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The Combined Effects of Diet, Environment and Genetics on Pigmentation in the Giant Tiger Prawn, *Penaeus monodon*.

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Abstract

The deep red colour of the Giant Tiger prawn, *Penaeus mondon*, is highly desired and fetches premium market prices. Prawn pigmentation is influenced by the interaction of a range of factors, including the amount of dietary carotenoid, the distribution of hypodermal pigments, and genetics. These aspects have been studied in isolation, but there is limited knowledge on how these components interact to influence prawn pigmentation. This study tracked the colour of prawns that had been fed four different levels of dietary astaxanthin (Axn) over 6 weeks, and then transferred to either black or white coloured tanks. The dietary influence on colour was slow and had only developed after 6-weeks. Meanwhile the effect of background colour was rapid, within 15 minutes. Results showed that diet and background colour work in combination to affect prawn colour. The poorest colour was recorded in prawns fed without dietary Axn and transferred to white substrates, and this colour was improved by the addition of dietary Axn. Animals fed without dietary Axn and exposed to black substrates showed an intermediate colour, and this was further improved by addition of dietary Axn. The best colour was recorded in prawns fed 100 mg/kg Axn and exposed to black substrates. The abundance of the epithelial pigment protein crustacyanin (CRCN) was not correlated with prawn colour, suggesting that this protein does not regulate the modifications in response to background colour. Finally, the effect of substrate exposure was assessed on farmed prawns, and indicated a small positive effect on colour during harvesting. These data demonstrate that while short term exposure to black substrates can have a positive effects on prawn colour, dietary Axn supplementation can both improve pigmentation of animals exposed to black substrates, and prevent the negative effects of exposure to white substrates.

Introduction

Many crustacean tissues attribute their colouration to the presence of various carotenoids, particularly those that provide external colouration. In addition to providing a protective camouflage to the animal, colour plays a major role in consumer acceptability, perceived quality and price paid for commercial crustacean species (Chien, Jeng, 1992; Erickson*, et al.*, 2007; Parisenti*, et al.*, 2011a; Shahidi*, et al.*, 1998). This colour may be embedded in the exoskeleton, or in pigment structures within the underlying hypodermal layer known as chromatophores (Rao, 1985). The most abundant carotenoid in crustacean tissues is astaxanthin (Axn) (Castillo*, et al.*, 1982; Lenel*, et al.*, 1978; Tanaka*, et al.*, 1976), where it is found in free, esterified and protein-bound forms. The amount and distribution of pigment is dependent upon a range of dietary, environmental and genetic factors that have been considered independent from one another, with each having been studied in isolation.

Several crustacean species have been shown to lose or not develop pigmentation if not supplied a diet with sufficient carotenoids (Dall, 1995; Daly*, et al.*, 2013; Tlusty, Hyland, 2005). Dietary astaxanthin supplementation is known to improve crustacean colour through the abundance of epithelial astaxanthin and astaxanthin esters (Barclay*, et al.*, 2006; Boonyaratpalin*, et al.*, 2001; Kumar*, et al.*, 2009; Supamattaya*, et al.*, 2005; Yamada*, et al.*, 1990). Crustaceans have the metabolic capacity to interconvert different carotenoids, such as canthaxanthin and ß-carotene, into astaxanthin (Negre-Sadargues, 1978; Schiedt*, et al.*, 1993). Dietary astaxanthin between 50-100 mg/kg fed for one month was sufficient to produce optimal pigmentation in a range of prawn species (Chien, Jeng, 1992; Petit*, et al.*, 1997; Yamada*, et al.*, 1990). Within the exoskeleton and hypodermal tissue of crustaceans, free astaxanthin is often also bound within a multimeric protein complex called crustacyanin (CRCN) (Wald*, et al.*, 1948). The interaction of CRCN and Axn modifies the naturally red carotenoid to blue or any other colour in the visible spectrum, producing the diverse array of colours seen in the exoskeleton of crustaceans (Cianci*, et al.*, 2002). During cooking, this interaction is disrupted, releasing the distinct red colouration of cooked seafood.

In response to various physiological cues, crustacean chromatophores expand and contract through hormones secreted from the eyestalk (Bagnara, Hadley, 1973; Rao, 2001). This rapid and reversible response strongly contributes to the degree of individual colouration, particularly for species with thin opaque shells like prawns (Fingerman, 1965). These cues can span aspects such as background colour, light source and photoperiod (Latscha, 1990; Rao, 1985). Short-term exposure to black substrates has been shown to improve prawn pigmentation through expansion of hypodermal chromatophores (Parisenti*, et al.*, 2011b; Tume*, et al.*, 2009). This expansion was linked with the accumulation of the colour protein CRCN in the hypodermal tissues (Wade*, et al.*, 2012).

The present study sought to determine whether the long-term beneficial effect of feeding high levels of dietary astaxanthin could be combined with the short-term beneficial effects of exposure to dark coloured substrates. In addition, this study sought to assess the ability of dietary carotenoid supplementation to overcome the negative effects of short-term exposure to white substrates. Whether the colour protein CRCN has a role in regulating colour change was also assessed. Raw or cooked prawn colour was monitored using digital images, expansion of chromatophores was assessed using microscopy, and CRCN protein abundance quantified by western blotting. Lastly, the ability to transfer this knowledge to industry was assessed during harvesting on farmed *Penaeus monodon*. Results demonstrated that there was a significant interaction between the dietary, environmental and genetic mechanisms that regulate crustacean colour.

Materials and Methods

Animal Handling

Live prawns, *Penaeus monodon* (*P. monodon*), were obtained from commercial farms and maintained at CSIRO Marine and Atmospheric Research (CMAR) laboratories at Bribie Island. For all trials, filtered seawater was heated then pumped through the tanks at 1.2 L min^{-1} maintaining water temperatures at 28˚C and salinity at 35 g/L. Animals were held in a total of 36 red tanks that held 80 L seawater in each. The experiment was conducted indoors under low artificial light conditions and a 12-12 light dark photoperiod. Animals across the experiment were within the size range 7.25 ± 1.34 g and were fed once per day on formulated diets different only in the amount of astaxanthin (Carophyll Pink, DSM Nutritional Products) as shown in Table 1. Red tanks were used in the initial diet trial as they had previously been observed to produce an intermediate coloured prawns.

