Optimising External Colour in Farmed Crustaceans, using *Penaeus monodon* as a model species.

Dr Nick Wade and Dr Brett Glencross

Project No. 2011/731

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

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Non-Technical Summary

2011/731 Optimising External Colour in Farmed Crustaceans, using *Penaeus monodon* as a model species.

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This project has developed and validated three novel methods to quantify the colour of uncooked or cooked prawns. These methods were used to define the colour variation that existed in farmed prawns, and determined it was largely due to variation between ponds. This effect is likely to produce significant variability in product colour and therefore quality across a single farm, as well as between different farms. A number of interacting factors may have a role in causing this variation, including diet, harvest methods, pond substrate, the pond algal community and possibly harvest stress. High levels of a pigment protein have previously been shown to be critical for producing consistently dark coloured prawns, and this study developed a highly sensitive assay to quantify this protein from pigmented tissues. However, the abundance of this protein was not correlated with variation in prawn colour, indicating that variation in this protein was not responsible for the colour variation in farmed prawns. This work also quantified the effects of different harvest practices on prawn colour, and demonstrated that extended periods of ice storage, freeze thawing of uncooked product or harvesting into white holding bins was detrimental to cooked prawn colour. Through collaboration with another Seafood CRC project, we were able to demonstrate that changes in pigment prior to processing are retained through cooking and storage. Based on these research findings, a series of pre-harvest recommendations have been developed to optimise colour in farmed prawns.

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PROJECT OBJECTIVES:

1. Assess the natural variation in uncooked and cooked colour of farm reared *Penaeus monodon*.
2. Quantify the underlying colour protein abundance and carotenoid content that may underlie this variability.
3. Investigate the ability of short-term dark substrate exposure to minimise the on-farm colour variability.

OUTCOMES ACHIEVED

- Quantification of the colour variation of farmed *Penaeus monodon* and proof that significant colour variation exists in farmed prawns.
- Demonstration that the levels of colour protein extracted and quantified from prawn tissues does not underlie the observed variation in prawn colour.
- Demonstration that prawns lose significant colour if exposed to white substrates, stored on ice or frozen prior to cooking during harvest.
- Demonstration that on-farm exposure to dark coloured harvest bins can improve cooked prawn colour.
- Indication that maintaining high dietary carotenoid levels prior to harvest is effective at producing consistently dark coloured prawns, but this benefit can easily be lost by using certain harvest methods.
- Consistently darker pigmented prawns with increased product quality, value and consumer acceptance.

LIST OF OUTPUTS PRODUCED

- Database of prawn colour measurements for more than 2000 individuals and the degree of colour variation quantified across these individuals.
- Presentation of results at APFA/ABFA conference in 2012 and 2013.
- A series of pre-harvest recommendations for optimising prawn colour, with a fact sheet summary to be produced for industry.
- Two peer-reviewed scientific papers in draft form.

ACKNOWLEDGEMENTS

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1 Introduction and Background

This application was developed to address a specific concern raised by the prawn aquaculture industry, and based on the expertise and strong research outcomes of CSIRO projects in the area of prawn colouration. Consistent, deep red coloured cooked prawns are highly sought after in the Australian market and accordingly fetch premium market prices. Past research has focussed on identifying the optimal dietary carotenoid inclusion levels and the duration of feeding for a number of species, both of which are essential for producing the best prawn colour. Despite these efforts, strong colour variation remains in farmed reared animals, both within and across ponds. Inclusion of carotenoids in prawn diets is expensive, and the benefits to prawn health and colour must be maximised to justify this investment. This project has been developed with this in mind, and seeks to define the amount of colour variation that exists in farmed prawns, as well as test a recently established method for manipulating prawn colour. As such, this project matches the objectives of the Seafood CRC Production Innovation program, and is a direct application of novel research findings to maximise existing industry production. Through the course of developing this application, industry members expressed that depth and consistency of colour was often an over-riding factor in their production. Despite improvements in production costs from things such as improved growth, feed conversion ratio or energy savings, if the colour quality was not maintained the product would not be well received and hence would not demand a high price.

1.1.1 Colouration in crustaceans

Carotenoids are central to producing colouration in many crustacean tissues such as the shell and epithelial tissue, eggs and ovaries, and hepatopancreas (Castillo et al., 1982; Dall et al., 1995a; Lenel et al., 1978). Crustaceans are unable to synthesise carotenoids and must obtain them from their diet (Lee, 1977), although they are able to transform ingested carotenoids into a limited range of closely related derivatives, for example β-carotene into astaxanthin (Axn) or modification of Axn into Axn esters (Mantiri et al., 1995; Negre-Sadargues, 1978; Tanaka et al., 1976). The predominant carotenoid responsible for colouration of hypodermal tissue and the exoskeleton is Axn, in association with a protein called crustacyanin (CRCN). When protein bound, the colour of the Axn is modified from red to any other colour in the visible spectrum, from red and orange to green, purple and blue (Wade et al., 2009). When cooked, this interaction is disrupted, releasing the red colour once again, and providing the distinct red colouration of cooked seafood.

Changes in crustacean colouration can be due to physiological or morphological mechanisms. Physiological mechanisms include carotenoid availability in the diet, background substrate colour, photoperiod, light intensity or temperature (Rao, 1985). Such changes are often rapid, reversible, rhythmic and under the control of eyestalk hormones (Kleinholz, 1961; Rao, 2001). By contrast, morphological colour changes are slower and more permanent, involving modifications of exoskeletal pigment concentration or composition. Although these changes may define cross-species differences in colour and patterning (Wade et al., 2009), we also know that morphological mechanisms respond to physiological cues such as background substrate colour (Wade et al., 2012).

1.1.2 Colouration of Prawns

Axn forms the predominant carotenoid in prawns (Katayama et al., 1971; Katayama et al., 1972), where it is found in the digestive gland, exoskeleton and hypodermis (the pigmented layer in between the exoskeleton and abdominal muscle). Colouration is dependant largely upon the amount of Axn present within these tissues (Boonyaratpalin et al., 2001; Menasveta et al., 1993), however large variations in colour have been observed. Incorporation of Axn in the diet at up to 200 mg/kg has been shown to be most effective for optimal colouration in *P. monodon* (Negre-Sadargues et al., 1983; Negre-Sadargues et al., 1993; Yamada et al., 1990), however dietary carotenoid concentration can be reduced by increasing duration of carotenoid intake from feed (R. Smullen, pers. comm.). Prawns reach a critical level for optimal colouration of around 20-30 mg/kg total body carotenoid content, however, although useful as a guide, total carotenoid content does not correlate well with subjective prawn colour grade scores (Tume et al., 2009). Along with colouration at harvest, dietary carotenoid deficiencies are thought to be responsible for certain diseases, such as Blue Color Syndrome (Howell and Matthews, 1991; Menasveta et al., 1993). The benefits of carotenoids also extend beyond colouration, and include improved survival, reproduction, growth, immune response and hypoxia tolerance (Yamada et al., 1990; Dall, 1995b; Linan-Cabello et al., 2002; Supamattaya et al., 2005; Kumar et al., 2009; Niu et al., 2012; Niu et al., 2014).

Most prawns have thin opaque shells, and colour is present in the hypodermal layer in pigment structures, known as chromatophores (Rao, 1985). These chromatophores are under hormonal control and their expansion and contraction strongly contributes to the degree of individual colouration, as well as allowing a rapid and reversible response to background colouration (Fingerman, 1965; Fingerman, 1966; Robison Jr and Charlton, 1973). Exposure to light or dark background substrates has been shown to alter the proportion of Axn within the hypodermis, where dark substrates triggered an increase in the proportion of free Axn, and light substrates caused an increase in Axn esters, while total carotenoid content was the same (Tume et al., 2009; Wade et al., 2012). This adaptive colour response in prawns is potentially useful to the prawn farming industry as a simple means of optimising prawn pigmentation and reducing individual colour variability. The type and distribution of specific Axn esters appears critical in this process.

1.1.3 Genetics of Colouration

Crustacyanin (CRCN) is a multimeric protein complex that was originally isolated from the exoskeleton of the clawed lobster *Homarus gammarus* (Wald et al., 1948). This large molecular weight complex, called α-crustacyanin is composed of eight β-crustacyanin subunits, which itself is a dimer formed by two types of CRCN subunits (A and C) in association with two Axn molecules (reviewed in Chayen et al., 2003). The interaction of CRCN with Axn within this complex changes the colour of Axn from red (λ_{max} = 472 nm in hexane) to purple as seen in β-crustacyanin (λ_{max} = 580 - 590 nm) or blue in the case of α-crustacyanin (λ_{max} = 632 nm) (Buchwald and Jencks, 1968). Elevation of levels of CRCN protein abundance in the hypodermis of *P. monodon* was shown to be the mechanism that underlies the adaptive response of prawns to black substrates (Wade et al., 2012). The presence of this protein in high levels was essential for producing high cooked prawn colour grade scores. Two CRCN genes have been identified across a range of crustaceans that encode for *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 CRCN gene expression plays in crustacean colour patterning, there was no role of CRCN gene expression in adaptive colouration in prawns (Wade et al., 2012).

1.1.4 Colour Measurement

Figure 1. The Lab system of colour notation.

The Commission Internationale de l'Eclairage (CIE) *'Lab'* system of colour notation (Publication CIE No 15, 2004) measures the absolute colour of a sample on a three dimensional scale of value, hue and chroma (Fig. 1). 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 hue, 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 lobsters (Melville-Smith et al., 2003; Tlusty, 2005; Wade et al., 2008), and also more recently to prawns (Wade et al., 2012). The use of digital images to quantify colour is widespread, and has been successfully used to quantify shell pigments in mangrove crabs (Todd et al., 2011).

1.2 Need

Consistency of colour as well as overall colour intensity are essential elements to seafood product acceptability, marketability and dollar value. This is particularly true for the deep red colour of cooked crustaceans. Farmed crustacean species commonly have suboptimal colour consistency and/or colour intensity. There is an industry driven need to therefore optimise colour consistency and intensity of farmed crustacean product.

Farmed crustacean colour is enhanced by a critical but costly feed additive, the carotenoid astaxanthin. The increased outlay in production is offset by gains in market value of between $2 to 5 / kg, in the case of prawns. To achieve consistent premium colour grade scores, our most recent research in Penaeid prawns and hard-shelled lobsters showed that the cooked colour of the animal is not related solely to the total carotenoid content of the animal, but it is essential for the carotenoid to be bound with high levels of a colour protein called crustacyanin (CRCN).

To expand our scientific knowledge in this area, future research needs to extend beyond carotenoid inclusion levels in diets. It must begin to explore natural variation in abundance of this novel colour protein complex or simple methods that can increase the protein abundance and enhance colour. This baseline information will support the development of commercial procedures that maximise crustacean colour consistency and intensity, allowing farmers to maximise product quality, price, marketability and acceptability.

1.3 Objectives

1. Assess the natural variation in uncooked and cooked colour of farm reared *Penaeus monodon*.
2. Quantify the underlying colour protein abundance and carotenoid content that may underlie this variability.
3. Investigate the ability of short-term dark substrate exposure to minimise the on-farm colour variability.

2 Methods

2.1.1 Summary of Experiments

A range of on farm experiments were performed to validate methods, define natural prawn colour variation, assess the effects of different harvest methods and test the impact of different substrates on cooked prawn colour. Farm samples incorporated the timing and practices of a commercial farm operation, and placed no demands on which ponds were harvested or the level of dietary carotenoid intake. Samples were taken at random and opportunistically from harvest bins before being measured across the various experiments. The use of colorimeters to quantify colour was prohibitively slow for some experimental objectives, and quantification of prawn colour from digital images was developed to enable rapid assessment of changes in prawn colour in response to background substrates. In addition, samples from a controlled tank based experiment, funded by CSIRO and DSM Nutritional Products, were used to assess pigment retention after harvest. The methods and source animals used to perform each experiment has been summarised in Table 1 below. Further details of each experiment are provided in the sections below.

Table 1 Overview of the animals and methods used for various experiments in this project.

