The Journal of Experimental Biology 215, 343-350

doi:10.1242/jeb.064592

RESEARCH ARTICLE

Mechanisms of colour adaptation in the prawn *Penaeus monodon*

Nicholas M. Wade[1,*], Mike Anderson[1], Melony J. Sellars[1], Ron K. Tume[2], Nigel P. Preston[1] and Brett D. Glencross[1]

[1]Commonwealth Scientific and Industrial Research Organisation (CSIRO) Food Futures Flagship, Division of Marine and Atmospheric Research, Ecosciences Precinct, Dutton Park, QLD 4102, Australia and [2]CSIRO Food and Nutritional Sciences, Kessels Road, Coopers Plains, QLD 4108, Australia

*Author for correspondence (nick.wade@csiro.au)

Accepted 3 October 2011

SUMMARY

Exposure of prawns to dark- or light-coloured substrates is known to trigger a strong colour adaptation response through expansion or contraction of the colouration structures in the prawn hypodermis. Despite the difference in colour triggered by this adaptive response, total levels of the predominant carotenoid pigment, astaxanthin, are not modified, suggesting that another mechanism is regulating this phenomenon. Astaxanthin binds to a specific protein called crustacyanin (CRCN), and it is the interaction between the quantities of each of these compounds that produces the diverse range of colours seen in crustacean shells. In this study, we investigated the protein changes and genetic regulatory processes that occur in prawn hypodermal tissues during adaptation to black or white substrates. The amount of free astaxanthin was higher in animals adapted to dark substrate compared with those adapted to light substrate, and this difference was matched by a strong elevation of CRCN protein. However, there was no difference in the expression of *CRCN* genes either across the moult cycle or in response to background substrate colour. These results indicate that exposure to a dark-coloured substrate causes an accumulation of CRCN protein, bound with free astaxanthin, in the prawn hypodermis without modification of *CRCN* gene expression. On light-coloured substrates, levels of CRCN protein in the hypodermis are reduced, but the carotenoid is retained, undispersed in the hypodermal tissue, in an esterified form. Therefore, the abundance of CRCN protein affects the distribution of pigment in prawn hypodermal tissues, and is a crucial regulator of the colour adaptation response in prawns.

Key words: carotenoid, aquaculture, pigment.

INTRODUCTION

Changes in crustacean colouration can be due to physiological or morphological mechanisms. Physiological mechanisms that influence prawn colour include carotenoid availability in the diet, background substrate colour, photoperiod, light intensity and temperature (Rao, 1985). Such colour changes are often rapid, reversible and rhythmic, and are associated with hormonally controlled expansion and contraction of pigment structures, known as chromatophores, contained within the hypodermal layer, the pigmented layer in between the exoskeleton and abdominal muscle (Kleinholz, 1961; Fingerman, 1966; Rao, 1985; Rao, 2001). Chromatophores strongly influence crustacean colour, particularly in prawns that have thin, opaque shells. Morphological mechanisms that influence prawn colour, in contrast, are considered to involve quantitative modifications of exoskeletal pigment concentration or composition, and hence are thought to be slower and more permanent. These changes have been associated with particular crustacean life history stages (Wade et al., 2008) or interspecific differences in colour and patterning (Wade et al., 2009). The traditional view is that physiological and morphological mechanisms of colour change are independent of each other; however, the relationship between them has not been investigated.

Astaxanthin is the predominant carotenoid in penaeid prawns (Katayama et al., 1971; Katayama et al., 1972) and dietary inclusion levels to produce optimal pigmentation in aquaculture have been determined for many species (Negre-Sadargues et al., 1983; Negre-Sadargues et al., 1993; Yamada et al., 1990; Chien and Jeng, 1992). Colouration is dependent upon the presence of astaxanthin within hypodermal tissues (Menasveta et al., 1993; Boonyaratpalin et al., 2001) and must reach a critical level for optimal colouration. However, total hypodermal carotenoid content does not directly correlate with cooked prawn colour and it is the proportions of free and esterified astaxanthin that change in prawns in response to background substrate colour (Tume et al., 2009).

Within the exoskeleton and hypodermal tissue, astaxanthin is often bound within a multimeric protein complex called crustacyanin (CRCN). This interaction modifies the naturally red carotenoid, producing the diverse array of colours seen in the exoskeleton of crustaceans (reviewed in Chayen et al., 2003). During cooking, this interaction is disrupted, releasing the distinct red colouration of cooked seafood. Two genes have also been identified across a range of crustaceans that encode CRCN-A and CRCN-C (Wade et al., 2009). Expressed predominantly in the outer layer of the hypodermis, the spatial regulation of the *CRCN* genes is thought to be a major contributor to the colours and patterns that an individual can produce (Wade et al., 2009). Despite the central role that the CRCN protein and gene expression play in crustacean colouration, any potential role of CRCN in adaptive colouration in prawns has not been investigated.

In the present study, we compared the differences in colouration, carotenoid content, CRCN protein abundance and *CRCN* gene expression between groups of prawns both fed high dietary

astaxanthin levels but subject to either long-term black or white background colours. We used this system to investigate the underlying mechanisms of colour production and colour adaptation in crustaceans, and investigate the potential interplay between physiological and morphological mechanisms of colour change.

MATERIALS AND METHODS

Experimental setup and sampling

Live *Penaeus monodon* (Fabricius 1798) were obtained from commercial farms and maintained at CSIRO Marine and Atmospheric Research (CMAR) laboratories at Cleveland, Australia. For all trials, filtered seawater was heated and then pumped through the tanks at 1.2 l min^{-1}, maintaining water temperatures at 28°C and salinity at 35 g l^{-1}. Animals were held indoors in 1.9 m diameter 2000 l tanks covered with twin-walled tinted polycarbonate sheets (Suntuf, Capalaba, QLD, Australia) to reduce light intensity by approximately 80%, and under natural photoperiod, which was approximately 12.5 h:11.5 h light:dark during the course of the experiment.

