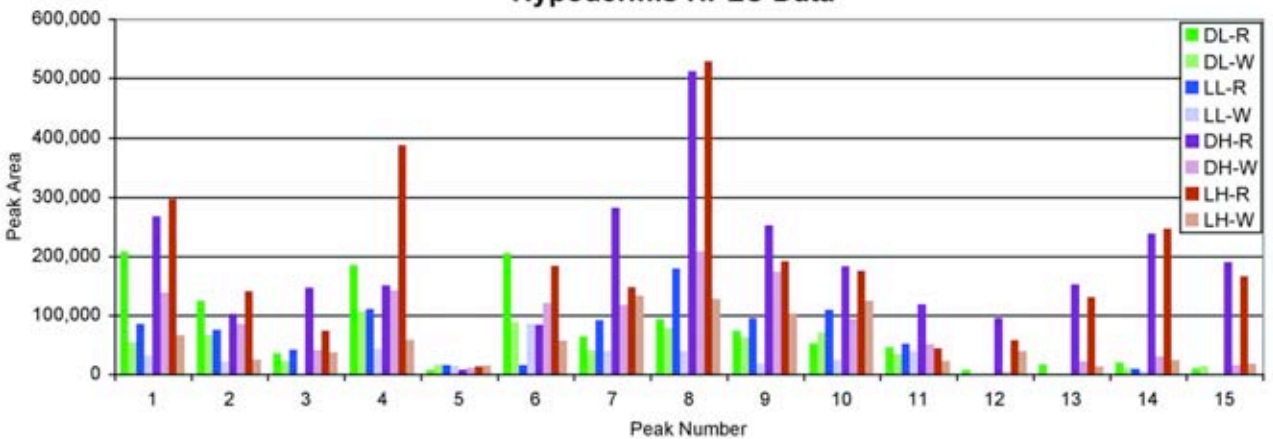
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Table S1. Tristimulus colour values and carotenoid content for wild-caught *Reds* and *Whites* and selected animals from each treatment in the 2002 and 2003 experiments.

	Tristimulus Values			Tristimulus Values			Carotenoid Content (µg/g)	
	L*	a*	b*	L*	a*	b*	Shell	Hypodermis
Wild-caught animals								
Reds	23.8114 ± 2.9918	10.7840 ± 2.4600	6.0565 ± 1.2927				4.85 ± 1.24	52.78 ± 7.23
Whites	37.4745 ± 2.2680	14.9718 ± 3.4734	11.6494 ± 1.6981				2.00 ± 0.19	32.97 ± 10.65
2002 Experiment	**Measurement 1**			**Measurement 2**				
DL-R	25.3835	10.3684	8.8131	22.0747	9.7934	5.4248	-	-
DL-W	22.9926	8.0392	5.3189	39.6610	11.8169	5.9641	-	-
LL-R	22.7461	11.8226	7.2172	22.0335	12.9846	7.7711	-	-
LL-W	29.2505	7.6280	6.8461	39.5871	11.6457	9.0353	-	-
DH-R	21.7400	9.8750	5.8400	22.3563	8.8978	5.4414	-	-
DH-W	30.0446	13.6739	10.6897	39.1994	14.5091	7.2753	-	-
LH-R	22.7150	14.2120	6.9024	23.1669	14.5276	6.9229	-	-
LH-W	26.4500	10.7526	8.5973	38.0473	17.1707	13.8098	-	-
2003 Experiment								
DL-R	20.6319	5.7826	3.5588	22.4021	6.8850	3.3615	5.72	43.61
DL-W	21.0251	8.3576	2.2574	28.3267	7.5624	6.9264	4.73	60.14
LL-R	22.7558	9.3068	3.5525	31.3218	7.5278	4.5831	2.36	68.70
LL-W	24.9323	10.5858	7.9844	45.2044	6.9665	13.6214	0.21	22.05
DH-R	20.5598	8.2766	4.3556	23.2306	7.0467	3.6538	1.47	96.42
DH-W	26.9193	14.4935	9.3649	36.3330	13.3906	10.2164	1.26	66.53
LH-R	21.1752	11.5505	5.5209	24.3467	13.4887	7.5940	1.56	192.42
LH-W	26.8413	11.6355	7.3249	45.1712	7.5647	12.5732	0.28	63.63

For wild caught *Reds* and *Whites*, L*a*b* values are derived from 14 and 13 animals, respectively, while n = 3 for carotenoid content as measured by absorbance at 470 nm. Individuals were selected as the darkest and lightest from each of the tank treatments, hence n = 1 for these values. R – Red; W – White; DL – dark substrate, low diet; LL – light substrate, low diet; DH – dark substrate, high diet; LH – light substrate, high diet.