Experiment	Colour Quantification Method Used	Animal Source	Dietary Carotenoid Level
Quantitative measurement of prawn colour	Colorimeter and digital images	single farm, single pond	50 ppm
Colour variation across farms and ponds	colorimeter	4 farms, up to 7 ponds	50 ppm
Effect of different harvest methods	colorimeter	2 farms, 2 ponds	50 ppm
Quantification of colour proteins	Digital images and western blot	single farm, 8 ponds	80 ppm
Exposure to black or white substrates	Digital images	single farm, 3 ponds	80 ppm

2.1.2 Quantitative and Subjective Measurement of Prawn Colour

Prawn colour was quantified using the average colour of the first three abdominal segments measured using three different methods. The first used a Minolta CR-400 Chroma Meter with an 8 mm aperture and D65 illumination at a 10˚ angle. The second used a HunterLab Mini Scan XE colorimeter with a 10 mm aperture and D65 illumination at a 45˚ angle. The third method used digital images taken at a distance of 40 cm using a Cannon D-400 (Cannon) fitted with an 18 mm lens, with fixed settings of ISO1600, aperture F22 and 1/100th sec shutter speed. Animals were photographed in a 38 x 50 cm light box illuminated with 2 x 8W 30 cm Fluroglow single reflector full spectrum aquarium lights (AquaOne). Average RGB values were calculated across a 3600 pixel square from the first three abdominal segments using ImageJ software (Schneider et al. 2012). Where necessary, image intensity was adjusted between photographs using the MacBeth ColorChecker that was positioned in each photograph (Supplementary Figure 1A). Subjective scoring was performed against both the Lineal Salmofan (DSM Nutritional Products) and

Australian Tiger Prawn Colour Chart (Aquamarine Marketing) under standardised illumination by experienced researchers.

Validation of the digital image method was performed by quantification of the MacBeth colour checker, Salmofan and prawn colour chart values measured from 10 independent photographs (Supplementary Figure 1, Supplementary Table 1). Comparison of the three different methods was performed using the colour values quantified from the same randomly selected 45 cooked prawns, each measured using three different methods. Due to the size of the animals, colour quantification for the 45 animals from digital images was performed across three photographs containing 15 animals each.

2.1.3 Colour Variation Within and Across Ponds and Across Farms

Prawn colour variation was assessed from different ponds from the one farm. Fifty prawns were selected at random from holding bins immediately after harvesting from different ponds. The average *Lab* Hunterlab Miniscan XE reading from the first 3 abdominal segments was used as the measure of colour for individual prawns. Individuals were tagged, colour measured uncooked, then cooked in commercial salt brine boilers and re-measured on the cooked prawns. All animals were from domesticated stocks of the same genetic origin, and fed the same commercial diet according to an optimal pigmentation regime that incorporated 50 ppm astaxanthin for at least 4 weeks before harvest and sampling. Comparison of prawn colour was also performed from a further four farms using 40 randomly sampled cooked animals and measured using a Minolta CR-400 chroma meter. Animals had been harvested from a mixture of different ponds and processed at each separate farm on the day of sampling. To assess colour variation between groups, each individual *L*, *a* and *b* colour value was standardised by subtracting the mean value of the entire group. These individual *delta L*, *delta a* and *delta b* values were used to assess the mean and variance for each group of animals. This also allowed effective comparison of measurements performed using different colorimeters despite their difference in absolute colour value.

2.1.4 Effect of Harvest Method on Colour

To measure the effect of harvesting prawns live in chilled seawater, prawns from the same pond were held live in aerated 12˚C filtered seawater in large covered 800 L bins. Twenty animals were collected immediately after harvesting, individually tagged and colour measured a using a HunterLab Miniscan XE. These same 20 animals were recovered and re-measured at 30 min, 1 hour, 2 hours and 4 hours after the initial measurement. Similarly, to measure the effect of harvesting prawns into an ice slurry, 20 prawns were individually tagged immediately after harvesting and colour measured a using a HunterLab Miniscan XE. These animals were held in an ice slurry and filtered seawater and the colour of each one re-measured every hour over an eight-hour period. The change in absolute colour over time for both these groups was calculated by subtracting the average initial *Lab* value from each of the measured *Lab* values of the 20 prawns at each time point. These individual *delta L*, *delta a* and *delta b* values were used for comparison over time.

To assess the effect of freeze-thawing on uncooked prawn colour, 50 prawns were colour measured uncooked using a HunterLab Miniscan XE, then frozen for one day, thawed at room temperature for 1 hour and colour re-measured. To assess the effect of ice slurry storage on cooked prawn colour, 50 cooked prawns were colour measured using a HunterLab Miniscan XE, then placed in an ice slurry for 14

hours, then colour measured again. The change in absolute colour for these treatments was calculated by subtracting the average initial *Lab* value from each of the measured *Lab* values of the 50 prawns after being re-measured. These individual *delta L*, *delta a* and *delta b* values were used for comparison before and after treatment.

2.1.5 Quantitative Measurement of Prawn Colour Proteins

The abundance of the colour protein CRCN was quantified from six individual prawns from eight different ponds, each sampled on the same day from the same farm. Individual prawns were photographed and uncooked colour quantified using digital images as outlined above. Epithelial tissue was dissected from the first and second abdominal segments, and homogenised in 2 ml water containing the Complete protease inhibitor cocktail (Roche) using a Precellys 24 (Bertin Technologies). Insoluble material was removed by centrifugation at 13 000 x g for 5 min at 4ºC, and the total soluble protein was denatured by adding SDS to a final concentration of 0.1% and then measured by BCA assay (Pierce). Equal amounts of protein were loaded in triplicate onto a 96-well dot blot apparatus (Bio-Rad) and drawn by vacuum onto Hybond LFP PVDF membrane (GE Healthcare). Membranes were blocked for 1 hour at room temperature in 5% skim milk powder in PBS 0.1% Tween20 (PBST) before incubation for 1 hour at RT with a rabbit anti-CRCN primary antibody diluted 1:2000 in blocking solution. Membranes were washed 3 x 10 min in PBST, then incubated for 1 hour at RT in goat anti-rabbit CY5 (GE Healthcare) diluted 1:2500 in blocking solution and finally washed 3 x 10 min in PBST. The fluorescent signal was detected on dried membranes using the Typhoon 9400 Imaging System (GE Healthcare) with laser power set at 600 PMT. The average spot intensity for each individual was quantified using the three triplicates across two independent membranes using the Quantity One 1-D Analysis Software, and the abundance of CRCN protein for each individual calculated relative to the average intensity of all the samples.

2.1.6 Pre-harvest Exposure to Black or White Substrates

To assess any effects of harvesting into different coloured bins on farm, prawns from the same pond were transferred to either a black or a white lined 800 L plastic bin containing aerated seawater at 12˚C. Sixty animals were sampled immediately after harvest, then cooked and colour measured using digital images or subjective scoring. Further groups of sixty animals were sampled from both bins at 30, 60, 120 or 180 minutes and cooked and colour measured. The average RGB colour of the twenty prawns in each of three digital images was quantified separately to give three replicate colour values at each time point. These average RGB values were used to create a colour square that represented the average abdominal colour of each group of twenty animals. The average RGB value of all sixty animals was used to assess the change in prawn colour over time. The average subjective colour grade score of the sixty prawns used also to assess colour change over time. This process was repeated three times at the same farm on separate days using animals from different ponds on each of the three days.

2.1.7 Post-harvest Retention of Pigment Through Freezer Storage

To investigate whether pre-harvest changes in prawn colour are retained through freezer storage, animals from a previous CSIRO funded experiment in collaboration with DSM Nutritional Products were used (Wade et al 2012b). A total of

402 animals that had been grown for six weeks on black or white tanks and fed four different dietary carotenoid levels were colour measured using a Minolta CR-400 chroma meter. The number of animals in each treatment group is shown in Table 2. Animals were held under optimal storage conditions below -30˚C for 217 days, then re-measured using the same colorimeter. These samples were measured in collaboration with Sue Poole and Carl Paulo from the Queensland Department of Fisheries and Forestry (QDAFF). The average *Lab* values for the animals in each of the 8 groups was used to assess colour retention before and after freezer storage.

Table 2 – Prawns measured to assess colour retention through freezer storage. Animals were held in either black or white coloured tanks for different periods of time while they were fed different amounts of dietary astaxanthin supplement. A total of 402 animals from an experiment previously run and funded by CSIRO and DSM Nutritional Products were used to assess whether improvements in pigment were retained during freezer storage.

Tank Colour	Astaxanthin supplement (ppm)	Days on supplement and number of prawns sourced			
		5 days	10 days	20 days	40 days
White	0	15	12	12	9
White	25	15	12	12	12
White	50	15	12	11	12
White	100	15	12	12	12
Black	0	15	12	12	12
Black	25	15	11	12	12
Black	50	15	12	11	12
Black	100	15	12	12	12

2.1.8 Statistical Analysis

Where required, statistical significance was assessed by single factor analysis of variance (ANOVA), followed by Tukey's HSD test allowing 5% error. F-Test for significant differences in variance between two groups was performed after Kolmogorov-Smirnov/Lilliefor Test for data normality. All statistical analyses were performed using StatPlus:Mac 2009 (AnalystSoft Inc, 2009).

3 Results

3.1 Methods for Measuring Prawn Pigmentation

3.1.1 Validation of Image Based Quantification

The quantification of prawn colour from digital images was performed using standardized illumination from a constant distance using optimized settings on a Canon 400-D digital camera. To allow photographs to be compared, each photo contained a Macbeth colour checker array, as well as the cooked prawn colour chart and lineal Salmofan (Figure 2). The average colour was quantified using ImageJ (Schneider et al. 2012) from within a square of equal size. Colour was standardized across photographs by adjusting the image colour until the measured RGB values of the Macbeth colour checker squares matched the actual RGB values for each colour. To validate this method, the average RGB values for each square of the Macbeth ColorChecker, the Lineal Salmofan and the first 3 segments of the prawns on the Prawn Colour Chart were also quantified using the average across 10 separate images. These average RGB values showed very little variation across photographs (Table 3) and were combined to produce the measured colours that reproduced the actual colours of the charts (Figure 2), as well as accurately reproduce the colours of the Salmofan and prawn colour chart (Table 4).

Table 3. Validation of image based quantification. Absolute RGB values for each MacBeth Colorchecker square compared with the values quantified from 10 independent photos. The combined RGB values produced the corresponding colours as shown in the table. The relationship between expected and measured RGB values is shown in figure 2A.

MacBeth ColorChecker	Expected Values				Measured Values			
	R	G	B		R	G	B	
dark skin	115	82	68		92 ± 1.33	58 ± 1.31	51 ± 1.39	
light skin	194	150	130		177 ± 1.29	123 ± 1.49	110 ± 1.29	
blue sky	98	122	157		87 ± 1.33	105 ± 1.41	160 ±1.70	
foliage	87	108	67		83 ± 2.31	101 ± 1.34	75 ± 2.23	
blue flower	133	128	177		134 ± 1.85	125 ± 1.92	182 ± 1.27	
bluish green	103	189	170		91 ± 2.98	176 ± 1.60	186 ± 1.68	
orange	214	126	44		181 ± 1.35	81 ± 2.47	38 ± 2.89	
purplish blue	80	91	166		70 ± 1.93	71 ± 1.75	141 ± 1.28	
moderate red	193	90	99		186 ± 1.60	73 ± 1.72	80 ± 1.50	
purple	94	60	108		88 ± 0.85	56 ± 0.56	107 ± 0.96	
yellow green	157	188	64		141 ± 0.95	182 ± 1.74	71 ± 2.21	
orange yellow	224	163	46		225 ± 1.74	146 ± 1.46	59 ± 1.50	
blue	56	61	150		42 ± 1.76	49 ± 1.53	120 ± 1.45	
green	70	148	73		40 ± 1.30	122 ± 1.50	74 ± 1.64	
red	175	54	60		183 ± 1.39	54 ± 1.99	53 ± 1.75	
yellow	231	199	31		221 ± 1.91	187 ± 1.87	53 ± 2.51	
magenta	187	86	49		199 ± 1.08	85 ± 1.87	142 ± 1.74	
cyan	8	133	161		36 ± 1.96	114 ± 1.67	173 ± 1.84	
white	243	243	242		238 ± 1.58	239 ± 1.78	230 ± 1.96	
neutral 8	200	200	200		204 ± 1.64	208 ± 2.20	200 ± 1.89	
neutral 6.5	160	160	160		159 ± 1.51	161 ± 1.92	161 ± 1.88	
neutral 5	122	122	121		125 ± 1.59	123 ± 2.03	125 ± 2.00	
neutral 3.5	85	85	85		89 ± 3.02	83 ± 3.48	92 ± 3.49	
black	52	52	52		51 ± 2.80	48 ± 3.21	51 ± 3.34	