Background colouration trial

To induce a strong difference in prawn colour, there were three replicate tanks painted black in colour and three replicate tanks painted white in colour, each containing 20 prawns. At the start of the experiment, animals of 13.9±4.2 g were stocked into experimental tanks and fed commercial prawn pellets (Ridley Grower, Ridley Aquafeed, Narangba, Australia) containing approximately 70 mg kg^{-1} Carophyll Pink (DSM Nutritional Products, Basel, Switzerland) to excess twice daily. Animals were maintained in these tanks for 6 weeks, which has been shown to provide optimal colouration in this species at this carotenoid concentration (R. Smullen, Ridley Aquafeed, personal communication). At the end of the trial, animals had grown to 22.8±4.6 g in size with 97% survival. Subsets of these animals were used to assess changes in: (1) colour, (2) hypodermal carotenoids, (3) abundance of hypodermal CRCN protein and (4) gene expression of CRCN in the hypodermis as outlined below.

Moult stage sampling

Penaeus monodon from the same population used to stock the background colouration trial were moult staged according to setal staging and epidermal withdrawal in uropods (Smith and Dall, 1985; Promwikorn et al., 2004). In total, six individuals from each of the seven classifications (A, B, C, D_0, D_1, D_2 and D_3) were sampled. Hypodermal tissue was dissected from each of the animals and used to assess the gene expression of CRCN across the moult cycle as outlined below.

Colour measurement

At 6 weeks, five animals from each of the six tanks in the background colouration trial were selected at random for colour assessment using a CR-300 colorimeter (Konica Minolta, Marunouchi, Tokyo, Japan) over a 1 cm diameter circle standardised under D65 illumination that most accurately replicates natural sunlight. The instrument uses the Commission Internationale de l'Eclairage (CIE) '*Lab*' system of colour notation (CIE, 2004) to measure the absolute colour of a sample on a three-dimensional scale of value, hue and chroma. The value of colour [or lightness (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 hue: positive a towards red, negative a towards green and positive b towards yellow, negative b towards blue.

Uncooked individuals were measured in triplicate along the lateral and dorsal sides of the first three abdominal segments and these measures were averaged to give one reading for each individual. Individual prawns were boiled for 2 min in water, and then re-measured in the same location to record the corresponding colour change induced by cooking. Subjective grade scoring was made under standardised fluorescent lighting using a Lineal Salmofan (DSM Nutritional Products, Kaiseraugst, Switzerland), which is the accepted international benchmark for prawn colour. Scores recorded for each animal were made in agreement by two researchers.

Carotenoid analysis and quantification

At 6 weeks, two animals from each of the six tanks in the background colouration trial were selected at random for carotenoid analysis and quantification. After removal of the exoskeleton, hypodermal tissue was dissected from the first three abdominal sections of uncooked prawns, freeze dried using an Alpha 1-2 freeze drier (Martin Christ, Osterode am Harz, Germany) and the dry masses of the tissues were recorded. Carotenoids were extracted from three different components of the target tissue: whole dissected hypodermal tissue, soluble protein extract from dissected hypodermal tissue, and the insoluble pellet from the dissected hypodermal tissue. Carotenoids were triple extracted from freeze-dried tissue by homogenisation, using an Ultraturrax TP 18-10 (IKA, Staufen, Germany), in equal volumes of iso-amyl alcohol and hexane. Pooled extracts were dried under nitrogen and resuspended in 200 µl of hexane, before being separated by HPLC using the methods and equipment of Tume et al. (Tume et al., 2009). Peaks of free astaxanthin, astaxanthin mono-esters or astaxanthin di-esters were quantified using Empower Pro software (Waters Corporation, Milford, MA, USA) with a peak width of 45 s and a threshold of 50–70 absorbance units. Peak areas were normalised against the dry mass of the original tissue extracted.

Protein analysis and quantification

At 6 weeks, hypodermal tissue was dissected from two individuals selected randomly from each of the six tanks in the background colouration trial. Tissue samples were homogenised into a neutral extraction buffer (150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris HCl pH 7.4, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, Complete mini-EDTA free protease inhibitors; Roche, Basel, Switzerland). The soluble fraction was retained and the insoluble material was washed in 5 ml MilliQ water, followed by two further washes in 1 ml MilliQ water. This washed material contained the majority of the blue colouration from the hypodermal tissue, and the proteins in this fraction were precipitated using 50% ammonium sulphate and centrifuged at 20,000 ***g*** for 15 min at 4°C. The dark blue pellet (λ_{max}=634 nm) was exchanged into phosphate-buffered saline using microcon 10,000 MWCO filters (Millipore, Billerica, MA, USA) and added back to the original soluble protein fraction to make the total soluble protein fraction. The insoluble pellet that remained was also used for carotenoid extraction as outlined above, along with the soluble fraction.

A detergent solution (1% Triton X-100, 0.5% IGEPAL CA-630 and 1% sodium dodecyl sulphate) was added to the total soluble protein fraction, which triggered a distinctive red-shift in the colour of the soluble protein fraction (λ_{max}=484 nm). Protein concentrations of the soluble protein extract were estimated using the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA), and 20 µg of total protein was separated on a precast 4–16% BisTris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). The Coomassie-stained gel was visualised on a Gel Doc XR gel

documentation system (Bio-Rad, Hercules, CA, USA) and bands were quantified using Quantity One analysis software (Bio-Rad). The four bands that had an average intensity of >700 optical density were selected for quantification across samples.

Gene expression analysis and quantification

At 6 weeks, RNA was extracted from hypodermal tissue from four individuals from each of the six tanks in the background colouration trial and from six individuals sampled for each of the seven moult stages. Total RNA was extracted using RNeasy RNA Extraction Columns and on-column DNase digestion (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Quality and quantity of all RNA samples were assessed by gel electrophoresis and on a Nanodrop spectrophotometer (Thermo Fisher Scientific), and were subsequently diluted to 100 ng μl^{-1}. An equivalent of 1 µg of total RNA was reverse transcribed following previously published protocols (Callaghan et al., 2010), which incorporated 400 pg of non-endogenous Luciferase RNA (Promega L4561, Venlo, The Netherlands) as an internal control. Expression of *pmCRCN-A* and *pmCRCN-C* as well as the control genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor-1 alpha (*EF1α*) and *Luciferase* were analysed in all samples by real-time PCR as described below.