Diet and Background Trial

The experimental dietary carotenoid treatments were composed of a total of nine replicate tanks for each of the four diets, and each tank contained six individually eyetagged *P. monodon*. The nine replicates were largely necessary to provide sufficient numbers of animals fed over a 6-week period to be tested in the background colour change experiment. The colour change of the animals due to diet was assessed using digital images at day 0, 14, 28 and 42, using animals from four replicate tanks from each treatment. An initial sample of three groups of six prawns was taken at random from the pool of individuals used to stock the experiment. At day 42 of the dietary trial, five animals from each treatment were randomly sampled from five separate tanks and stored at -20˚C for later sampling. The experimental background colour treatments were composed of two 100 L tanks made of either black or white plastic. Animals from one dietary treatment were pooled, then divided evenly between the two coloured tanks. Animals were then removed and photographed at 15min, 30min, 1 hour and 2 hours, with the exception of the no carotenoid treatment that was not sampled at 15 minutes. Animals exposed to black or white substrates for 2 hours from each

diet treatment were frozen immediately and stored at -20˚C until used for microscopy analysis and protein quantification.

Farm Trial

To assess any effects of harvesting prawns into different coloured bins on farm, several hundred 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. Animals had consistently received feed containing 80 mg/kg Axn for a minimum of 4 weeks prior to harvest. Sixty animals were sampled immediately after harvest, then cooked and colour measured using digital images and 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.

Colour Measurement and Microscopy

Colour of uncooked or cooked prawns was quantified using the average colour of the first three abdominal segments measured using digital images and ImageJ software (Schneider*, et al.*, 2012), as used previously (Wade*, et al.*, 2014). Each photograph from the Diet and Background Trial contained six animals from each of the four treatments (Supplementary Figure 1), or from both the black and white treatments at the same time point (Supplementary Figure 2). Each photo from the Farm Trial contained 20 individuals, and three photographs were used for each time point in each trial. Where necessary, image intensity was adjusted between photographs using the MacBeth ColorChecker that was positioned in each photograph (data not shown). The average RGB values for each animal were used to display a single coloured square, representative of the average colour for

all animals in that treatment. 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. For all microscopy samples, the first abdominal segment was removed from uncooked animals, the shell removed and chromatophores photographed using a Leica M165C stereo microscope fitted with a Canon EOS 5D digital camera. The four remaining intact abdominal segments were cooked, and used to assess the subjective change in cooked colour in response to background colour over 2 hours.

Protein Quantification

After microscopy analysis, epithelial tissue was dissected from the first abdominal segment of animals from the 0 and 100 mg/kg Axn treatments exposed to black and white substrates (n=4 for each treatment). Tissue was homogenised in 2 ml water containing the Complete protease inhibitor cocktail (Roche) using a Precellys 24 tissue homogenizer (Bertin Technologies). Insoluble material was removed by centrifugation at 13 000 x g for 5 min at 4˚C, 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 (Wade*, et al.*, 2009) 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. To validate this method, total protein from all individual extractions was pooled in equal quantities and then loaded in triplicate onto three replicate membranes in a linear concentration gradient. The average spot intensity for the validation and for each individual was quantified using the average intensity of three triplicates across two independent membranes using

the Quantity One 1-D Analysis Software. For comparison across treatment groups, the abundance of CRCN protein for each individual was calculated relative to the average intensity of all the samples.

Statistical Analysis

Where comparison between individual measurements was required, statistical significance was assessed by single factor analysis of variance (ANOVA), followed by Fischer's test allowing 5% error. All statistical analyses were performed using StatPlus:Mac 2009 (AnalystSoft Inc).

Results

Prawn Colour Change in Response to Diet

The different carotenoid inclusion levels produced a strong change in average abdominal colour (Figure 1), a clear visual difference in uncooked prawn colour (Supplementary Figure 1), and quantifiable differences in the RGB values between the different treatments (Table 2). There were many significant differences between the RGB values across the various groups, only some of which are highlighted here. The R values for all treatments became significantly higher over time when compared with the values at time zero, but there was no significant difference in R value between any of the treatments at day 42. However, the G and B values at day 42 for the 100 mg/kg treatment were significantly lower than those from the 0, 25 and 50 mg/kg treatments. In addition, the G and B values of the 0 and 25 mg/kg treatment at day 42 had become significantly higher than their corresponding values at day 0, 14 and 28.

Prawn Colour Change in Response to Background Colour

After the 6-week feed trial using red tanks and four different dietary carotenoid inclusion levels, animals from the same treatment were exposed to either black or white substrates and the change in their uncooked colour quantified over time (Figure 1). As expected, prawn colour changed rapidly in response to substrate colour, all within 15 minutes with the exception of the 0 mg/kg treatment which was not measured at that time. In general, RGB values from animals exposed to white substrates were significantly higher compared with the values before the treatment started (Table 3). This was visible in the change in the average abdominal colour over time (Figure 1), and the difference in colour of prawns exposed to black (Supplementary Figure 2A,C,E,G) or white (Supplementary Figure 2B,D,F,H) substrates. However, in the 100 mg/kg Axn treatment exposed to white substrates, the R and B values were not significantly different from their pre-exposure values. In addition, only the 100 mg/kg Axn treatment showed a significant decrease in G and B values after exposure to black substrates relative to the pre-exposure values (Figure 13). Exposure to black substrates caused no significant change in RGB values of any other treatment.