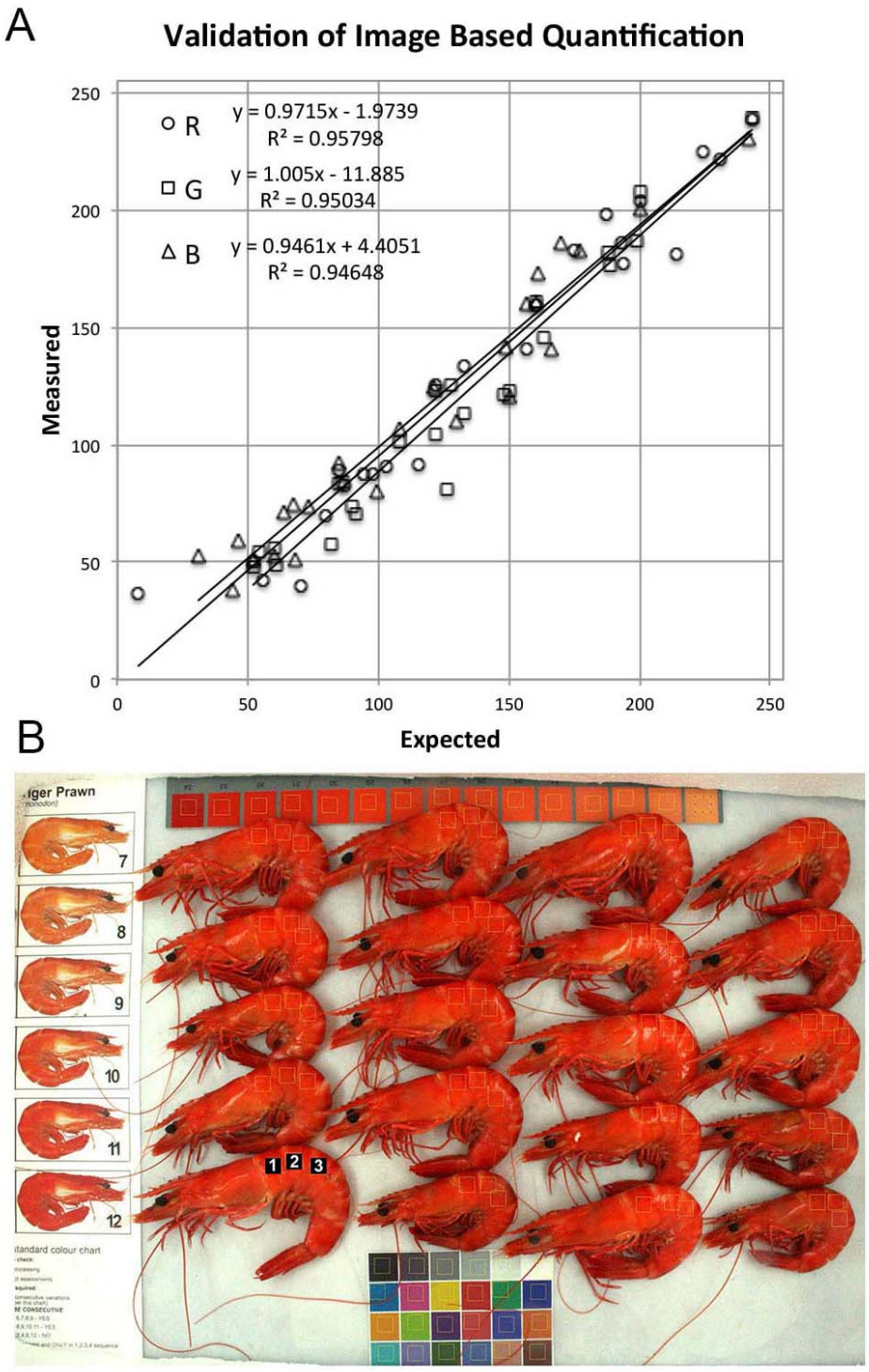

Figure 2. Validation of Colour Quantification from Digital Images. Photos were taken under standardized light and camera settings, and included the Prawn Colour Chart, Salmofan and MacBeth colour checker array as colour references. A. Images were normalized so that the measured colour values of the Macbeth colour checker were strongly correlated with the absolute RGB colour values for each square. B. The colour of each individual prawn was quantified from an average of 3 equally sized squares (as shown on one of the animals). The RGB colour values for the Salmofan and Prawn Colour chart measured from 10 different photographs showed little variation between photographs and the measured colours reproduced the chart colours very well.

Table 4 Validation of using digital images to quantify prawn colour. Each RGB value represents the average RGB value of an equal sized square that was quantified from either the salmofan or prawn colour chart photographed across 10 individual photos. The RGB values were then used to produce the colour square on the right.

	Average			
Salmofan	R	G	B	
20	251 ± 0.79	148 ± 3.45	83 ± 2.77	
21	254 ± 0.28	132 ± 2.45	76 ± 1.94	
22	254 ± 0.34	118 ± 2.19	62 ± 1.56	
23	251 ± 0.89	105 ± 2.00	57 ± 1.33	
24	250 ± 0.93	92 ± 2.23	48 ± 1.46	
25	252 ± 0.72	84 ± 1.99	41 ± 1.29	
26	249 ± 0.95	75 ± 1.42	34 ± 1.08	
27	252 ± 0.66	70 ± 1.80	28 ± 1.07	
28	245 ± 1.79	58 ± 2.01	23 ± 1.02	
29	247 ± 1.73	53 ± 2.20	21 ± 1.20	
30	239 ± 2.43	45 ± 2.10	21 ± 1.47	
31	235 ± 2.49	42 ± 1.65	20 ± 1.41	
32	233 ± 2.27	43 ± 1.58	23 ± 1.33	
33	227 ± 2.59	37 ± 1.73	18 ± 1.40	
34	195 ± 2.74	26 ± 1.88	18 ± 1.77	
	Prawn Colour Chart			
	R	G	B	
PCC 7	244 ± 1.93	118 ± 1.64	51 ± 1.61	
PCC 8	245 ± 1.56	113 ± 3.19	60 ± 2.51	
PCC 9	245 ± 1.46	99 ± 2.57	58 ± 2.00	
PCC 10	246 ± 1.30	78 ± 1.81	45 ± 1.33	
PCC 11	244 ± 1.65	66 ± 2.48	44 ± 1.98	
PCC 12	242 ± 1.92	58 ± 1.73	44 ± 1.46	

3.1.2 Comparison of Quantitative Methods to Measuring Prawn Colour

Regardless of the method, individual prawn colour for each animal was quantified using the average of the first 3 segments of the animal. There was considerable difference between the average *Lab* values for the prawns measured with the different colorimeters (Table 5). This was expected given the different light incident angles that the machines have for measurement. Despite the difference in absolute values, a very strong relationship between the *Lab* values was observed for individual prawns measured with the two colorimeters (Figure 3A). However, a simple linear model was not sufficient to convert *Lab* values from one machine to the *Lab* values of the other (data not shown).

Table 5. Absolute colour values of cooked prawns using 3 different methods.

	Measured Values		
Minolta CR-400	*L*	*a*	*b*
Prawns 1-45	52.67 ± 0.49	33.47 ± 0.51	39.13 ± 0.45
HunterLab Miniscan XE	*L*	*a*	*b*
Prawns 1-45	44.95 ± 0.49	36.15 ± 0.54	35.71 ± 0.58
ImageJ	R	G	B
Prawns 1-45	219.76 ± 3.36	67.68 ± 2.18	39.16 ± 1.41

Colour quantification from digital images was very accurate and reproducible, and the colours of the MacBeth colorchecker (Figure 2A), as well as the Salmofan and prawn colour chart (Table 4) were reliably reproduced from 10 different images. Similarly, image quantification also reliably reproduced the colour scales of the Lineal Salmofan and the Australian Tiger Prawn Colour Chart (Table 4), which are the internationally recognised subjective methods for grading prawn colour. However, when the RGB values from the images were converted to *Lab* values (converted RGB) there was no relationship with the Lab values measured using the colorimeters (Figure 3B). Our results define the average colour values of prawns measured using the different methods. They demonstrate that quantitative differences in individual prawn colour could be detected by either colorimeter or digital images. However, at present the values from different techniques cannot be accurately interconverted, particularly those from digital images.

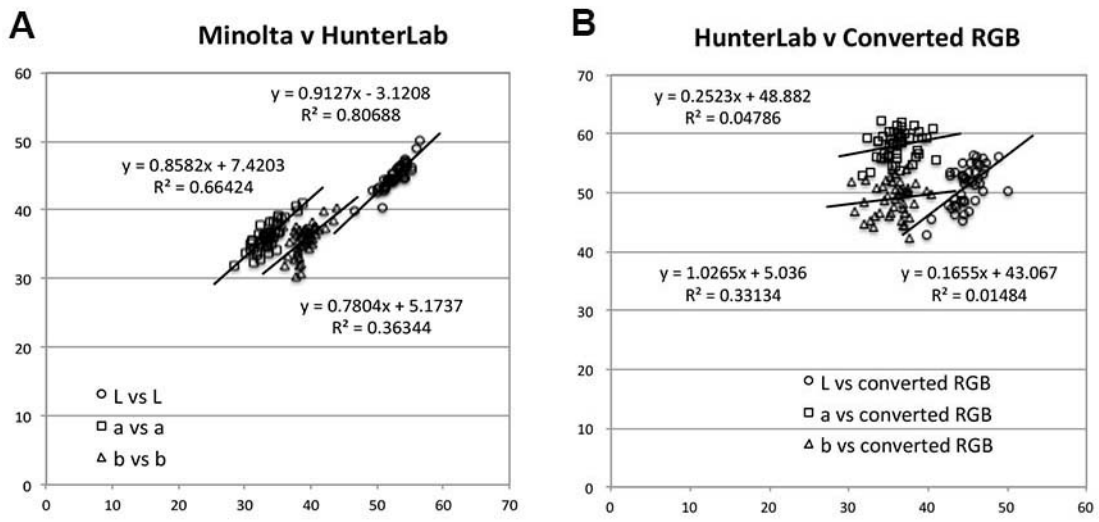

Figure 3. Comparison of quantitative methods of measuring prawn colour. The colour of the same 45 cooked prawns was quantified using two different colorimeters and also digital images. A. Comparison of the absolute *Lab* values taken using a Minolta CR-400 and a HunterLab Miniscan XE colorimeter. B. Comparison of the absolute *Lab* values taken using a Minolta CR-400 colorimeter and the absolute RGB values quantified from digital images that had been converted to Lab values using mathematical formulas.

3.2 Variation in Colour of Farmed *Penaeus monodon*.

3.2.1 Colour Variation Within and Across Ponds

Farmed prawns showed considerable variation in uncooked and cooked colour. The mean *Lab* values were significantly different between animals from different ponds, for both their uncooked colour and their cooked colour. For uncooked prawns, the *L* values were particularly informative. Data showed that the L values of uncooked prawns from ponds 2 and 3 were significantly higher than those from ponds 1 and 4 (Table 6, Figure 4), which indicated that prawns from ponds 2 and 3 were significantly lighter in colour than those from ponds 1 and 4. The mean *a* and *b* values of cooked prawns were most informative, and significant differences were observed between animals from different ponds (Table 6, Figure 5). Higher *a* and *b* values indicated the presence of more red and yellow hues, respectively, and therefore the more highly pigmented prawns. Groups of prawns from different ponds also showed different amounts of colour variation within each group. The variance of *L* and *a* values of cooked colour was significantly higher for some ponds than for others (Table 6), and was reflected by the greater spread of the interquartile range for some ponds (Figure 5). This indicated there was a greater amount of individual colour variation in some ponds compared with others. Interestingly, *b* values did not show any significant differences in variance between any of the ponds.