Real-time PCR primers specific to *pmCRCN-A*, *pmCRCN-C* and control genes (Table 1) were designed with CLC Main Workbench (CLC Bio, Aarhus, Denmark). Real-time PCR reactions were carried out using 1× SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA, USA), 0.2 µmol l^{-1} of each primer and the equivalent of 7.5 ng of reverse-transcribed RNA. Amplification cycle conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 40 s at 60°C, and completed by a dissociation melt-curve analysis. Reactions were set up in triplicate using the epMotion 5070 (Eppendorf, Hamburg, Germany) and run on a 7500 Real-time PCR system (Life Technologies Corporation). Removal of gDNA contamination was verified by gene-specific PCR amplification of an equivalent amount of DNase treated RNA sample that had not been reverse transcribed. Optimal template concentration and PCR efficiencies for each primer pair were calculated using a fivefold serial dilution of a mixture of cDNA. Normalisation was performed against the internal *Luciferase* control gene using the $\Delta\Delta C_q$ method as this method has been shown to be the least biased approach (De Santis et al., 2010; Zhong et al., 2008). For comparison between treatments, expression of targets genes is shown as relative fold change against the *Luciferase* control.

Statistical analysis

Where comparison between individual measurements was required, such as uncooked colour, cooked colour, carotenoid levels, protein abundance or gene expression levels, statistical significance was assessed by single-factor ANOVA, followed by Fisher's exact test allowing 5% error. A linear regression was also used to determine whether the uncooked *L*, *a* or *b* values were able to predict the substrate the animals had been exposed to (*P. monodon* black *versus* *P. monodon* white). When comparing the amount of colour change in prawn exposed to the two substrates, the distance between two points in the three-dimensional colour space, known as ΔE, was calculated for each individual using the following formula:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}. \quad (1)$$

RESULTS AND DISCUSSION

Colour measurement

Uncooked colour

Exposure to black or white substrates over the experimental period caused a striking difference in colour of uncooked *P. monodon* (Fig. 1). Using the '*Lab*' colour space, *a* (positive=red, negative=green) and *b* (positive=yellow, negative=blue) define the colour, whereas *L* (lightness) defines the intensity of that colour. For the animals measured in this study, the *L* value ranged between 30 and 40; the *a* value was close to zero, indicating very little red or green; and the *b* value was negative, indicating the presence of blue (Fig. 2A). In general, *P. monodon* from the dark substrate were bluer and darker than their counterparts held on a white substrate. It was possible to distinguish between treatment groups of *P. monodon* on the basis of any of the *L*, *a* or *b* values and, when averaged, the measured values were all significantly different ($P<0.001$; Table 2). Despite this clear difference in average *L*, *a* or *b* values, when individuals were plotted (Fig. 2B–D), only the *L* value showed a clear delineation between the black and white substrate treatments (Fig. 2C,D). This indicated that the absolute colour of uncooked animals could not accurately differentiate between treatment groups, but the intensity of that colour was consistently different. A linear regression was also used to determine whether the uncooked *L*, *a* or *b* values were able to predict the substrate the animals had been exposed to (*P. monodon* black *versus* *P. monodon* white). We observed a significant regression for the uncooked *L* value ($P<0.001$), but this regression was not significant for either *a* or *b*.

Colour changes induced by cooking

The visible colour change induced by cooking was large (Fig. 1), with distinct increases in the *L* (lightness), *a* (redness) and *b* (yellowness) values of *P. monodon* on either the black or white substrate (Fig. 3A, Table 2). The uncooked colour began near zero or low negative values on the *a* and *b* scales, but once cooked, these values reached between positive 15 and 30 on both the redness and yellowness scales, reflecting the strong observed colour change. The

Table 1. Primers used in this study for quantitative real-time PCR amplification of specific gene targets

Primer name	Primer sequence (5′–3′)	Gene target	Accession number
PmCRCNA_RACE_F2	CTGCGTGTATTCCTGCATTG	PmCRCN-A	FJ498898
PmCRCNA_RACE_R1	AGGCACACCTGTCAATCGCTG	PmCRCN-A	FJ498898
PmCRCNC_RACE_F2	GTATCGGCGGTGATGGTA	PmCRCN-C	FJ498904
PmCRCNC_RACE_R1	GGAGTAGATGCAGGAGAAGTTC	PmCRCN-C	FJ498904
GAPDH F1	GAGGTGGTGGCTGTGAATGA	PmGAPDH	AI770197
GAPDH R1	GCCTTGACCTCCCCCTTGT	PmGAPDH	AI770197
EF1α F1	TCGCCGAACTGCTGACCAAGA	PmEF1α	DQ021452
EF1α R1	CCGGCTTCCAGTTCCTTACC	PmEF1α	DQ021452
LUC F1	GGTGTTGGGCGCGTTATTTA	Luciferase	Promega L4561
LUC R1	CGGTAGGCTGCGAAATGC	Luciferase	Promega L4561

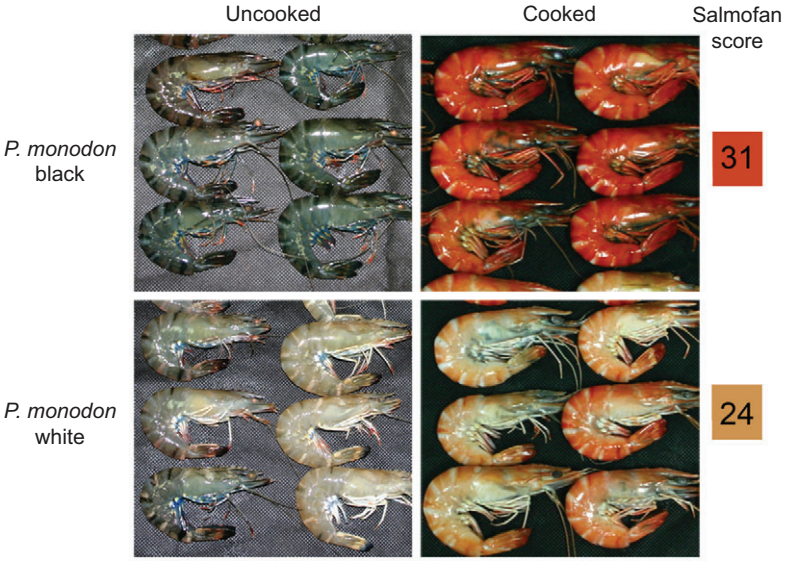

Fig. 1. Uncooked and cooked individual *Penaeus monodon* exposed to black or white substrates. Representatives of each treatment group, cooked and uncooked, showed a distinct colour difference that translated into a strong difference in Salmofan score. Note: relative positions of each individual in photos do not match.