Prawn Colour Change in Response to Diet and Background Colour

In combination with the effects over time, differences specific to diet were observed between the four treatments after 120 minutes exposure to black or white substrates. After 120 min exposure to white substrates, animals from the 25 mg/kg Axn treatment had significantly higher R, G and B values than the other treatments, closely followed by the no carotenoid treatment (Table 3). G and B values for the 50 and 100 mg/kg Axn treatments were significantly lower than the G and B values of the 0 and 25 mg/kg Axn treatments, indicating the presence of more blue and green pigments. Overall, results indicated that animals on low carotenoid diets became significantly lighter than those on high carotenoid diets after exposure to white substrates. The reverse was true after exposure to black substrates. Although there was very little difference in RGB values of animals from the 0, 25 and 50 mg/kg Axn treatments after 120 minutes of exposure to black substrates, the 100 mg/kg Axn treatment had significantly lower G and B values.

Cooked Prawn Colour Change in Response to Diet and Background Colour

When cooked and subjectively colour scored, both short-term exposure to background substrate colour and increased dietary carotenoid levels improved cooked prawn colour (Table 4). As might be expected, animals exposed to white substrates with the no dietary carotenoid recorded the lowest colour grade scores (colour chart 6.9, salmofan 25.6). There was then a significant improvement in the colour of animals fed any level of carotenoid and exposed to white substrates (Table 4). Exposure to black substrates without dietary carotenoids produced an intermediate colour score (colour chart 8.9, salmofan 28.9), similar to that of the highest carotenoid diet exposed to white substrates treatment. The addition of carotenoids in the diet of animals exposed to black substrates produced a further significant improvement in colour grade score (Table 4). The highest colour grade score (colour chart 11, salmofan 32.6) was recorded in the animals fed 100 mg/kg Axn and exposed to black substrates.

Effects on Epithelial Chromatophores and CRCN Protein Abundance

Prawn epithelial tissue was studied under light microscopy from representative animals across the different dietary treatments after 42 days of feeding. Visualising the effect quantified in Figures 1 and Table 3, prawn epithelial

chromatophores were more disperse and appeared to contain more pigment (Figure 2). Considerable variation existed between the colour of individual prawns, and it should be noted that these images were selected to visualise the effects quantified using digital images. Visualised under a dissecting microscope, prawn epithelial chromatophores were observed to expand or contract in response to exposure to black or white substrates, respectively (Figure 2). However, the expansion of blue colour was not restricted to the chromatophores themselves, and extended into the tissue between individual chromatophores (Figure 2).

Quantification of fluorescence intensity demonstrated that antibody based CRCN protein detection was linear across this range of protein concentrations (Figure 3A). The CRCN antibody did not recognise any proteins extracted from muscle tissue (C) as a negative control (Figure 3A). This clearly demonstrated that CRCN protein could be quantitatively detected from a complex mixture of proteins extracted from prawn epithelial tissue. However, no significant differences were observed in the amount of CRCN protein between the 0 and 100 mg/kg Axn diet treatments, or between animals exposed to black or white substrates for two hours (Figure 3B). A large amount of variation was recorded in the abundance of CRCN protein between individuals.

Prawn Colour Change in Response to Background Colour on Farms

This study performed the first on-farm attempts to quantify the utility of exposure to black substrates to improve prawn colour. 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 (Table 5). However, the effect was quite small, only half a colour grade score, and was only observed in two of the three trials conducted after several hours of exposure. The difference in cooked colour was largely due to a loss of colour in animals held in white bins, and there was a general trend for all animals to become slightly darker during holding in the bins, regardless of the colour of the bin.

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 4). 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.

Discussion

Combined Effects of Diet and Background Colour Exposure on the Colour of Prawns

As observed in most crustaceans (Barclay*, et al.*, 2006; Boonyaratpalin*, et al.*, 2001; Kumar*, et al.*, 2009; Supamattaya*, et al.*, 2005; Yamada*, et al.*, 1990), this study showed that prawns not receiving dietary carotenoid became paler while those receiving the highest dietary carotenoid became darker in colour. Prawns also rapidly respond to the colour of their surroundings (Parisenti*, et al.*, 2011b; Tume*, et al.*, 2009), and in this study became paler when exposed to white substrates and darker when exposed to black substrates. The unique aspect of this study demonstrated that diet and background colour work in combination to affect prawn colour. The increase in RGB values across all treatments showed that white substrates had a strong negative effect on uncooked prawn colour (Table 3), but these effects were minimised by including dietary carotenoid. The inclusion of 100 mg/kg Axn in diets prevented significant elevation of RGB values in response to white substrates (Table 3). After exposure to black substrates, only the highest carotenoid levels produced an improvement in uncooked colour. Animals in the 100 mg/kg treatment were the only ones to record a significant decrease in G and B values when exposed to black substrates. This indicated that animals fed 100 mg/kg Axn and exposed to black substrates had the darkest overall colour, and that only the animals receiving the highest carotenoid diet responded to the black substrate exposure.

The combined effects of diet and substrate were also very clear using subjective scoring of cooked prawns. These data showed that the colour of animals exposed to white substrates was improved by dietary carotenoids, similar to the colour of animals (Table 4). Diet then caused a further improvement in the colour of animals exposed to black substrates (Table 4). The retaining of additional astaxanthin (presumably mono-esters) in epithelial tissue (Figure 2) may underlie the ability of prawns to resist the negative effects of exposure to white substrates. Based on the results from the uncooked animals, the negative effects of exposure to white substrates might be a larger contributor to overall prawn colour score than the positive effect of exposure to black substrates, although this response may be affected by the initial colour of the animals. Overall, these

data clearly demonstrate that diet and substrate colour work in combination to improve prawn pigmentation.

The absolute RGB values recorded in this study can be greatly impacted by the red colour of the tanks used for the 6-week trial. This can also influence the ability of animals to respond to exposure to black or white substrates. The increase in R value in all groups over time may indicate that, regardless of the treatment, the animals continued to adapt to their background over time and this effect was occurring equally in all treatments. Tanks of another colour, such as mid-grey, may provide a better intermediate colour in future work. Nonetheless, the effects of this study are clear, in that animals became lighter or darker in colour after exposure to white or black substrates, respectively.