Table 6. Absolute Lab Colour Values for Farmed Prawns from Different Ponds

	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Pond 6	Pond 7	All Ponds
Uncooked Mean								
L	14.25^{a}	18.76^{b}	18.54^{b}	12.75^{a}	15.70^{a}	16.83^{b}	15.36^{a}	16.02
a	1.67	1.43	2.72	1.62	1.76	2.45	2.33	2.00
b	3.57	3.63	6.08	4.36	3.11	5.34	3.36	4.21
Uncooked Variance								
L	4.54	5.11	7.78	4.40	10.17	7.63	10.72	11.75
a	1.72	1.25	0.76	1.16	2.62	2.39	1.61	1.89
b	10.68	5.97	9.35	9.03	7.42	11.69	9.65	4.05
Cooked Mean								
L	40.51^{a}	41.64^{b}	41.57^{c}	39.63^{a}	40.44^{a}	40.49^{a}	42.62^{d}	40.98
a	40.10^{a}	38.32^{bcg}	38.74^{bcg}	42.80^{d}	36.13^{ef}	36.42^{ef}	37.84^{g}	38.62
b	40.00^{ad}	37.72^{b}	35.68^{cg}	40.29^{ad}	30.88^{e}	33.18^{fg}	34.55^{cfg}	36.04
Cooked Variance								
L	2.34^{a}	5.40^{b}	2.34^{c}	4.81^{d}	4.58^{cd}	8.35^{ce}	5.22^{bcde}	5.65
a	6.13^{a}	10.87^{b}	6.00^{ac}	8.34^{ab}	10.41^{b}	10.11^{b}	7.18^{abc}	12.80
b	10.68	9.08	9.35	13.41	11.48	11.69	9.65	21.17

Superscripts denote significant ($P<0.05$) differences among ponds within a parameter. Lack of any superscripts within a row indicates that there were no significant differences among any of those measurements for that parameter.

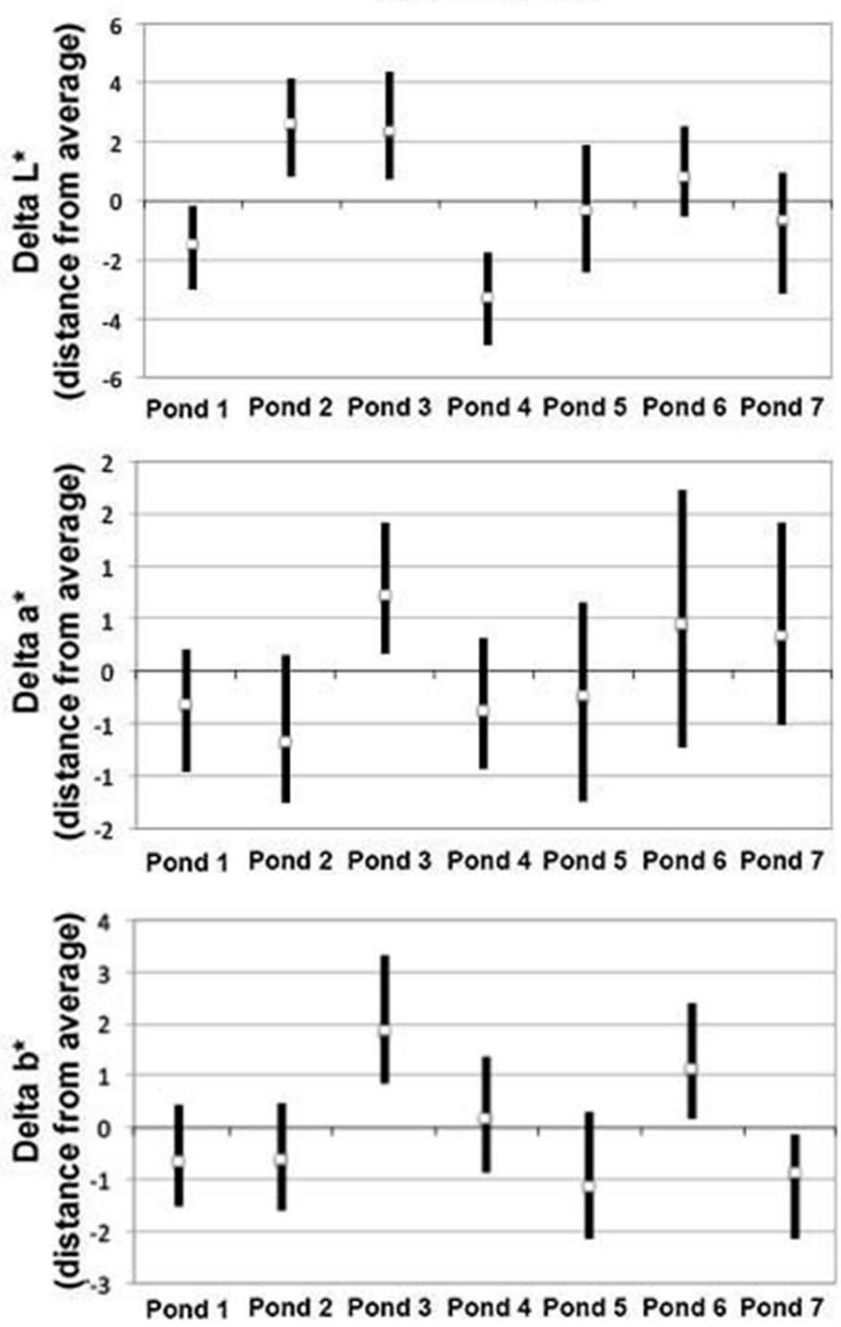

Figure 4. Colour Variation Within Uncooked Prawns Across Ponds. The median (square) and Q1-Q3 interquartile range (bars) distribution of delta *Lab* values from fifty uncooked prawns from 7 different ponds. The delta *Lab* values were calculated as the difference in the value of each individual from the average of all the animals across the 7 ponds.

Some weak correlations were observed between uncooked *Lab* values and cooked *Lab* values, the best of which was a negative correlation between uncooked *L* value and cooked *a* value ($r^2 = 0.161$). This indicated that an increase in *L* value of an uncooked prawn would result in a decrease in the *a* or 'redness' value of the cooked prawn. This is best demonstrated in Pond 4, where the average *L* value of uncooked prawns was low (Figure 4), but this translated into a higher *a* and *b* values when cooked (Figure 5) and therefore a much redder prawn colour.

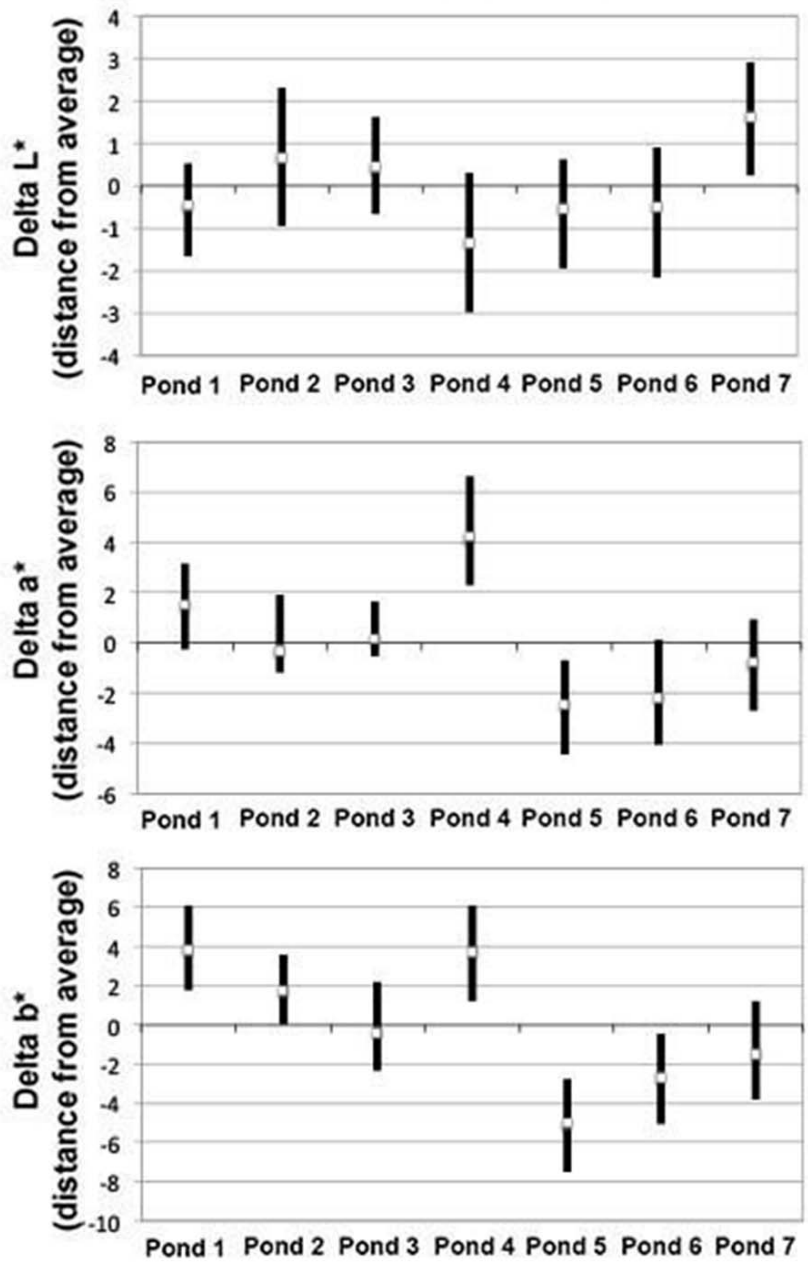

Figure 5. Colour Variation Within Cooked Prawns Across Ponds. The median (square) and Q1-Q3 interquartile range (bars) distribution of delta *Lab* values from fifty cooked prawns from 7 different ponds. The delta *Lab* values were calculated as the difference in the value of each individual from the average of all the animals across the 7 ponds.

3.2.2 Colour Variation Across Farms

When comparing cooked prawn colour across farms, similar variation was observed. The absolute *a* and *b* values of cooked prawns was significantly different between animals from a further four farms showed marked variation in their cooked *Lab* values (Figure 6 A-C). Using Farm 1 as an example, the high *L* value combined with a low *a* value indicated that on average a paler and less red animal was recorded from that farm. This was also combined with a large variation in that colour, as shown by the variation in *L* and *a* values. Meanwhile, the above average *a* and *b* values recorded for animals from Farm 4 indicated much more pigment was present.

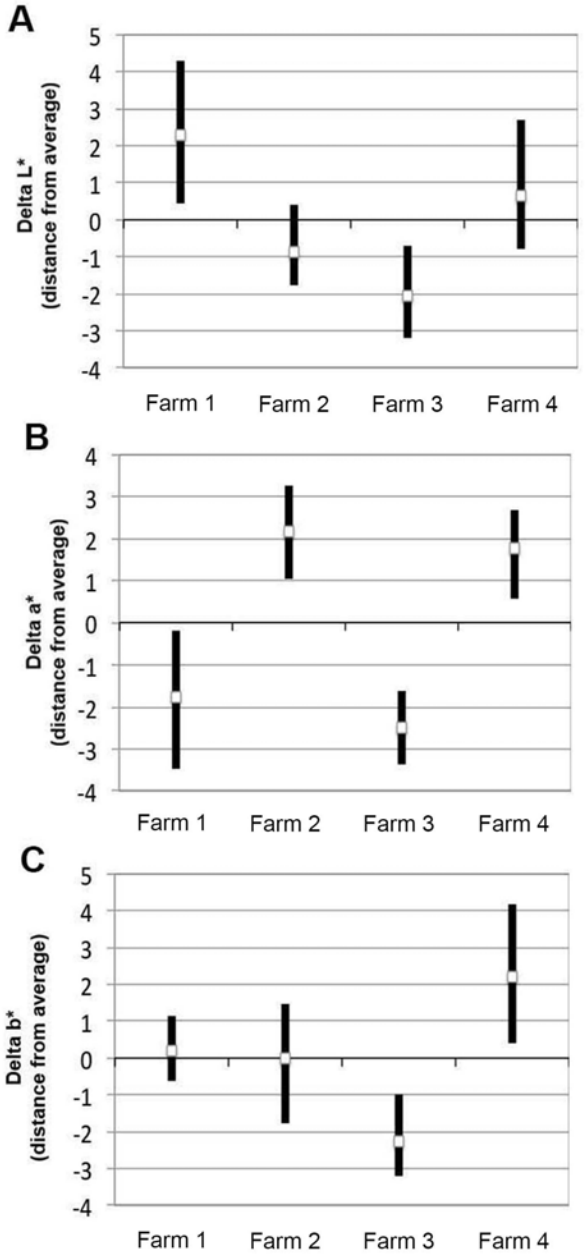

Figure 6. Colour Variation Across Farms. The median (square) and Q1-Q3 interquartile range (bars) distribution of delta *Lab* values from forty uncooked prawns from 7 different ponds when uncooked (A) and cooked. The delta *Lab* values were calculated as the difference in the value of each individual from the average of all the animals across the 4 farms.