ΔE was much smaller for *P. monodon* held on a white background compared with those held on a black background, indicating that these animals did not change colour as much during cooking, which was also reflected in the difference in subjective Salmofan colour chart score (Table 2).

Regardless of whether *P. monodon* were held on the black or the white background, the direction of this colour change was the same (indicated by the slope of the lines, Fig. 3A), even though the ΔE value was different. This indicates that the colour seen in animals from black and white substrates is produced by the same mechanism, but the intensity of this colour has changed. This is consistent with the *L* value being the distinguishing component of the colour scale, indicating that the colour itself is very similar but the intensity or lightness of that colour is different.

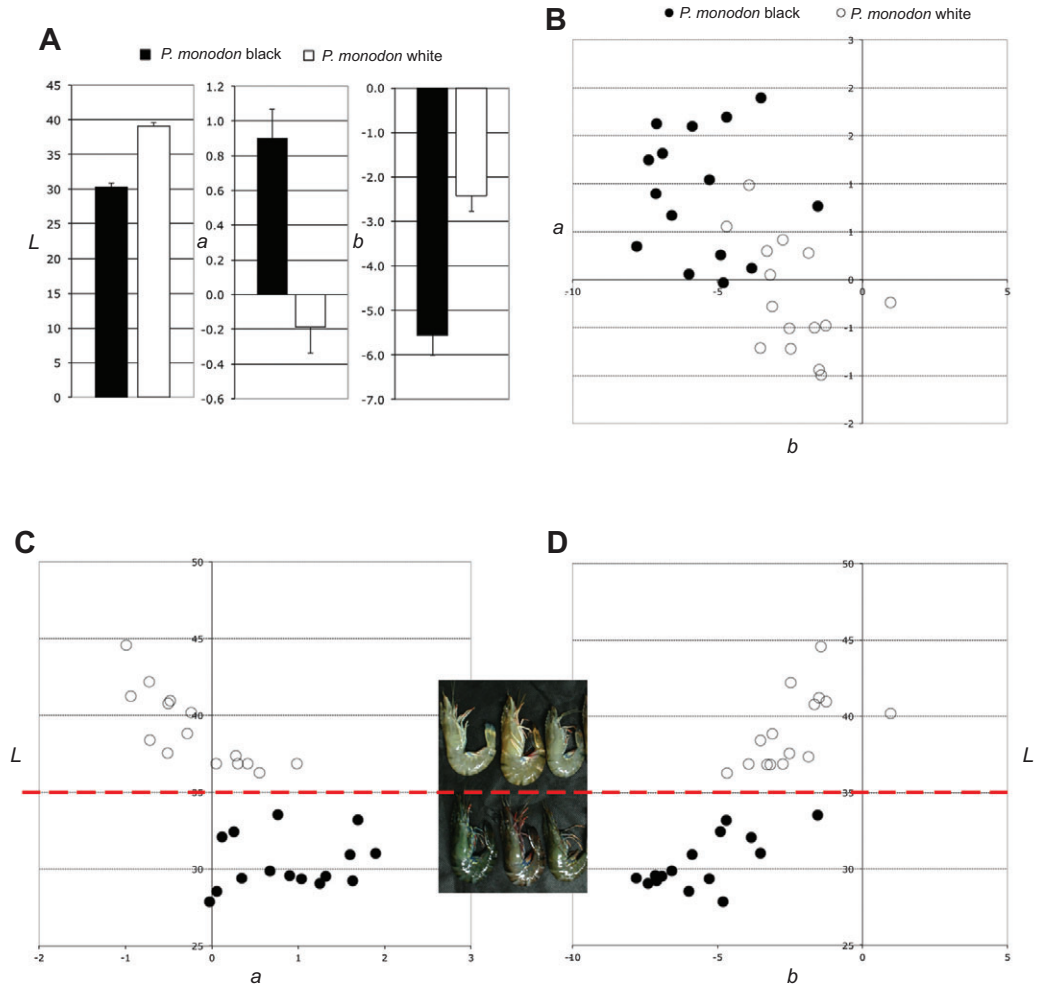

Fig. 2. Colour differentiation of uncooked *P. monodon*. (A) Average values of *L*, *a* and *b* for different treatment groups. Lightness, represented by *L*, has a scale of 0 (pure black) to 100 (pure white). The two components *a* and *b* determine the hue and distinguish opposing colours, where positive *a* is red, negative *a* is green, positive *b* is yellow and negative *b* is blue. The amount of these hues is known as chroma (or saturation). Treatment groups: black bars, *P. monodon* on black background; white bars, *P. monodon* on white background. (B–D) Two-dimensional plots of individual colour data from uncooked animals. *L* was the best differentiator of uncooked colour and was the only component that could predict the background colour. Treatment groups: filled circles, *P. monodon* on black background; open circles, *P. monodon* on white background.

Table 2. Colour space values for uncooked *Penaeus monodon* held on black or white substrates and the corresponding colour change induced by cooking

	Measured colour uncooked			Measured colour cooked			
Substrate	*L*	*a*	*b*	*L*	*a*	*b*	Salmofan colour score
Black	30.38±0.45	0.90±0.17	–5.56±0.44	44.26±0.34	31.02±0.60	26.70±1.08	31.13±0.31
White	39.05±0.64	–0.19±0.15	–2.41±0.35	53.70±0.82	15.89±1.11	18.39±0.57	23.80±0.63

a, red–green scale; *b*, blue–yellow scale; *L*, lightness. See Materials and methods, Colour measurement, for more details.
Significant differences (P<0.001) were observed for *L*, *a* and *b* values between the treatment groups *P. monodon* black and *P. monodon* white. These differences were consistent for measurements on uncooked or cooked individuals, and this cooked colour difference was also reflected in significantly different (P<0.001) observed colour score values.