CRCN Protein Abundance and Mechanisms of Colour Change

Past studies have shown that long-term exposure to black substrates led to a significant increase the abundance of epithelial CRCN protein, and a significant decrease in response to white substrates (Wade*, et al.*, 2012). This may indicate that the amount and distribution of this protein is critical to achieving optimal cooked colour (Wade*, et al.*, 2012). The current study used a specific CRCN antibody that is able to detect the CRCN protein whether it is bound to free Axn or not. Here we attempted to discover whether the amount of CRCN protein was controlling short-term adaptive colouration responses, as had been the indication from long-term substrate exposure. Results showed no relationship between the amount of CRCN protein and the colour of animals exposed to black or white substrates for 2 hours (Figure 3). When biochemically purified from crustacean shells, CRCN protein was present at a defined 1:1 stoichiometric relationship with free astaxanthin (Zagalsky, 1985). However, these studies specifically purified and analysed the pigmented protein and not the amount of CRCN protein present in whole tissue extracts. Given that the CRCN protein is not pigmented unless bound to the carotenoid, the total abundance of the CRCN protein would not be measured by purifying and quantifying the pigmented protein alone. Our study indicates that CRCN protein may be present in prawn epithelial tissue in varying amounts without being bound to free Axn. This is

similar to the presence of Axn in the epithelial tissue in esterified form without being bound to CRCN. Combined, studies suggest that CRCN protein abundance is not directly related to external prawn colour, and is unlikely to be the mechanism by which the increase in prawn pigmentation during two-hours exposure to black substrates is regulated.

Effects of Background Colour Exposure on the Colour of Farmed Prawns

Past studies have shown that the methods used during harvesting farmed prawns can have a significant impact on cooked prawn colour, and hence price of farmed product (Wade*, et al.*, 2014). The response of prawns to background colour (Parisenti*, et al.*, 2011b; Tume*, et al.*, 2009) also suggests that harvesting animals into black bins has the potential to rapidly improve prawn colour, but also that harvesting into white bins may adversely affect cooked prawn colour. The results of the current study demonstrated that post-harvest exposure to different coloured substrates can affect prawn colour (Figure 4), but only a mild disparity in colour was observed using subjective scoring (Table 5). In some cases exposure to white substrates produced significantly paler prawns but black substrates had no effect. Meanwhile in other cases, black substrates produced significantly darker prawns but white substrates had no effect.

As demonstrated by the effect of the initial colour of the animals after 6 weeks on red tanks, the disparity in response to black or white substrates in farmed prawns may be similarly influenced by differences in animal colour between ponds. Significant variation in the colour of farmed prawns has been demonstrated between different ponds (Wade*, et al.*, 2014), and this may influence the response to substrate colour. An animal that is already very dark may not show a strong response to dark substrates compared with an animal that is less pigmented before exposure. In addition, the colour of *P. monodon* has been observed to become redder when subjected to thermal and hypoxic stress, but this effect was reversible when the stress was removed (de la Vega*, et al.*, 2007). This stress response may also be evident during harvesting, the effects of which are likely to cause uncooked prawns to become significantly redder and hence produce a darker cooked prawn colour. Anecdotal evidence suggests that

this may be occurring (N. Wade unpublished data), although this effect has not been quantified.

Overall, results of the farm-based trial demonstrate that this method can produce darker, more desirable prawns that can fetch higher market prices. It has the potential to correct the colour of prawns from ponds known to produce poor pigmentation, and equalise the variation between ponds during harvesting and processing. It is equally important to ensure that the harvest method is not causing a significant loss of colour prior to cooking. However, the practicalities of intentionally holding large numbers of animals for long periods of time may restrict its application.

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Figure 1. Prawn colour change in response to diet and background colour. Each colour square represents the average RGB colour of all prawns from each treatment. Colour was quantified from digital images at day 0, 14, 28 and 42 during the feeding trial, and then at 15, 30, 60 and 120 minutes after exposure to either black or white substrates.