3.2.3 Effect Harvest Method on Colour of Prawns

Animals that had been held in covered and aerated 800 L bins for different periods of time showed very little change in cooked colour after up to four hours holding prior to cooking (Figure 7A). Only the *b* value of animals sampled at 30 min and the *L* value of animals sampled at four hours were significantly different from the values of animals sampled at other times. Subjective scores showed that animals retained scores of between 9 and 10 on the Prawn Colour Chart, and 29 on the Salmofan throughout holding (data not shown). Prawns are known to rapidly respond to the colour of their surroundings, such as the colour of holding bins (Tume et al., 2009), and the effect of different coloured bins was assessed in section 4 of this study. The initial focus was to isolate this potential background colour effect, and used bins that had lids to completely block the light while animals were being held. This method was shown to be highly effective at preserving prawn colour during holding prior to cooking for at least 4 hours.

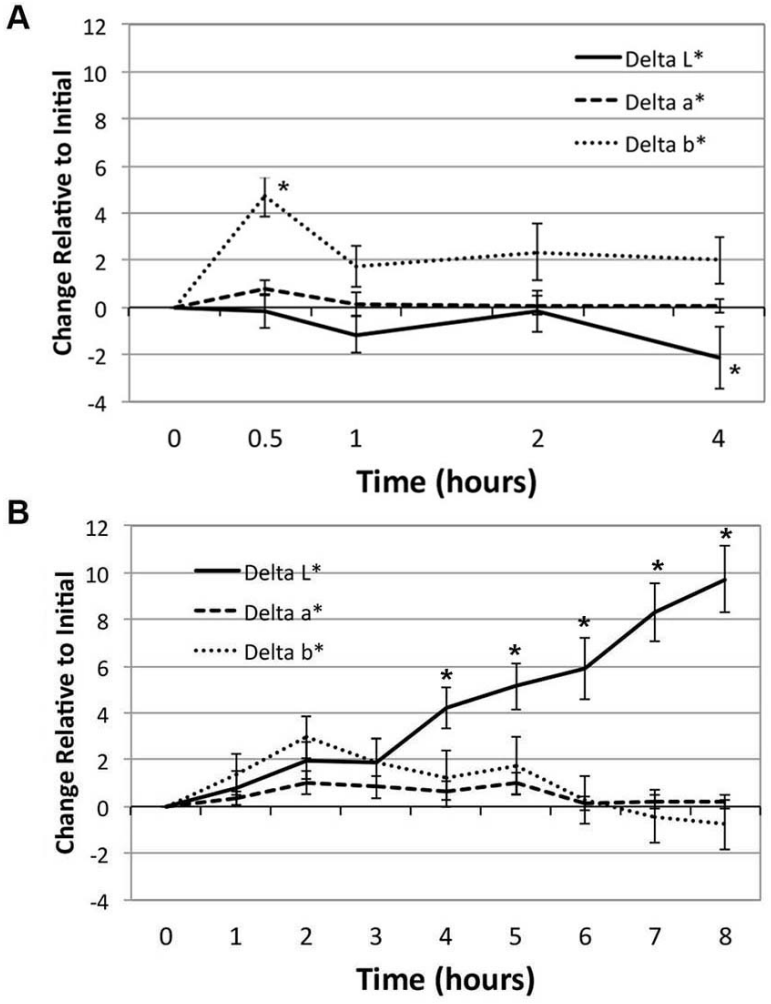

Figure 7. Colour change in uncooked prawns held live in chilled seawater or on ice. A. Prawns harvested from the same pond were held in large 800 L aerated bins that contained seawater at 12˚C. Twenty animals were taken at random immediately after harvesting and at four different times after harvest and uncooked colour measured using a HunterLab colorimeter. B. The same twenty uncooked animals were tagged and colour measured using a HunterLab colorimeter over an eight hour period of storage in an ice slurry. Results are shown as delta *Lab*, which is the difference in absolute *Lab* colour value at each time point relative to the initial sample. * denote significant (P<0.05) differences in *Lab* value from the initial measurement.

When the same 20 prawns were colour measured over time during ice storage, there was a significant increase in the *L* value after 4 hours while the *a* and *b* values were unaffected (Figure 7B). Although it was not possible to predict the precise cooked colour from the measured uncooked *Lab* values, it was possible to use the negative correlation recorded earlier between uncooked *L** value and cooked *a** value to infer the effect on cooked colour. This demonstrated that prawns that recorded a higher uncooked *L** values were not only lighter in colour before cooking, but would record lower a* values when cooked and were therefore appear less pigmented. By measuring the same prawns at different times and through different treatments, this study eliminated the variability between individual prawns and showed that uncooked prawns that were either held on ice for periods longer than 4 hours or frozen and then thawed become paler in colour. The effect of freeze thawing uncooked product was a similar magnitude to that seen over 8 hours of ice storage, and both were shown to result in a less pigmented product and a lower colour grade score.

3.2.4 Effect of Ice Storage or Freezing Prior to Cooking on Colour of Prawns

Two groups of fifty uncooked prawns were tagged and colour measured and used to assess the effect of ice storage or freezing on prawn colour. Results showed that after 14 hours of ice storage, the *L* value of uncooked prawns had increased significantly, along with a significant increase in the amount of colour variation within the *L* values (Figure 8A). This result was the same as the effect seen with the twenty prawns measured over time in the previous section, although the increase in *L* value was not as pronounced on this occasion. Similarly, 50 prawns that were measured uncooked, then frozen and measured again, also recorded significantly higher uncooked *L* values and a significant increase in the variance of the *L* values (Figure 8B).

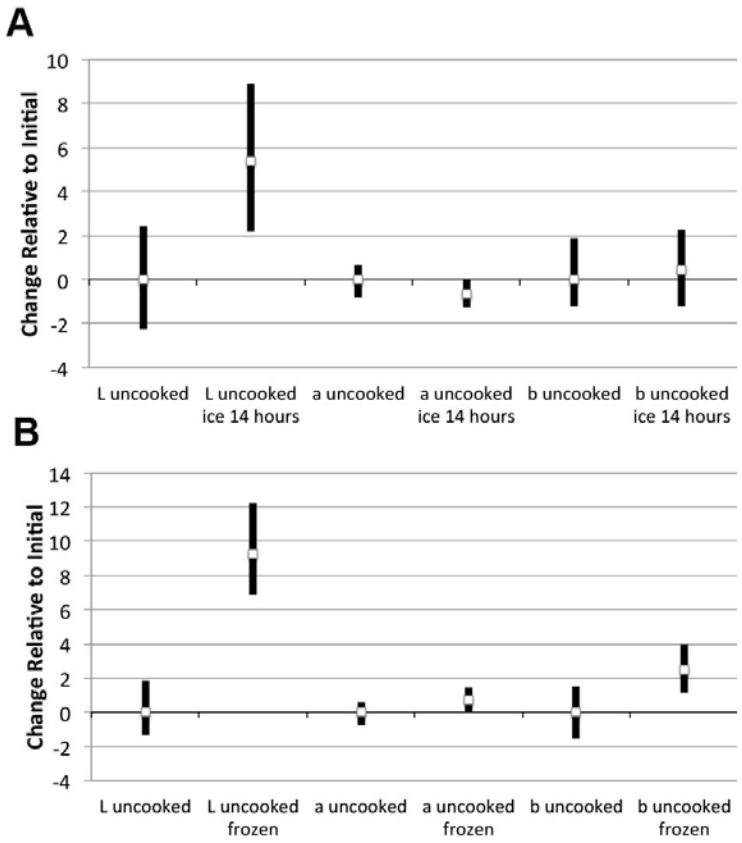

Figure 8. Colour change in uncooked prawns stored on ice or frozen. The median (square) and Q1-Q3 interquartile range (bars) distribution of delta *Lab* values from 50 uncooked prawns were recorded before and after 14 hours of ice storage (A), or before and after freezing (B). The *delta L*, *delta a* and *delta b* values were calculated as the difference in the value of each individual after the treatment relative to the average of all the animals before the treatment. Both methods had a negative influence on prawn colour as animals recorded a significant increase in *L* values, and a significant increase in the variance of those *L* vales.

3.2.5 Effect of Ice Storage After Cooking on Colour of Prawns

Cooked prawns are often preserved in salted ice slurry overnight to improve flavour and stored shelf-life. However, the effect of this treatment on colour has not been quantified. The same 50 prawns were measured immediately after cooking and again after 14 hours in an ice slurry. Results showed there was a small but significant increase in the *a* and *b* values of cooked prawns after being held in an ice slurry (Figure 9). This indicated the presence of more red and yellow hues, and demonstrated this treatment was having a positive effect on prawn colour. Colour preservation during frozen storage has been the focus of another extensive study, and demonstrated that cooked colour can be best preserved at stable temperatures below -30˚C (Sue Poole, personal communication).

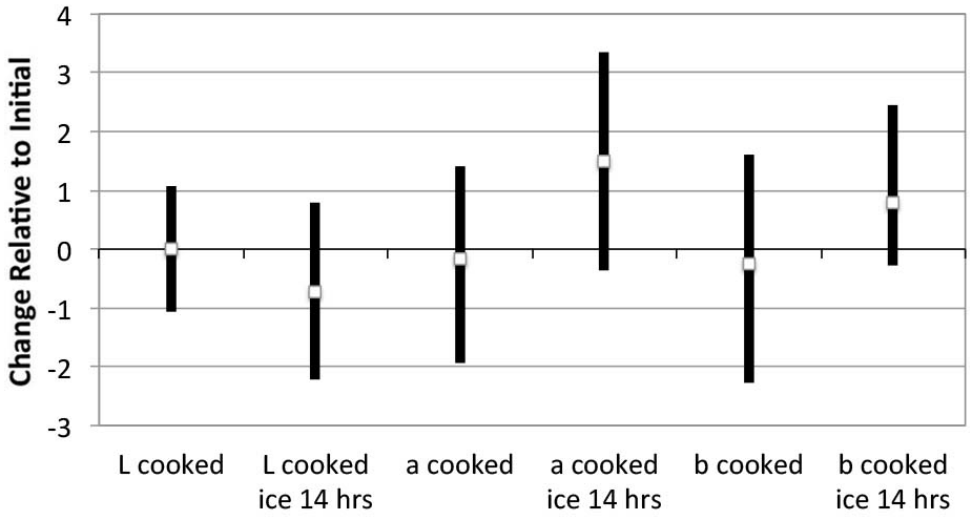

Figure 9. Colour change in cooked prawns after 24 hours ice storage. The median (square) and Q1-Q3 interquartile range (bars) distribution of delta *Lab* values from 50 cooked prawns were recorded before and after 14 hours of ice storage. The *delta L*, *delta a* and *delta b* values were calculated as the difference in the value of each individual after the treatment relative to the average of all the animals before the treatment. Once prawns had been cooked, ice storage had a positive influence on prawn colour as animals recorded a small but significant increase in both *a* and *b* values.

3.3 CRCN Protein Quantification

In addition to quantifying the variation in external colouration, this project seeks to understand the role that a specific colour protein, known as crustacyanin (CRCN), has in the observed variation in colour of farm reared prawns. Our past research has produced a highly specific and sensitive antibody against this protein and used it to investigate its role in crustacean colour production (Wade et al., 2009), as well as specifically to investigate changes in prawn colour (Wade et al., 2012a). In this project, the attempts to develop an indirect Enzyme-Linked ImmunoSorbent Assay (ELISA) using the CRCN antibody were not successful (data not shown). However, this project was able to successfully develop a novel, highly sensitive fluorescence based method for detecting CRCN abundance.

3.3.1 Validation of Protein Quantification

Total protein from all individual extractions was pooled in equal quantities and then loaded in triplicate onto three replicate membranes in a linear gradient. Quantification of fluorescence intensity demonstrated that CRCN protein detection was linear across this range of protein concentrations (Figure 10). The CRCN antibody did not recognise any proteins extracted from muscle tissue as a negative control (C). This clearly demonstrated that CRCN protein could be quantitatively detected from a complex mixture of proteins extracted from prawn epithelial tissue.