Carotenoid analysis and quantification

Carotenoids were extracted from dissected hypodermal tissue and separated by HPLC. Astaxanthin accounted for >90% of the total carotenoids (data not shown), and peaks evaluated were only quantified from the free, mono-ester or di-ester forms of astaxanthin. Results on whole tissue showed that free astaxanthin levels were lower in *P. monodon* held on a white substrate compared with those on a black substrate (Fig. 4A), although these changes were not significant. There was also a corresponding increase in esterified astaxanthin in prawns exposed to white substrates, with astaxanthin mono-esters being the predominant form in these animals (55%). These trends were similar to those found by previous work in this species (Tume et al., 2009), which showed that exposure to black or white substrates triggered a change in the proportion of free astaxanthin, from 51.9 to 12.8%, with a corresponding increase in mono-esterified astaxanthin, from 20.8 to 66.2%. Discrepancies between these two data sets may be attributable to slight differences in the methodology of carotenoid extraction and analysis, such as not including the exoskeleton with the hypodermal fraction in this study or differences in peak threshold values. The similar trend seen in this study reinforced the conclusions of Tume et al. (Tume et al., 2009), indicating that esterified forms of astaxanthin play an important role in regulating colouration in *P. monodon*. This response has also been observed in natural colour morphs of the lobster *Panulirus cygnus* (Wade et al., 2005), suggesting that this mechanism is not restricted to prawns and potentially exists throughout decapod crustaceans.

In order to further investigate the role of free and esterified astaxanthin, the hypodermal tissue was separated into a soluble protein fraction or an insoluble pellet fraction. The pellet fraction showed no difference in quantitative levels of any carotenoid class across the two treatments, and was mostly (>90%) composed of astaxanthin esters (Fig. 4B). In contrast, the free astaxanthin content of the soluble fraction was 3.6-fold lower in prawns exposed to white substrates compared with those exposed to black substrates (P<0.002), and this fraction contained predominantly (80–85%) free astaxanthin (Fig. 4C). These data demonstrate that astaxanthin esters (mono- and di-esters) are contained in an insoluble pellet fraction of the hypodermis, and the majority of the free astaxanthin is found in the soluble protein fraction and shows quantitative differences in response to changes in background substrate colour. This indicates that the soluble protein fraction may be central to the colour changes that are observed in response to background substrate colour in *P. monodon*.

Protein analysis and quantification

The soluble protein fraction was isolated from hypodermal tissue from prawns exposed to both black and white substrates. The colour was initially extracted under native conditions and had a peak absorbance in the blue spectrum at 634 nm, which was very similar to the characteristics of the protein previously identified in this species (Nur-E-Borhan et al., 1995) and also to the properties of α-crustacyanin isolated from lobster shells (Wald et al., 1948; Buchwald and Jencks, 1968). This native extract was denatured using detergents, which triggered a distinctive colour shift from 634 to 484 nm, also typical of disrupting the CRCN–carotenoid interaction. Once purified, the soluble protein fraction had a strong difference in the total absorbance at 484 nm (A_{484}) mg^{-1} protein

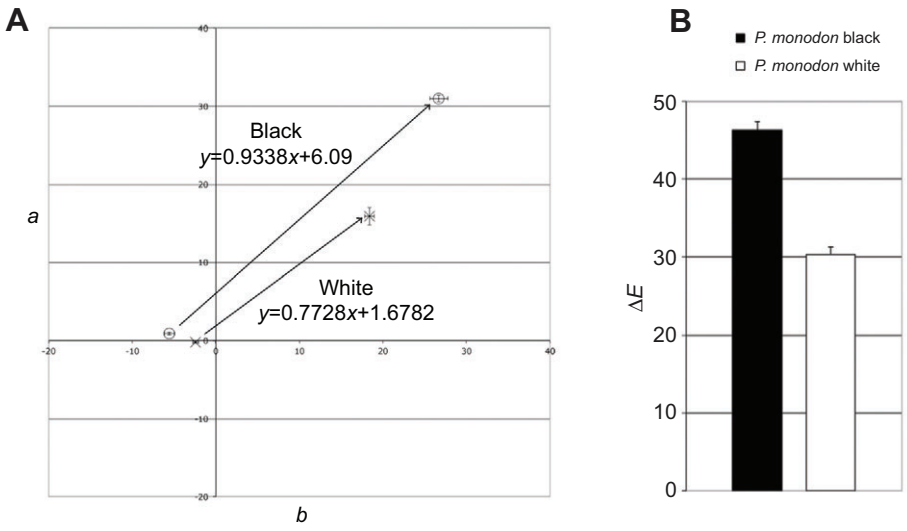

Fig. 3. Effect of exposure to black or white substrates on cooked colour of *P. monodon*. (A) Two-dimensional plot of the average movement within the *a versus b* colour space for each group during cooking. As a result of cooking, each group shifted into strongly positive *a* and *b* values, indicating the presence of red and yellow hues. The direction of colour change of the prawn exposed to black or white substrates was the same. (B) The distance between two points in three-dimensional space (ΔE) showed that the colour change of the prawns exposed to white substrates did not approach that of the prawns exposed to black substrates, indicating a strong difference in the intensity of the cooked colour.