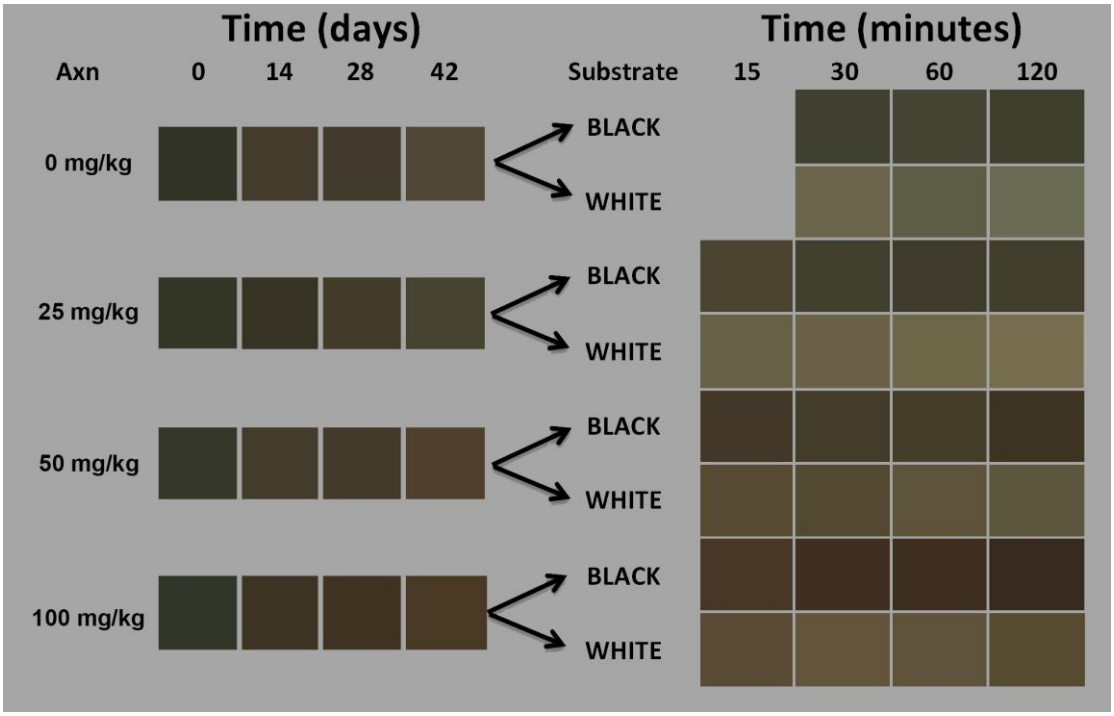


Figure 2. Expansion and contraction of epithelial chromatophores in response to diet and background substrate. Epithelial tissue from prawns fed different levels of dietary astaxanthin (0, 25, 50 and 100 mg/kg) for 6-weeks (T0), and then exposed for 2 hours to black (T120 Black) or white (T120 White) substrates. Images show the dorsal surface of the first abdominal segment from each prawn, and the inset a magnified view from the same region of each segment.

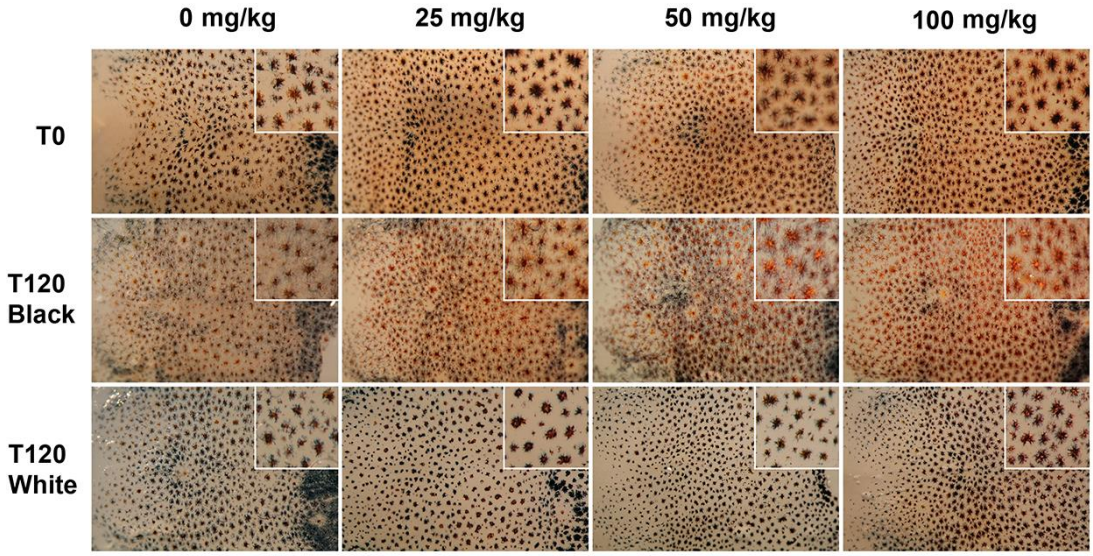


Figure 3. Quantification of CRCN protein from epithelial tissue. Total protein extracted from uncooked prawn epithelial tissue was pooled across all individuals in equal quantities. Different amounts (2-10 µg) of total epithelial protein and 10 µg of total muscle protein (C) was loaded onto a PVDF membranes in triplicate, and the amount of the colour protein CRCN was quantified using a highly specific anti-CRCN antibody.