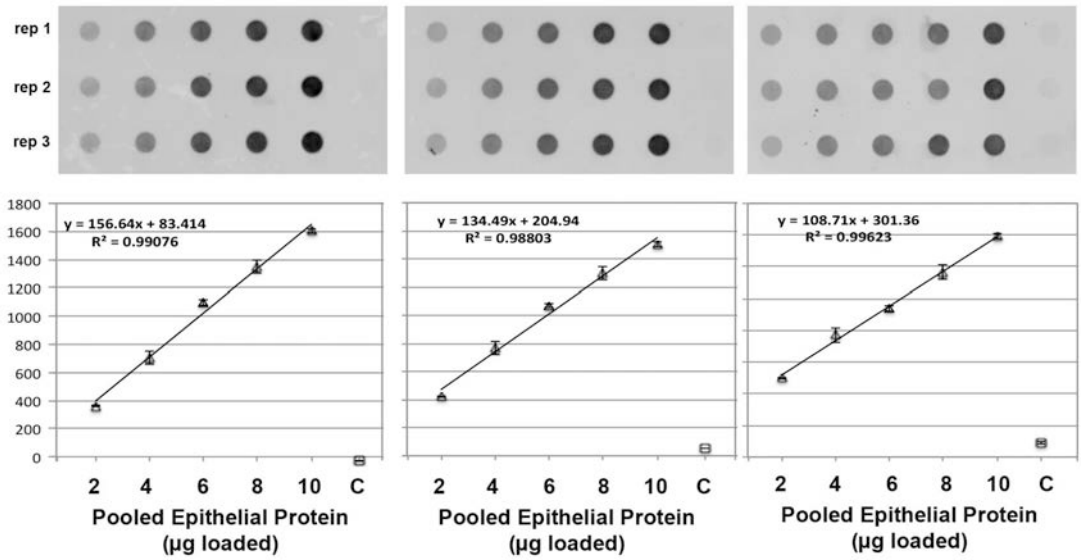

Figure 10. Validation of colour protein quantification in prawn tissues measured by western blot. Total protein extracted from uncooked prawn epithelial tissue was pooled across all individuals in equal quantities. Different amounts (2-10 µg) of total epithelial protein and 10 µg of total muscle protein (C) was loaded onto a PVDF membranes in triplicate, and the amount of the colour protein CRCN was measured using a highly specific anti-CRCN antibody. Results from three replicate membranes showed that this method was an extremely sensitive and reproducible method to quantify amounts of CRCN protein from individual animals.

3.3.2 CRCN Protein Variation in Farmed Penaeus monodon.

The colour of uncooked prawns sampled on the same day from eight different ponds was quantified from digital images (Figure 11A). From the same individuals, total protein was extracted from dissected epithelial tissue samples and the abundance of CRCN protein was quantified. For both RGB values and protein abundance, values were standardised relative to the average of the entire group, and log transformed to show whether individual variation was above or below the average of all individuals. Results demonstrated that there was significant variation in the average colour of individual prawns (Figure 11A) and also in the amount of CRCN protein quantified between individuals (Figure 11B). The amount of CRCN protein was 4-5 fold higher in some animals compared with the amount in other animals.

The RGB values and the amount of CRCN protein from six individuals sampled from the same pond was compared relative groups of individuals from other ponds (Table 7). The CRCN protein abundance and RGB values for each individual was then expressed as an average across all samples (Figure 12). Results demonstrated the amount of CRCN protein showed some variation across ponds, although there was no significant difference between groups of animals from different ponds. Some significant differences in RGB value were recorded between ponds, similar to the variation in colour observed between ponds measured in Section 2. However, ponds that recorded higher RGB values (such as Pond 2) did not record higher CRCN protein abundance. Similarly, ponds that recorded higher CRCN protein abundance (such as pond 6) did not record high RGB values.

Table 7. Absolute quantification of uncooked prawn colour and CRCN protein content across ponds.

	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Pond 6	Pond 7	Pond 8	ALL Ponds
Image Based Colour Quantification									
R	39.22 ±2.42^{ad}	50.97 ±3.03^{b}	33.09 ±1.99^{ad}	43.40 ±2.90^{bc}	36.36 ±2.63^{bd}	38.17 ±3.39^{bd}	35.67 ±3.93^{bd}	33.14 ±1.71^{d}	39.22 ±2.42
G	39.21 ±2.47^{ac}	43.76 ±2.92^{a}	32.90 ±1.53^{bc}	41.64 ±4.27^{a}	32.65 ±1.77^{bc}	37.72 ±4.01^{ac}	35.10 ±4.61^{ac}	31.74 ±2.13^{bc}	38.84 ±1.20
B	29.84 ±2.08^{ac}	33.44 ±2.47^{a}	24.81 ±1.13^{bc}	33.34 ±3.75^{a}	24.22 ±1.46^{bc}	28.97 ±3.53^{ac}	27.10 ±3.42^{ac}	23.68 ±1.94^{bc}	28.18 ±1.01
Protein Quantification (arbitrary fluorescence intensity units)									
CRCN	274 ±38	295 ±103	427 ±154	355 ±101	378 ±131	563 ±89	278 ±78	177 ±77	343 ±37

Values shown are mean ± SEM for 6 individuals per pond. Superscripts denote significant ($P<0.05$) differences among ponds within a parameter. Lack of any superscripts within a row indicates that there were no significant differences among any of those measurements for that parameter.

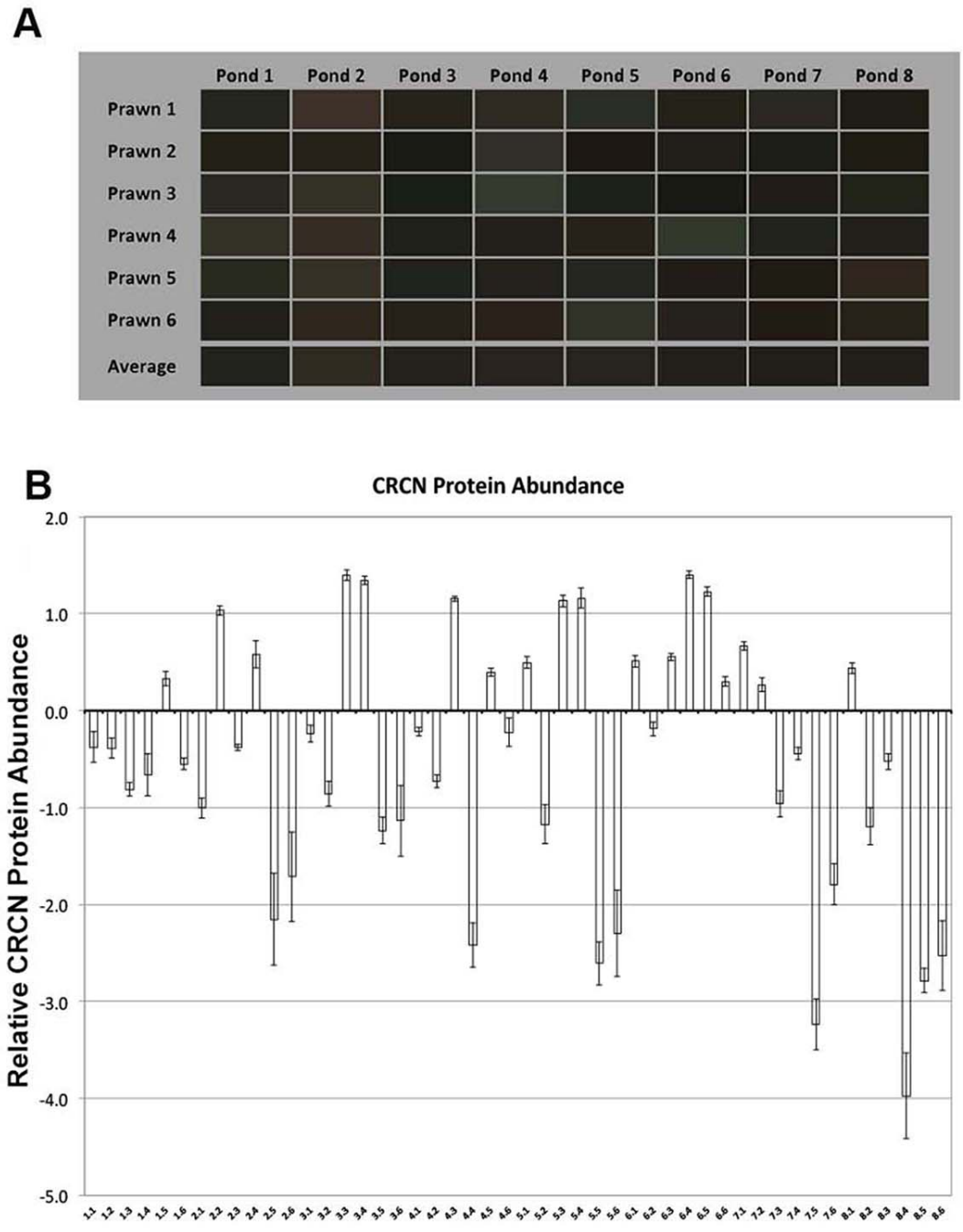

Figure 11. Colour protein abundance in prawn tissues measured by western blot. A. Absolute uncooked colour was quantified from digital images of individual prawns, each box represents the average RGB colour across the first 3 abdominal segments. B. Total protein was extracted from uncooked prawn tissues, and the abundance of the colour protein CRCN was quantified across six replicates for each sample. Individual variation was assessed relative to the average fluorescence intensity across all samples.

The relationship between RGB value and CRCN protein abundance was investigated in more detail, and the CRCN protein abundance for each individual was plotted against the corresponding R, G or B colour value (Figure 13). There was no relationship between the quantified RGB colour values and the abundance of CRCN protein for each individual (Figure 13). Although the method of quantifying CRCN protein was successful, results demonstrated that there was no relationship between the variation of individual colour and the variation in CRCN protein abundance.

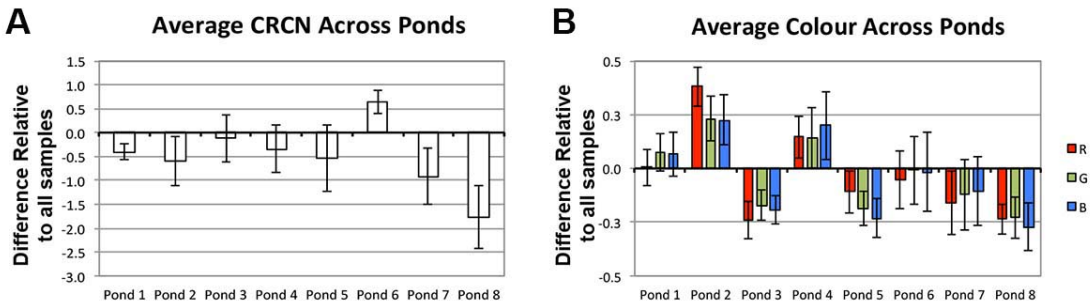

Figure 12. Colour protein abundance of prawns from different ponds measured by western blot. A. CRCN protein abundance was combined for the six different individuals and compared across eight different ponds. B. RGB values were combined for the six different individuals and compared across eight different ponds.

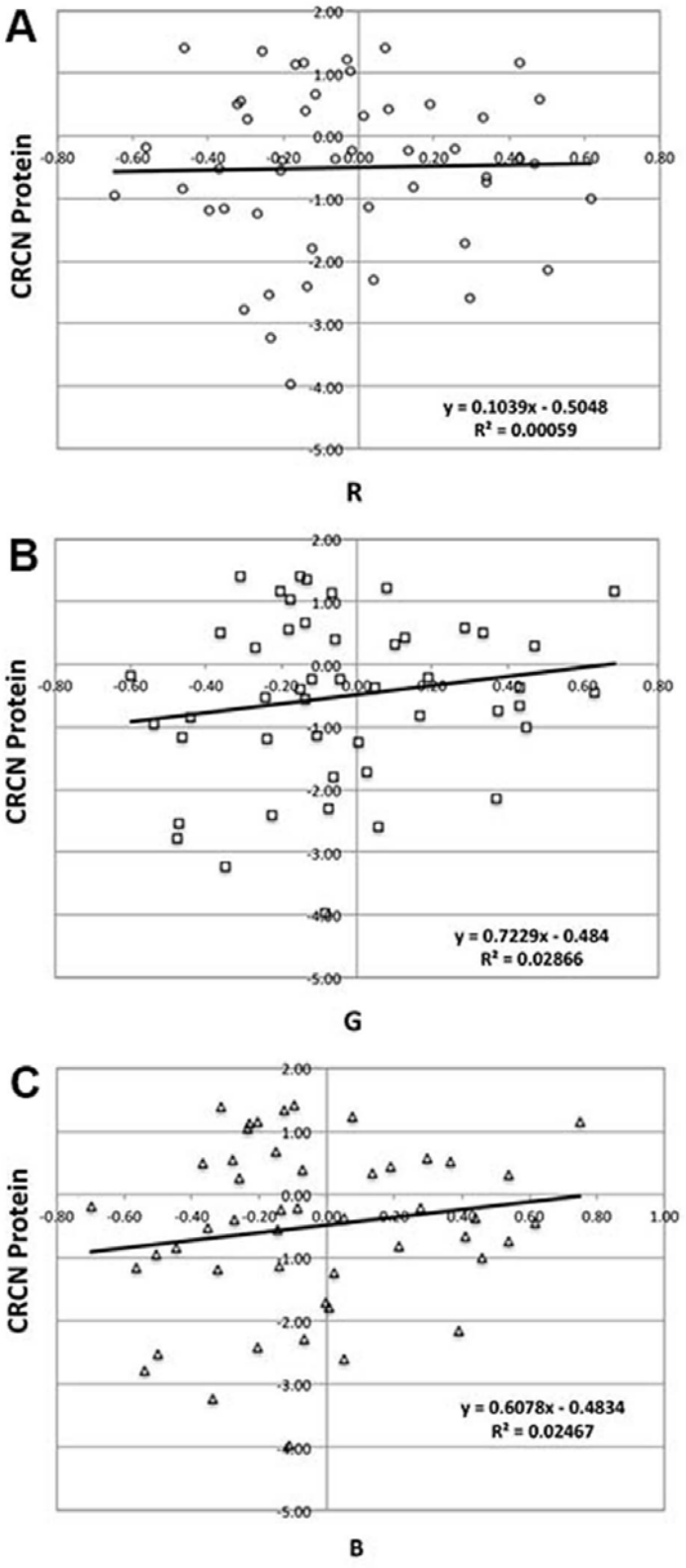

Figure 13. Relationships between RGB value and CRCN colour protein abundance in prawn tissues measured by western blot. Individual RGB values and CRCN protein abundance values were standardised relative to the average across the entire group. The relationship between R or G or B and the abundance of CRCN protein was compared.