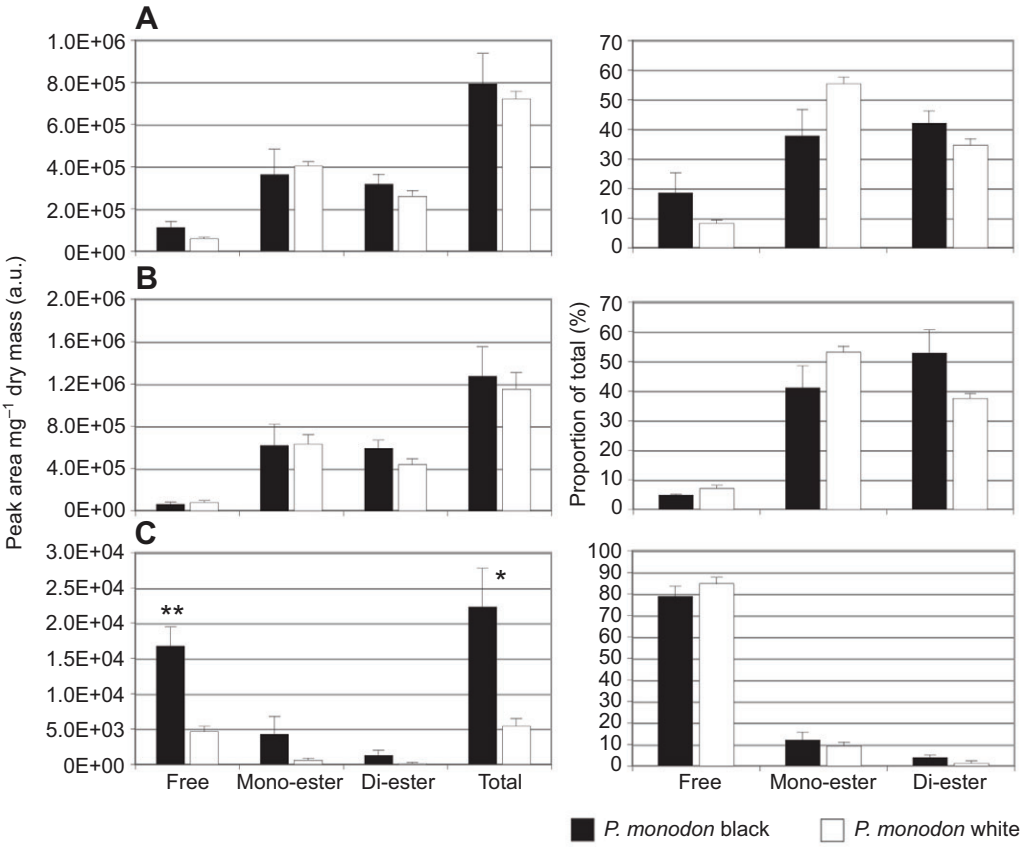

Fig. 4. Carotenoid quantification from hypodermal tissue from *P. monodon* exposed to black or white substrates. Relative quantification and percent distribution of free astaxanthin and astaxanthin esters for (A) whole hypodermal tissue, (B) insoluble pellet from hypodermal tissue and (C) the soluble protein fraction from hypodermal tissue. Total carotenoids extracted from whole tissue was similar in prawns exposed to either black or white substrates, but there was a greater proportion of astaxanthin esters in prawns exposed to white substrates. Although this effect was not significant, this trend was the same as significant effects observed in previous studies (Tume et al., 2009). The insoluble fraction contained almost exclusively astaxanthin esters, and there was no quantitative difference between substrates in any carotenoid group. Meanwhile, the soluble protein fraction contained greater than 80% free astaxanthin, and there was a significant difference in the relative quantity of free astaxanthin between prawns exposed to black or white substrates. **P*<0.02; ***P*<0.002.

between the black (0.283±0.035) and the white (0.108±0.010) treatments.

Equal amounts of these protein samples were separated on the basis of size on a polyacrylamide gel, and total proteins were visualised using a total protein stain (Fig. 5). Of the bands quantified, there was a distinct quantitative difference in a 21 kDa protein across prawns exposed to black or white substrates (Fig. 5), with no change in the quantity of bands 65, 43 or 39 kDa in size. This 21 kDa protein was recognised by a specific CRCN antibody (data not shown), and is the same size as CRCN from this species (Nur-E-Borhan et al., 1995) as well as other crustaceans (Zagalsky, 1985). However, it is important to note that the A and C subunits cannot be distinguished, neither on the basis of size using one-dimensional gel electrophoresis, nor by the CRCN antibody, which is likely to detect both subunits with equal

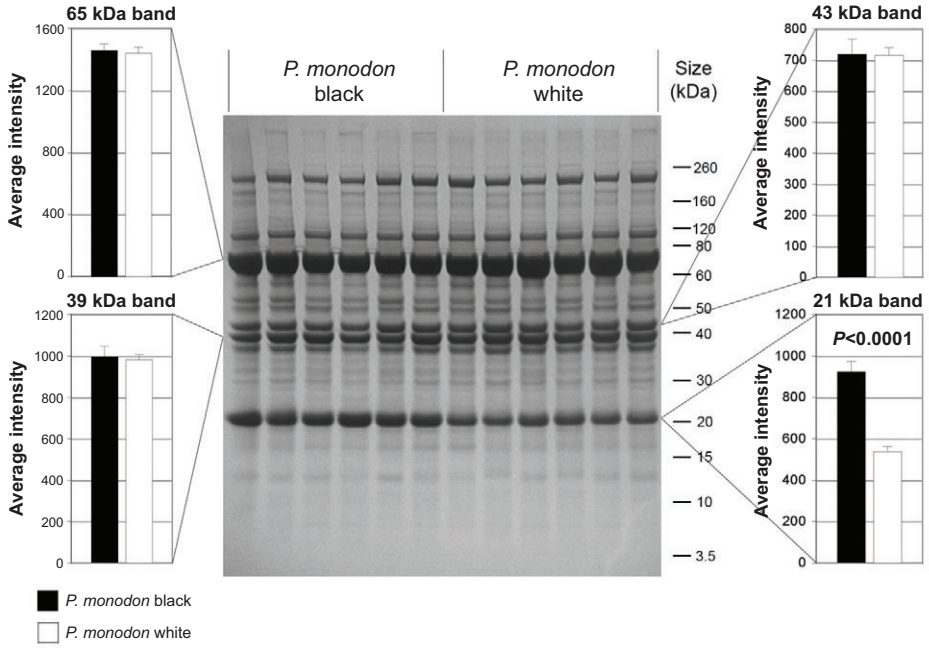

Fig. 5. Quantification of soluble protein extracts from *P. monodon* hypodermal tissue exposed to black or white substrates. Equal amounts of soluble protein extract from six prawns exposed to either black or white substrates were separated on the basis of size on a 4–16% polyacrylamide gel. Band intensity was quantified for four bands of different sizes as shown. The total abundance of one band of approximately 21 kDa in size was significantly different between the treatments. This band was the same size as the CRCN protein from other crustaceans, and also cross-reacted with a specific anti-CRCN antibody (data not shown).