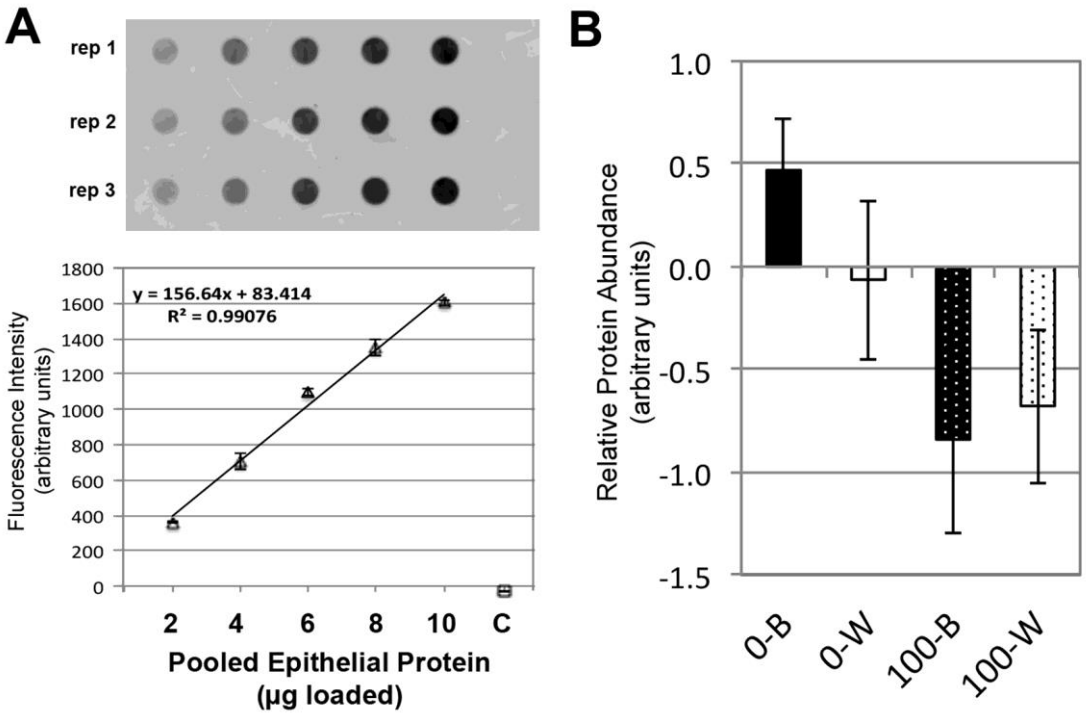
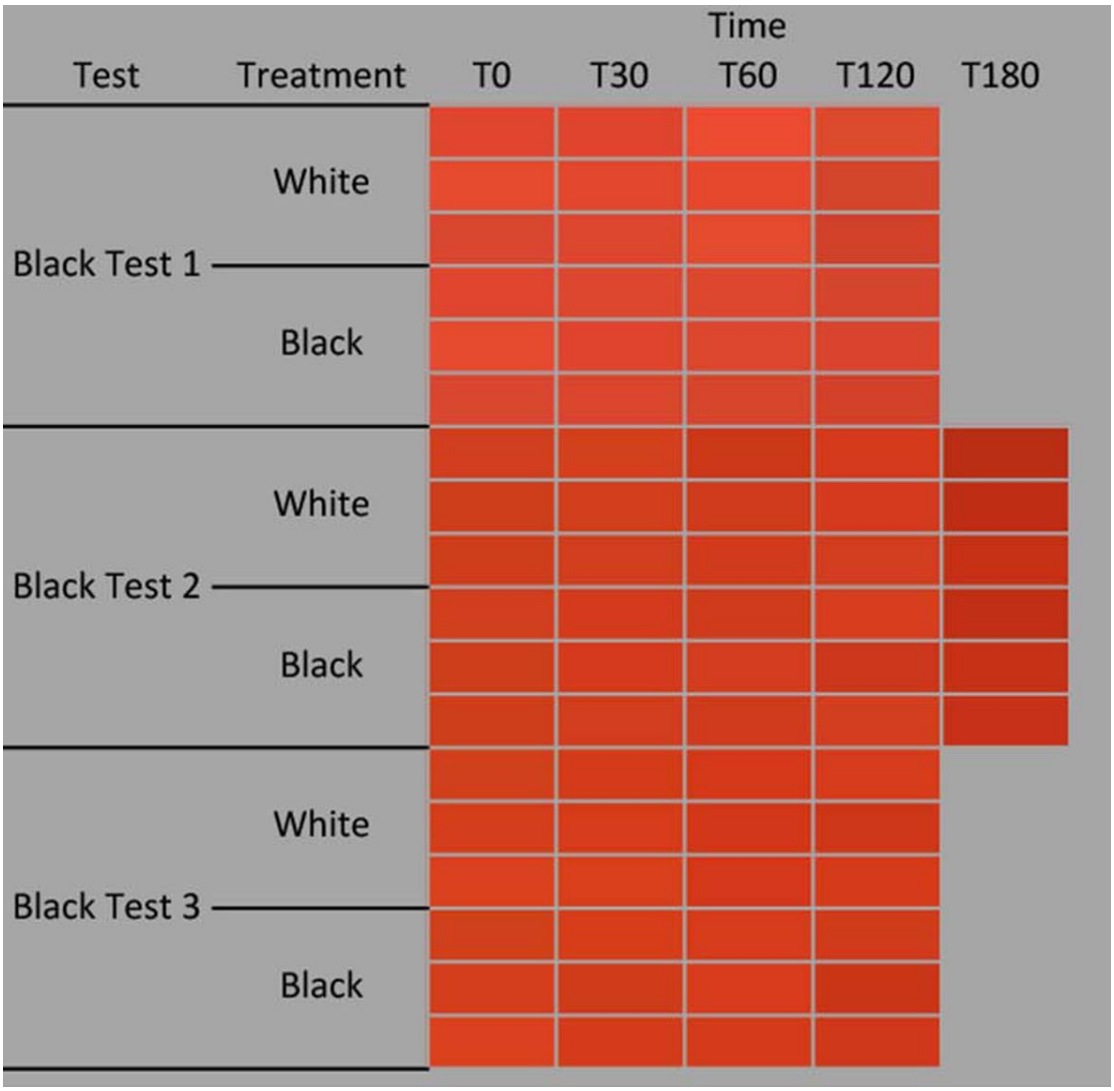


Figure 4. The effect of exposure to black and white substrates on the colour of farmed prawns. Three replicate photographs, each containing 20 prawns, were used to quantify the average colour of each prawn from digital images, and then grouped according to the various treatments. Each of the coloured 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.



586 Table 1. Experimental diet formulations and proximate composition.

Formulation (%)	**0 mg/kg**	**25 mg/kg**	**50 mg/kg**	**100 mg/kg**
Fish Meal	45.0%	45.0%	45.0%	45.0%
Gluten (wheat)	5.0%	5.0%	5.0%	5.0%
Flour	46.28%	46.255%	46.23%	46.18%
Lecithin	1.0%	1.0%	1.0%	1.0%
Fish Oil	1.5%	1.5%	1.5%	1.5%
Carophyll Pink (10%)	0.000%	0.025%	0.050%	0.100%
Cholesterol	0.10%	0.10%	0.10%	0.10%
Banox E	0.02%	0.02%	0.02%	0.02%
Vit C (Stay C)	0.10%	0.10%	0.10%	0.10%
Vit premix	0.20%	0.20%	0.20%	0.20%
Min premix	0.30%	0.30%	0.30%	0.30%
Yttrium	0.50%	0.50%	0.50%	0.50%
TOTAL	**100%**	**100%**	**100%**	**100%**
Proximate Composition as measured				
Moisture Content (%)	1.19	1.89	1.86	1.94
Total Protein (%)	40.40	39.88	40.10	41.07
Total Lipid (%)	6.57	9.37	8.05	7.97
Ash (%)	12.62	12.17	12.51	12.74
Carotenoids (mg/kg)	5.64	12.84	35.21	71.58

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Table 2. Average uncooked RGB colour values of prawns over time at different carotenoid inclusion levels.