3.4 Pre-harvest Methods to Improve Pigmentation in Farmed *Penaeus monodon*

3.4.1 On Farm Effects of Exposure to Different Coloured Substrates

Previous controlled experiments have demonstrated the significant positive effects that can be achieved through short-term exposure to black backgrounds (Tume et al. 2009; Wade et al. 2012a). Within this project we performed the first on-farm attempts to quantify the usefulness of this technique for improving prawn colour. Three separate experiments were conducted using customized harvest bins made from either black or white plastic (Figure 14). Cooked prawn colour was tracked over time using subjective colour grade scoring and colour quantification from digital images. Results from subjective scoring showed that there was a significant difference in colour between animals that had been held in black bins compared with those that had been held on white bins (Figure 14). However, the effect was quite small, only half a colour grade score, and was only observed in two of the three trials conducted. The difference in cooked colour was largely due to a loss of colour in animals held in white bins, and indicates that harvesting animals into white bins will adversely affect cooked prawn colour. There was a general trend for all animals to become slightly darker during holding in the bins, regardless of the colour of the bin.

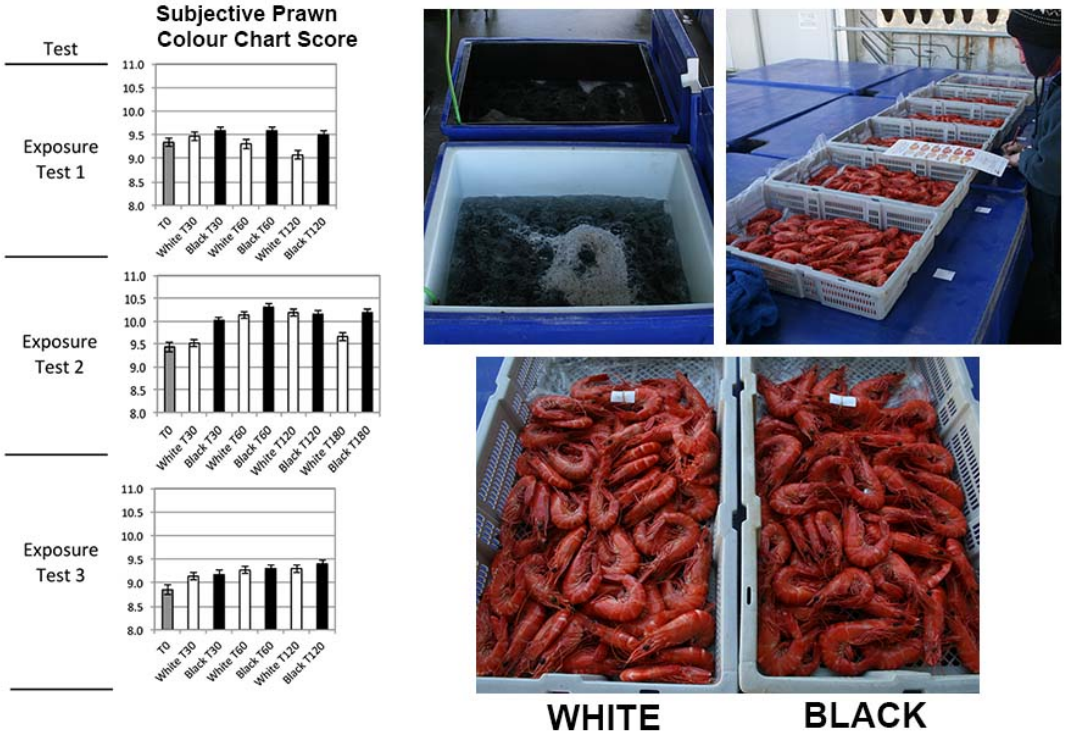

Figure 14. On farm subjective colour grade scoring of prawns exposed to black or white substrates. Each test was performed on separate days using aerated harvest bins that were made of either black or white plastic and contained filtered seawater. Prawns directly harvested from the same pond were divided across the two bins and retained for various lengths of time under natural illumination. At each time point shown, 60 animals were removed from each bin, cooked independently and subjectively colour grade scored. Average grade scores for each group showed some positive effect of retaining prawns in black bins as opposed to white bins.

At each time point, three coloured squares were used to visually represent the average colour of 20 prawns from each of three digital images at each time point. Quantification of prawn colour using digital images showed that prawn colour changed the longer they were held in bins, although the visual colour change was subtle (Figure 15A). Using this method, the prawn colour appeared to get darker over time, regardless of the colour of the bin they were held in. This darkening of colour over time was also observed with subjective scores, up until the final time point where significant differences in prawn colour were observed. Interestingly, the image quantification did not highlight the same loss of pigment we observed in subjectively scored animals that were held in white bins.

The absolute RGB values from the 60 individuals across the three photographs were also used to assess the colour change after 120 or 180 minutes. Significant differences in the R, G or B values of animals were observed over time (Figure 15B). Similar to the subjective scoring result, the response was slightly different for each of the three experiments. Overall, results showed that each of the RGB values quantified at the 120 or 180 minutes was significantly lower than the value at the beginning, regardless of bin colour. The combined effect was a slightly darker abdomen colour as shown in the combined RGB colour squares.

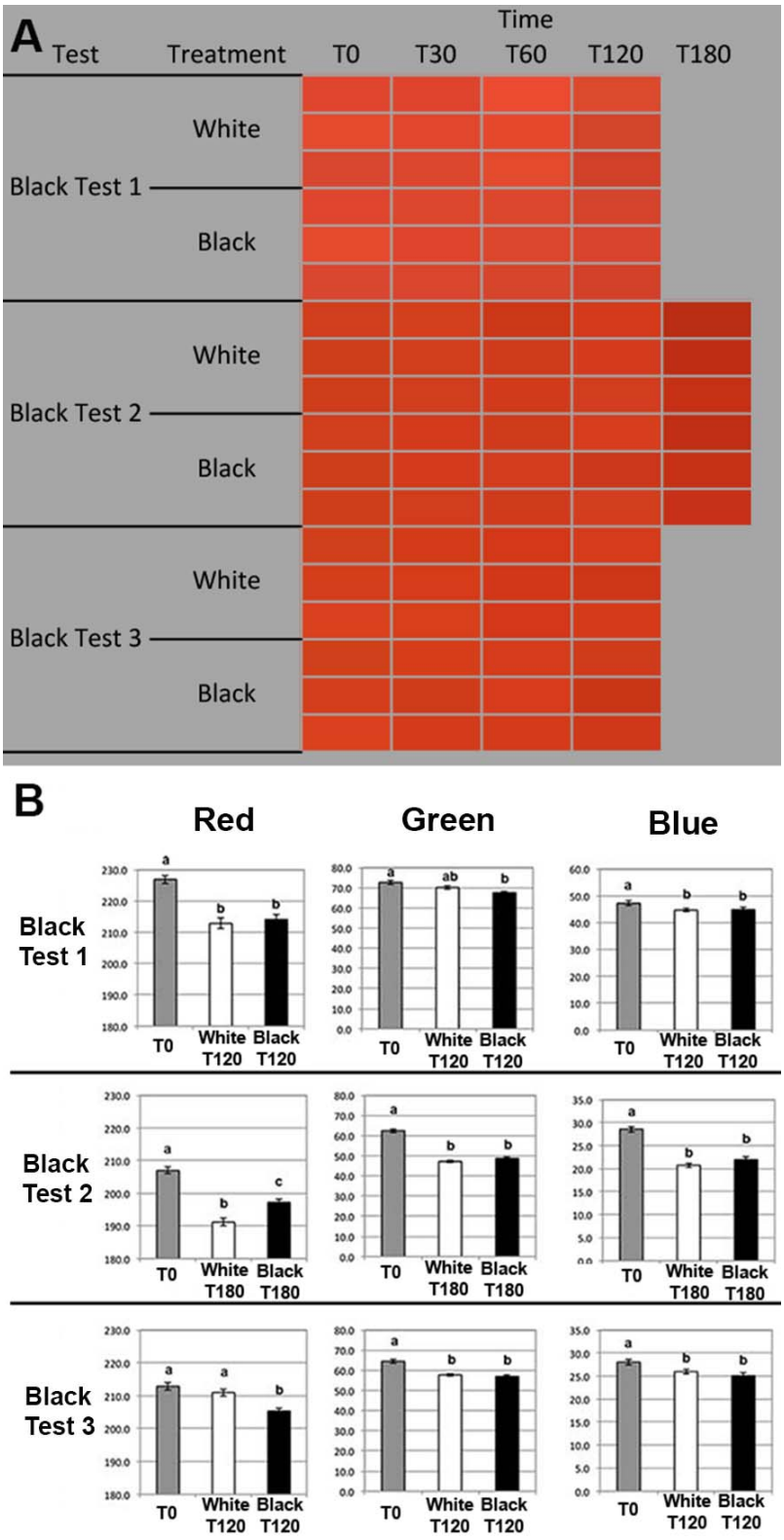

Figure 15. On farm colour quantification of prawns exposed to black or white substrates. A. Three replicate photographs each containing 20 prawns were used to quantify the average colour of each prawn from digital images using the method outlined above, and then grouped according to the various treatments. Each of the coloured boxes shown represents the average RGB colour for the 20 prawns from each photograph, and shows how the average colour of the prawns changed over time. B. The average R, G and B values of all prawns measured at time zero, compared with the corresponding values after being held in black or white tanks for 120 (T120) or 180 (T180) minutes.

3.5 Post Harvest Collaborative Extension

3.5.1 Retention of Pigment Through Freezer Storage

Lastly, within this project collaborative links were created with Dr Sue Poole's Seafood CRC project 2010/707 "Loss minimisation in farmed prawns through improvements in storage life and colour". A series of experiments were designed and carried out using resources and staff from both CSIRO and QDAFF. CSIRO's expertise in prawn colour manipulation prior to harvest was combined with QDAFF's expertise in assessing post harvest colour retention. CSIRO had independently run and funded an experimental trial on the effect of combining dietary astaxanthin supplementation with exposure to black or white substrates, in collaboration with DSM Nutritional Products. The samples shown in Table 5 were used in this experiment to assess the retention of pigment through freezer storage.

For all 402 animals at the beginning of this trial, prawn colour was quantified using colorimeter measures of the first three abdominal segments and legs (data not shown), as well as subjectively scored (Figure 16A). Animals were held for 217 days at -30˚C and re-measured. Results from our previous work had demonstrated that prawns grown in black or white tanks rapidly produced a major separation in prawn colour, within 5 days (Wade et al. 2012b, Figure 16A). The effect of dietary astaxanthin supplementation was more subtle, with effects only appearing after 40 days (Wade et al. 2012b, Figure 15A). At that time, there was a clear improvement of colour grade scores in prawns that had been fed higher levels of dietary carotenoid. The same animals were quantified and colour grade scored again after 217 days of freezer storage. Results showed that there was very little change in prawn colour during this time under optimal storage conditions, and prawn colour at the end was virtually identical to their colour prior to storage (Figure 16B). These results clearly demonstrate that improvements in pre-harvest pigmentation through dietary astaxanthin supplementation or black substrate exposure are retained through cooking and freezer storage.