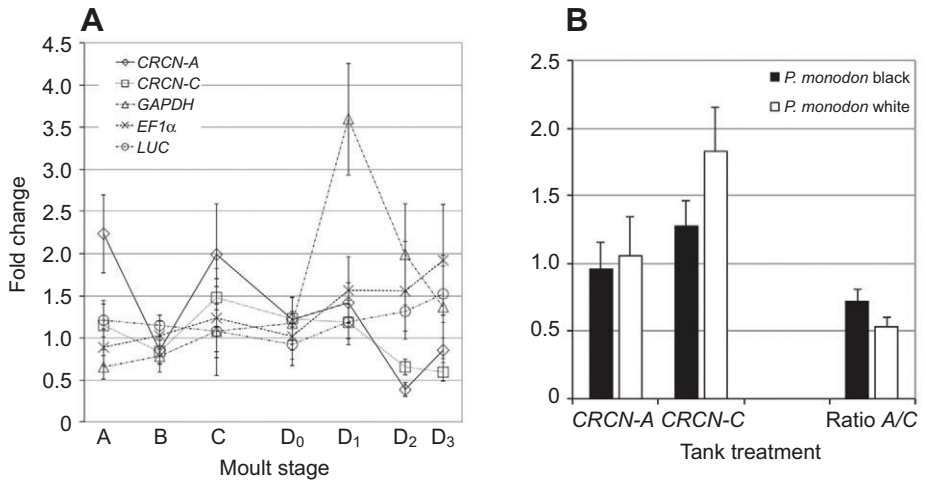

Fig. 6. Quantification of *CRCN-A* and *CRCN-C* gene expression. (A) Changes in gene expression of *CRCN-A* and *CRCN-C* across stages of moulting in *P. monodon*, in comparison with the control genes *GAPDH*, *EF1α* and *Luciferase* (*LUC*). Very small fold changes were observed for the *CRCN* genes across the prawn moult cycle. This was not the case for *GAPDH*, which showed a sharp spike in expression during the D_1 stage of the moult cycle. (B) Relative fold-change expression levels of *CRCN-A* and *CRCN-B* genes in *P. monodon* exposed to black or white substrates. There was no significant difference in expression level of either *CRCN-A* or *CRCN-C*.

affinity. Relative proportions of the subunits within the CRCN complex may be worthy of investigation using more sensitive techniques, such as two-dimensional gel electrophoresis.

These data show a clear difference in the abundance of the colour protein CRCN as a result of exposure to a black or white substrate. Differences in CRCN protein abundance are consistent with the measured difference in free astaxanthin from the soluble protein fraction, indicating that CRCN bound with free astaxanthin is producing a large proportion of the colour in prawn hypodermis, and is central to the regulation of colouration in prawns. At this point it is not possible to say whether the observed difference in CRCN protein abundance is due to degradation or relocation to another tissue. Detection of CRCN protein in other prawn tissues in response to background treatment has not been attempted. No knowledge exists on the rate at which CRCN protein is accumulated or lost during the rapid expansion or contraction of chromatophore pigments.

Gene expression analysis and quantification

To investigate the genetic control of colouration in prawns, the expression of the two *CRCN* genes was measured across the moult cycle and also in response to black or white substrates. Expression of the *CRCN* genes was stable across the moult cycle in *P. monodon* (Fig. 6A). Relative fold changes across the moult stages were very small, and both genes were evenly expressed in all stages. The *CRCN-A* gene showed the greatest variation in expression, with two small peaks of expression at the A and C stages, as well as a short period of lower expression at D_2 stage. Although not as strong, the *CRCN-C* gene also showed two small peaks in expression at the C stage, and weaker expression at pre-moult stages D_2 and D_3.

Initial evidence using northern blots suggested that there might have been a short post-moult (A stage) activation of *CRCN* expression in the freshwater prawn *Macrobrachium rosenbergii* (Wang et al., 2007). Here we demonstrate very little change in expression across the moult cycle or in response to background substrate colour in *P. monodon*. We conclude that these subtle changes in *CRCN* gene expression level are unlikely to be attributable to a biological function in prawns. The control gene *GAPDH* showed a strong spike of expression during pre-moult stage D_1 (Fig. 6A), suggesting that this gene undergoes strong increases in expression at specific times in the moult cycle. This gene is commonly used as a control gene for quantitative PCR, but these data suggest that this gene should not be used for normalisation of gene expression in prawns.

Results in *P. monodon* adapted to black or white substrates showed that fold-change differences in the expression level of either *CRCN-A* or *CRCN-C* were very small, and both genes were evenly expressed (Fig. 6B). There was a slight increase in *CRCN-C* expression in those animals exposed to the white substrate; however, this was not significant. Despite a marked colour difference and a strong change in CRCN protein abundance, expression of either *CRCN* gene showed little variation in prawns exposed to either black or white substrates. The ratio of the expression of the two genes also remained constant. The lack of transcriptional regulation of the CRCN gene suggests a post-translational regulation mechanism or translocation of CRCN protein.

Conclusions

The data presented here demonstrate that the basis of the adaptive background colour response, and the regulation of crustacean colouration, goes beyond a simple expansion or contraction of the pigment structures. This study showed that there is clear interplay between physiological and morphological mechanisms of colouration in crustaceans. When adapted to a dark substrate, prawns with dilated chromatophores also accumulate CRCN protein bound with free astaxanthin within hypodermal tissues. Conversely, in white-substrate-adapted animals with constricted chromatophores, the CRCN protein is depleted, but the astaxanthin is retained in the hypodermal tissue in esterified form. This adaptive response to background colour has a profound impact on the intensity of the prawn's external appearance, and this is reflected in the cooked prawn colour and corresponding colour grade score. The abundance of the CRCN protein is central to the adaptive colour response in prawns, yet there is a distinct lack of regulation of this process at the gene expression level.

LIST OF SYMBOLS AND ABBREVIATIONS

a	red–green scale
b	blue–yellow scale
CIE	Commission Internationale de l'Eclairage
CMAR	CSIRO Marine and Atmospheric Research
CRCN	crustacyanin
CSIRO	Commonwealth Scientific and Industrial Research Organisation
EF1α	elongation factor-1 alpha
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
L	lightness
ΔE	distance between two points in the three-dimensional colour space

ACKNOWLEDGEMENTS

The authors would like to thank Gold Coast Marine Aquaculture for supply of experimental animals, Nick Polymeris for his assistance with experimental tank setup and animal husbandry, Joanne Mountford for her assistance with carotenoid quantification and Simon Tabrett for his helpful discussion on the regulation of prawn colouration.

FUNDING

This work is supported by funding from DSM Nutritional Products and the CSIRO Food Futures Flagship.

REFERENCES

Boonyaratpalin, M., Thongrod, S., Supamattaya, K., Britton, G. and Schlipalius, L. E. (2001). Effects of β-carotene source, *Dunaliella salina*, and astaxanthin on pigmentation, growth, survival and health of *Penaeus monodon*. *Aquac. Res.* **32**, 182-190.