Day	0 mg/kg			25 mg/kg			50 mg/kg			100 mg/kg		
Average	R	G	B	R	G	B	R	G	B	R	G	B
0	49.9 ± 2.4	51.5 ± 1.8	39.1 ± 2.1	51.5 ± 3.3	54.6 ± 3.7	40.9 ± 2.9	52.6 ± 2.4	55.4 ± 3.5	42.5 ± 2.5	51.0 ± 2.9	54.8 ± 2.2	41.8 ± 2.5
14	67.4 ± 4.6	60.1 ± 4.0	44.2 ± 3.6	56.5 ± 2.3	51.1 ± 2.4	36.7 ± 2.6	67.0 ± 3.1	58.8 ± 2.6	43.4 ± 2.2	62.8 ± 1.9	52.2 ± 1.0	35.8 ± 0.5
28	65.7 ± 1.4	59.7 ± 2.3	44.5 ± 3.3	64.6 ± 4.1	58.8 ± 2.7	40.9 ± 2.6	67.2 ± 2.4	58.1 ± 2.0	41.6 ± 1.6	64.2 ± 0.6	51.7 ± 0.9	34.2 ± 0.7
42	78.5 ± 5.0	73.2 ± 1.5	53.7 ± 2.4	71.2 ± 4.8	67.6 ± 6.2	47.5 ± 5.6	79.9 ± 6.4	66.5 ± 4.3	46.2 ± 2.4	71.6 ± 4.0	57.8 ± 2.5	37.5 ± 1.3

Table 3. Average uncooked RGB colour values of prawns over time after exposure to black or white substrates.

Time	0 mg/kg			25 mg/kg			50 mg/kg			100 mg/kg		
White	R	G	B	R	G	B	R	G	B	R	G	B
0	78.5 ± 5.0	73.2 ± 1.5	53.7 ± 2.4	71.2 ± 4.8	67.6 ± 6.2	47.5 ± 5.6	79.9 ± 6.4	66.5 ± 4.3	46.2 ± 2.4	71.6 ± 4.0	57.8 ± 2.5	37.5 ± 1.3
15	n.d.	n.d.	n.d.	103.9 ± 4.2	97.2 ± 4.7	71.2 ± 4.6	86.1 ± 5.0	73.6 ± 3.2	50.2 ± 2.4	89.8 ± 9.0	75.0 ± 7.2	54.1 ± 4.5
30	88.6 ± 3.6	88.6 ± 2.6	66.4 ± 2.8	106.9 ± 5.1	97.0 ± 4.3	71.7 ± 3.0	82.5 ± 4.9	71.9 ± 4.2	50.3 ± 2.3	99.1 ± 6.3	84.6 ± 4.7	58.9 ± 3.3
60	106.3 ± 9.3	100.7 ± 7.0	75.1 ± 4.1	110.1 ± 3.8	102.5 ± 2.1	73.1 ± 0.8	94.1 ± 10.2	84.9 ± 8.6	60.3 ± 4.9	94.9 ± 8.9	82.8 ± 7.3	57.9 ± 5.9
120	91.9 ± 5.5	92.9 ± 4.7	71.4 ± 2.9	120.2 ± 5.9	110.5 ± 3.9	78.2 ± 2.8	92.8 ± 2.2	87.6 ± 1.5	61.2 ± 1.8	88.3 ± 5.5	75.5 ± 3.5	49.3 ± 1.3
Black	R	G	B	R	G	B	R	G	B	R	G	B
0	78.5 ± 5.0	73.2 ± 1.5	53.7 ± 2.4	71.2 ± 4.8	67.6 ± 6.2	47.5 ± 5.6	79.9 ± 6.4	66.5 ± 4.3	46.2 ± 2.4	71.6 ± 4.0	57.8 ± 2.5	37.5 ± 1.3
15	n.d.	n.d.	n.d.	75.4 ± 1.4	68.2 ± 3.7	49.2 ± 3.7	66.7 ± 3.9	56.2 ± 3.6	39.6 ± 2.7	71.8 ± 2.3	55.4 ± 3.8	38.9 ± 3.0

30	74.5 ± 5.2	75.7 ± 3.0	57.4 ± 2.4	64.3 ± 4.1	63.6 ± 2.8	45.8 ± 1.5	67.3 ± 2.2	59.6 ± 2.0	41.7 ± 2.1	63.6 ± 1.1	46.3 ± 1.5	33.3 ± 1.5
60	63.5 ± 1.5	65.5 ± 2.4	50.1 ± 1.7	62.8 ± 2.6	59.5 ± 3.7	42.8 ± 2.8	68.8 ± 1.8	60.7 ± 2.5	42.4 ± 2.3	62.3 ± 4.4	47.0 ± 4.9	32.3 ± 3.4
120	69.4 ± 4.0	69.7 ± 5.4	51.0 ± 5.4	66.1 ± 3.4	61.7 ± 6.0	44.4 ± 6.0	61.8 ± 4.2	53.3 ± 2.7	37.4 ± 1.3	55.9 ± 3.3	43.3 ± 1.4	30.5 ± 1.4

Table 4. Subjective cooked colour grade scores of prawns fed different levels of dietary carotenoid and exposed to black or white substrates for two hours

Background	White				Black			
Carotenoid (mg/kg)	0	25	50	100	0	25	50	100
Prawn Colour Chart	6.9 ± 0.48^{a}	8.3 ± 0.30^{b}	8.8 ± 0.17^{b}	8.8 ± 0.13^{b}	8.9 ± 0.31^{b}	9.9 ± 0.15^{c}	10.5 ± 0.23^{cd}	11.0 ± 0.25^{d}
Salmofan	25.6 ± 0.62^{a}	27.5 ± 0.48^{b}	28.9 ± 0.42^{b}	28.6 ± 0.31^{b}	28.9 ± 0.66^{b}	31.1 ± 0.34^{c}	31.3 ± 0.53^{cd}	32.6 ± 0.40^{d}