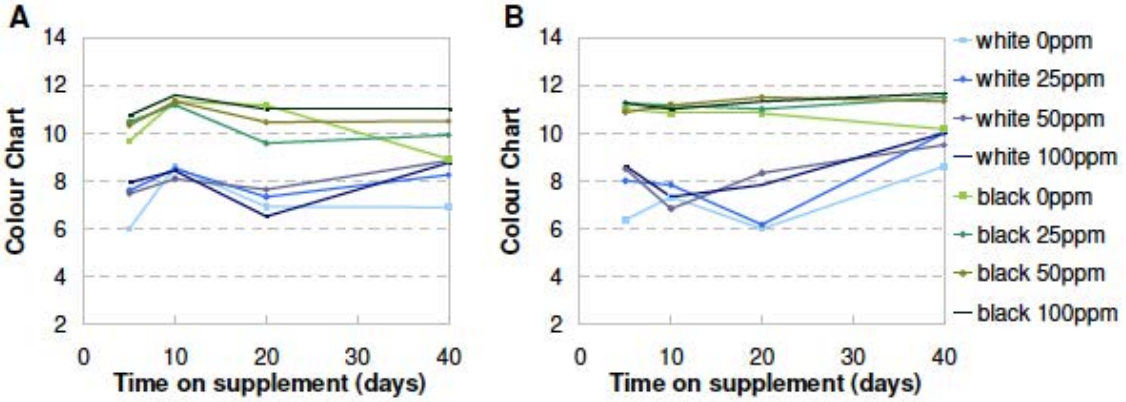

Figure 16. Retention of pigmentation improvements through freezer storage. A. Prawn colour chart scores of prawns prior to the freezer storage trial. Animals that were grown in black tanks (green lines) recorded significantly higher colour grade scores than those grown in white tanks (blue lines) across all time points. The effect of dietary astaxanthin supplementation was most evident after 40 days. Animals fed the highest levels of astaxanthin showed the best colour grade score, while animals that were not fed carotenoid at all recorded the lowest scores. B. Prawn colour chart scores of the same animals after 217 days of freezer storage. Colour grade scores after storage were very similar to those recorded after storage, and indicated that improvements to prawn pigment from dietary astaxanthin or exposure to black substrates was retained through freezer storage.

4 Discussion

The focus of this study was initially to develop methods to quantify prawn colour, then to identify and quantify any variation in pigmentation that existed across farmed animals, whether this variation was linked to levels of colour protein in the epithelial tissue and whether the pigmentation could be improved by exposure to dark substrates during harvesting.

4.1 Method Development and Validation

Accurate, reproducible and quantitative methods have been developed in this study for the analysis of prawn colour. The use of colorimeters or digital images was equally successful at quantifying colour in both uncooked and cooked prawns. Not unexpectedly, different colorimeters that use a different incident light angle during measurement (Hunterlab Miniscan XE 45˚, Minolta CR-400 10˚) recorded different absolute colour values for the same animals. However, based on the relationship between the two readings a simple conversion algorithm would be potentially accurately convert absolute values from one machine to another. The conversion of digital image RGB values to *Lab* values was not successful, demonstrating that multiple methods of colour measurement should not be combined within a single experiment. To partially overcome the problems of using different measurement systems in different experiments, this study standardised the readings from any one of the methods against the average of all the animals from a particular experiment. This allowed not only comparison within groups measured with the one instrument, but also a form of common reference for samples measured with different methods. Finally, using the large number of prawn colour measurements created in this study, it would be possible to create an algorithm to predict cooked colour from uncooked colour, although this would require further development and validation.

4.2 Colour Variation in Farmed Prawns

Based on the significant variation in colour observed between different ponds, it is likely that pond to pond variation is contributing the largest component of the colour variation in farmed prawns. Some ponds also appeared to contain animals with greater amounts of colour variation than others. Despite animals from different ponds sharing genetic background and dietary carotenoid content, a further series of factors can be potential causes of this variation. These may include such things as pond substrate, the density of algal bloom and types of algal species within each pond. The observed variation in the samples from different farms may be due to a number of other farm specific conditions such as those listed above, but also including different pigmentation regimes in feeds or processing methods that could not be accounted for in this study. To identify the causative factors of this variation is a much more complex undertaking, given the interaction of an array of different factors that are present within ponds.

4.3 Effects of Harvest Methods on Prawn Colour

This study was able to show that processing animals as quickly as possible after harvest, and storage of cooked product on ice after processing was beneficial to the colour of the product. This effect may be related to clarification of the shell and improved visualisation of the epithelial pigments. Meanwhile, storage of uncooked product on ice for periods beyond 4 hours or freezing-thawing of uncooked product were detrimental to cooked prawn colour. This effect may be cause by the

degradation or removal of the CRCN pigment from the epithelial tissue, which is highly water soluble.

How consistent various harvesting and processing techniques are across the industry is not known, yet this may be having a profound impact on the cooked product quality and price. In many cases, the practices employed across the industry may be adapted to specific circumstances at each farm, and the practicality of harvesting large quantities of product. The findings of this study extend beyond the Australian prawn industry, and would be applicable to other commercially produced prawn species. The same pigment improvements have been observed in *Penaeus vannamei* in response to black substrate exposure (Parisenti et al., 2011b). In addition, these research findings may translate to the wild harvest fishery sector, which currently freezes all product uncooked immediately after capture. These aspects would be worthy of further research consideration.

4.4 Quantification of CRCN Protein Abundance in Farmed Prawns

Our past research has shown that high levels of the protein crustacyanin (CRCN) are associated with the highest prawn colour grade scores (Wade et al 2012). Quantification of CRCN has not been This study developed a highly sensitive method for measuring the abundance of the crustacyanin protein within prawn epithelial tissues, and this method was used to quantify the variation in CRCN protein across individuals from different ponds. Our results demonstrated that the abundance of CRCN protein varied in different individuals, although the observed variation in farmed prawn colour was not correlated with the abundance of CRCN protein. It appears that the colour variation that exists in farmed prawns may be a result of not only variation in epithelial colour proteins, but a combination of a number of interacting factors that are poorly understood. To identify the specific cause of this variation would require a much more detailed and larger study.

4.5 Pre-harvest Improvement of Prawn Colour

Harvesting animals into white bins adversely affected prawn colour, although this affect was small and was only recorded using subjective scoring in two of the three experiments. Colour quantification from images showed that prawns held in bins became slightly darker over time, irrespective of the colour of the bin, again with some variation between experiments. Variability between the responses in the three experiments may be a result of the variability in the initial colour of the prawns, which in turn may be related to sourcing the animals from different ponds. Some ponds may contain darker prawns that became lighter in white bins, as we saw in experiment 1, while others contain lighter prawns that become darker, irrespective of the treatment. This result may possibly be attributed to differences in other factors such as harvest and handling stress, although there is very little scientific understanding of the effects of stress on prawn colour. Anecdotal evidence suggests that stress makes prawns darker, but this is difficult to replicate experimentally and has not been attempted.

The result of this study also highlighted the fact that there are likely to be factors other than abdomen colour alone in determining the subjective colour grade score, such as legs and pleopods, that were not quantified from digital images. This concept is supported by the freezer storage research of our colleagues at Queensland Department of Agriculture Forestry and Fisheries (QDAFF), where colorimeters showed that the first area to experience pigment loss was the legs (Sue Poole, personal communication). This resulted in an overall lower subjective colour

grade score for that animal, despite there being very little colour difference in the abdominal segments measured using the colorimeter. It would be possible to use the image-based method to quantify the colour from different parts of the animal, or indeed the entire animal. However, this would be more susceptible to shadows and reflections in the photograph or the orientation of the animal in the image.

A significant factor that can contribute to the lack of response to dark substrates may in part be due to the high degree of pigmentation of the prawns in the experiment. The animals used in these colour change trials were all extremely well pigmented before beginning the trial, having been fed a dietary astaxanthin supplement of 80 ppm and recorded average colour grade scores of between 9 and 10. Many farms have been adapting their practices in recent years based on our previous research recommendations to improve harvest colour, mainly through dietary pigment supplements, and attaining average colour grade scores of 8-9. Given poorly pigmented animals are difficult to source commercially, it was not possible to test the background adaptation method on animals that had average (7) to poor (5-6) colour grade scores. It is likely that holding animals in black bins that are already scoring 9 to 10 on the prawn colour chart will not produce as large an improvement in pigmentation compared with animals that initially only score 6 or 7. This effect has been recently been tested in a tank based trial performed at CSIRO.

4.6 Retaining Pigment Improvements Through Storage

In order to justify any changes to pigment management or harvest processes, it was vital to establish whether the changes in prawn colour prior to harvest were retained through cooked storage. This was possible by bringing together two complementary projects at CSIRO and QDAFF respectively. The results of this component unequivocally showed that, if stored under the appropriate conditions, prawns maintained their cooked colour. This was true for both pre-cooking improvements in pigmentation as well as pre-cooking deteriorations in colour.

5 Benefits and Adoption

The benefits of this project are the development of three reliable and quantitative methods of measuring the colour of farmed *Penaeus monodon*, including the ability to measure and quantify uncooked product. Additional benefits are an understanding of how much variation exists in the colour of farmed prawns and whether potentially expensive infrastructure changes to harvest methods. Certain harvest methods were beneficial to cooked colour, while others were detrimental. Irrespective of that effect, we now understand that those pre-harvest changes in colour are retained in the cooked product and in storage.

Recommendations:

- Dietary pigmentation levels should be maintained for at least 4 weeks prior to harvest at between 50-80 ppm
- Animals should be cooked as rapidly as possible after harvest
- Where possible, animals should be harvested into dark coloured bins
- Harvesting uncooked product into ice should be avoided
- Pre-harvest pigments can be modified by the colour of harvest bins
- If current harvest methods produce pigment scores that exceed 9, major infrastructure changes to incorporate black substrate exposure may not be worthwhile
- Holding cooked product on ice for 24 hours is encouraged and improves colour as well as flavour.

6 Further Development

- Using the data already collected, it would be possible to create a predictive algorithm to convert the colour values of uncooked prawns into cooked colour values. This would enable farmers and researchers to understand the effects of various pre-harvest treatments on the cooked colour without destructively sampling the product.
- Perform a survey of current harvest and processing methods. Although a standardised method for cooking is present across the industry, particularly for different sized animals, the way animals are handled prior to cooking is likely to be extremely variable.
- Individual farms may wish to investigate the variability across all ponds at their farm, in order to have an understanding of which of their ponds are producing dark or light coloured prawns, and how that might vary from day to day. This knowledge would assist farms with their dietary pigmentation and harvest management, and whether to undertake some sort of intervention to improve pigment before harvest.
- Investigate the effects of harvest stress on pigmentation. This study identified that there are likely to be a number of pre-harvest factors that interact to affect cooked prawn colour, with stress being a major one. Small amounts of stress may benefit cooked prawn colour, but there is little understanding of how harvesting might cause a change in epithelial pigments.
- Extension of these outcomes to wild harvest fishery. Measure the amount of potential pigment loss that occurs due to freezing uncooked product, and how that varies across different prawn species. If pigment loss is occurring, some novel methods may be able to be developed that prevent the deterioration of pigment during freezing.

7 Planned Outcomes

Public Benefit Outcomes

More darkly pigmented product for consumers
Higher quality product and greater satisfaction

Private Benefit Outcomes

Increased product value and maximise profit
Increased market penetration and acceptance of product
Methods that can produce darker pigmented prawns
Understanding of how harvest methods affect cooked colour

Linkages with CRC Milestone Outcomes

This project provides knowledge and understanding to maximise the product quality and price of commercially farmed prawns
Improvements to industry profitability and economic sustainability

8 Conclusions

In conclusion, this project has developed novel methods to quantify the colour of uncooked or cooked prawns. The amount of colour variation that exists between ponds and across farms is likely to produce significant variability in product colour and therefore quality. This work also quantified the affects of different harvest practices on prawn colour, and allows adoption of best practice methods to optimise product colour.

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