Buchwald, M. and Jencks, W. P. (1968). Optical properties of astaxanthin solutions and aggregates. *Biochemistry* **7**, 834-843.

Callaghan, T. R., Degnan, B. M. and Sellars, M. J. (2010). Expression of sex and reproduction-related genes in *Marsupenaeus japonicus*. *Mar. Biotechnol. (NY)* **12**, 664-677.

Chayen, N. E., Cianci, M., Grossmann, J. G., Habash, J., Helliwell, J. R., Nneji, G. A., Raftery, J., Rizkallah, P. J. and Zagalsky, P. F. (2003). Unravelling the structural chemistry of the colouration mechanism in lobster shell. *Acta Crystallogr. D Biol. Crystallogr.* **59**, 2072-2082.

CIE (2004). *Colorimetry*, 3rd edn. Publication CIE No. 15, 79 pp. Vienna, Austria: Commission Internationale de l'Eclairage.

De Santis, C., Smith-Keune, C. and Jerry, D. R. (2010). Normalizing RT-qPCR data: are we getting the right answers? An appraisal of normalization approaches and internal reference genes from a case study in the finfish *Lates calcarifer*. *Mar. Biotechnol. (NY)* **13**, 170-180.

Fingerman, M. (1966). Neurosecretory control of pigmentary effectors in crustaceans. *Am. Zool.* **6**, 169-179.

Katayama, T., Hirata, K. and Chichester, C. O. (1971). The biosynthesis of astaxanthin-IV. The carotenoids in the prawn, *Penaeus japonicus* Bate (Part I). *Bull. Jpn. Soc. Sci. Fish.* **37**, 614-620.

Katayama, T., Kitama, T. and Chichester, C. O. (1972). The biosynthesis of astaxanthin in the prawn, *Penaeus japonicus* Bate (Part II). *Int. J. Biochem.* **3**, 363-368.

Kleinholz, L. H. (1961). Pigmentary effectors. In *The Physiology of Crustacea*, *Sense Organs, Integration and Behaviour*, Vol. 2 (ed. T. H. Waterman), pp. 133-169. New York: Academic Press.

Menasveta, P., Worawattanamateekul, W., Latscha, T. and Clark, J. S. (1993). Correction of black tiger prawn (*Penaeus monodon* Fabricus) coloration by astaxanthin. *Aquac. Eng.* **12**, 203-213.

Negre-Sadargues, G., Castillo, R. and Lenel, R. (1983). Endocrine elements and metabolism of carotenoid pigments in crustacea. *Bull. Soc. Zool. Fr.* **108**, 277-288.

Negre-Sadargues, G., Castillo, R., Petit, H., Sance, S., Martinez, R. G., Milicua, J., Choubert, G. and Trilles, J. (1993). Utilization of synthetic carotenoids by the prawn *Penaeus japonicus* reared under laboratory conditions. *Aquaculture* **110**, 151-159.

Nur-E-Borhan, S. A., Okada, S., Watabe, S. and Yamaguchi, K. (1995). Carotenoproteins from the exoskeleton and the muscular epithelium of the black tiger prawn *Penaeus monodon*. *Fish. Sci.* **61**, 337-343.

Promwikorn, W., Kirirat, P. and Thaweethamsewee, P. (2004). Index of moult staging in the black tiger prawn (*Penaeus monodon*). *Songklanakarin J. Sci. Technol.* **26**, 765-772.

Rao, K. R. (1985). Pigmentary effectors. In *Integuments, Pigments and Hormonal Processes*, Vol. 9 (ed. D. E. Bliss and L. H. Mantel), pp. 395-462. New York: Academic Press.

Rao, K. R. (2001). Crustacean pigmentary-effector hormones: chemistry and functions of RPCH, PDH, and related peptides. *Am. Zool.* **41**, 364-379.

Smith, D. M. and Dall, W. (1985). Moult staging the tiger prawn *Penaeus esculentus*. In *Second Australian National Prawn Seminar* (ed. P. C. Rothlisberg, B. J. Hill and D. J. Staples), pp. 85-93. Cleveland, Australia: CSIRO.

Tume, R. K., Sikes, A. L., Tabrett, S. and Smith, D. M. (2009). Effect of background colour on the distribution of astaxanthin in black tiger prawn (*Penaeus monodon*): effective method for improvement of cooked colour. *Aquaculture* **296**, 129-135.

Wade, N. M., Goulter, K. C., Wilson, K. J., Hall, M. R. and Degnan, B. M. (2005). Esterified astaxanthin levels in lobster epithelia correlate with shell colour intensity: potential role in crustacean shell colour formation. *Comp. Biochem. Physiol.* **141B**, 307-313.

Wade, N. M., Melville-Smith, R., Degnan, B. M. and Hall, M. R. (2008). Control of shell colour changes in the lobster, *Panulirus cygnus*. *J. Exp. Biol.* **211**, 1512-1519.

Wade, N. M., Tollenaere, A., Hall, M. R. and Degnan, B. M. (2009). Evolution of a novel carotenoid-binding protein responsible for crustacean shell color. *Mol. Biol. Evol.* **26**, 1851-1864.

Wald, G., Nathanson, N., Jencks, W. P. and Tarr, E. (1948). Crustacyanin, the blue carotenoprotein of the lobster shell. *Biol. Bull.* **95**, 249-250.

Wang, M. R., Zhu, X. J., Yang, J. S., Dai, Z. M., Mahmood, K., Yang, F. and Yang, W. J. (2007). Prawn lipocalin: characteristics and expressional pattern in subepidermal adipose tissue during reproductive molting cycle. *Comp. Biochem. Physiol.* **147B**, 222-229.

Yamada, S., Tanaka, Y., Sameshima, M. and Ito, Y. (1990). Pigmentation of prawn (*Penaeus japonicus*) with carotenoids. I. Effect of dietary astaxanthin, beta-carotene and canthaxanthin on pigmentation. *Aquaculture* **87**, 323-330.

Zagalsky, P. F. (1985). Invertebrate carotenoproteins. *Methods Enzymol.* **111**, 216-247.

Zhong, Q., Zhang, Q., Wang, Z., Qi, J., Chen, Y., Li, S., Sun, Y., Li, C. and Lan, X. (2008). Expression profiling and validation of potential reference genes during *Paralichthys olivaceus* embryogenesis. *Mar. Biotechnol. (NY)* **10**, 310-318.