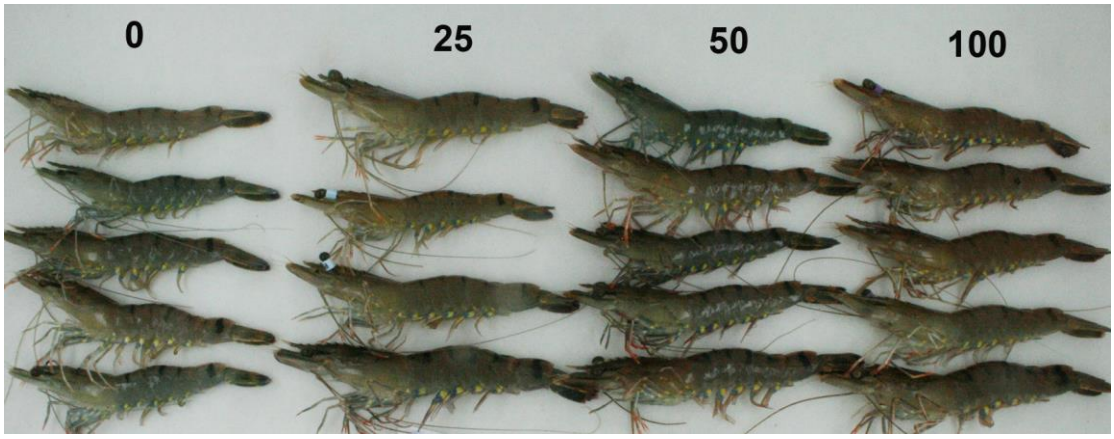
Superscripts denote significant (P <0.05) differences between measured values across groups.

Table 5. Subjective colour grade scores of farmed prawns exposed to black or white substrates for different lengths of time.

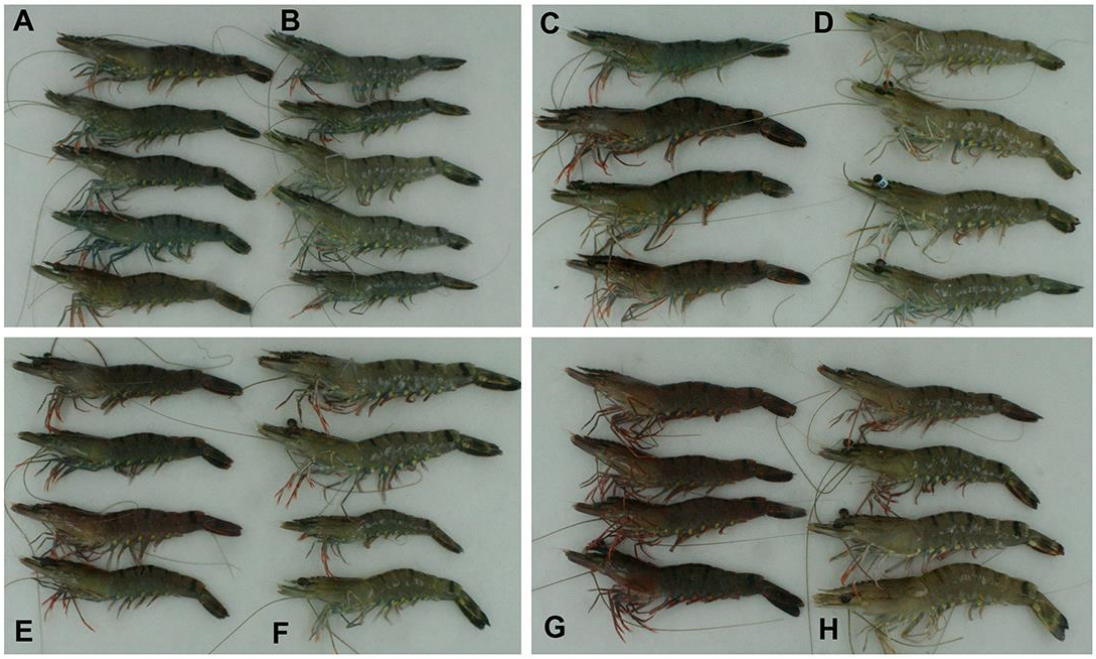
	T0	White T30	Black T30	White T60	Black T60	White T120	Black T120	White T180	Black T180
Prawn Colour Chart									
Test 1	9.3 ± 0.09^{ab}	9.5 ± 0.09^{ab}	9.6 ± 0.08^{ab}	9.3 ± 0.10^{a}	9.6 ± 0.08^{b}	9.1 ± 0.09^{c}	9.5 ± 0.08^{ab}		
Test 2	9.4 ± 0.11^{a}	9.5 ± 0.08^{a}	10.0 ± 0.07^{b}	10.1 ± 0.07^{b}	10.3 ± 0.08^{c}	10.2 ± 0.09^{b}	10.2 ± 0.08^{b}	9.7 ± 0.09^{a}	10.2 ± 0.07^{b}
Test 3	8.9 ± 0.11^{a}	9.1 ± 0.08^{b}	9.2 ± 0.09^{b}	9.3 ± 0.07^{b}	9.3 ± 0.08^{b}	9.3 ± 0.08^{b}	9.4 ± 0.08^{b}		
Salmofan									
Test 1	29.4 ± 0.12^{a}	29.5 ± 0.12^{ab}	29.8 ± 0.10^{b}	29.5 ± 0.13^{a}	29.7 ± 0.10^{a}	28.8 ± 0.14^{c}	29.5 ± 0.10^{a}		
Test 2	29.5 ± 0.16^{a}	30.0 ± 0.13^{ab}	30.8 ± 0.12^{b}	30.8 ± 0.13^{b}	31.3 ± 0.13^{b}	30.8 ± 0.16^{b}	30.9 ± 0.13^{b}	30.2 ± 0.15^{a}	31.0 ± 0.13^{b}
Test 3	29.1 ± 0.15^{a}	29.9 ± 0.15^{b}	29.9 ± 0.15^{b}	30.0 ± 0.13^{b}	30.2 ± 0.14^{b}	30.1 ± 0.16^{b}	30.1 ± 0.15^{b}		

Superscripts denote significant (P <0.05) differences between measured values across groups.

Supplementary Figure 1



Supplementary Figure 2



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