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Colour change of greenlip abalone (*Haliotis laevigata* Donovan) fed formulated diets containing graded levels of dried macroalgae meal

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ABSTRACT

This study evaluated the effects of supplementing dried macroalgae meal on the shell, foot and lip colour in terms of colour hue, saturation and brightness and pigment concentration in greenlip abalone *Haliotis laevigata*. Two species of dried macroalgae meal (*Ulva* sp. and *Gracilaria cliftonii*) at four levels (0% basal diet, 5%, 10% and 20%) of dietary inclusion were fed to the abalone (2.89 ± 0.01 g; shell length 22.41 ± 0.06 mm) for 92 days. The abalone fed *G. cliftonii* meal developed brown-red colour on the shell and the increased inclusion of algal meal resulted in darker brown shells, whereas abalone fed *Ulva* sp. meal or the basal diet exhibited light green shells. Although foot hue and foot brightness were not influenced by the type and level of inclusion of dried algal meal, the foot colour saturation of abalone fed ≥10% macroalgal meal was significantly higher than those fed the basal diet. Abalone developed a green lip when fed ≥10% of *G. cliftonii* meal, whereas lip colour did not change when fed *Ulva* sp. meal inclusion, compared to the basal diet, and exhibited milky lip colour. Although diet pigments varied with the species of macroalgae and inclusion level, β-carotene was the major pigment in the tissue of abalone fed all test diets and its content increased significantly with the inclusion level of macroalgae in the diet. Abalone fed the basal diet had significantly lower tissue β-carotene than those fed the diets with macroalgal inclusion. This study suggests that the inclusion of ≥5% *G. cliftonii* can produce a brown-red shell and the inclusion of ≥10% *G. cliftonii* intensifies the green colour of the lip. The brown-red colour mark on the shell by feeding macroalgal meal may be used as a harmless shell-marking method for ranching, stock enhancement or growth study in the wild.

Statement of relevance: Feed manipulation can change abalone shell, foot and lip colour.

1. Introduction

The colour of abalone meat, shell, lip or epidermal tissues has been used to identify abalone species (Olley and Thrower, 1977; Mottet, 1978; Brown, 1995), assess abalone market quality (Brown et al., 2008) and distinguish abalone stocks in the wild and on farm (Mottet, 1978; Gallardo et al., 2003). However, shell colours vary and are affected by the diet, environmental condition and habitat (Bautista-Teruel and Millamena, 1999; Gallardo et al., 2003; Allen et al., 2006; Mayfield et al., 2014). For example, the shell colouration of greenlip abalone in Australia is orange in the Great Australian Bight region, green in the Spencer Gulf region, while it is red in northwest Tasmania (Mayfield et al., 2014). Thus, it is difficult to identify greenlip abalone based on shell colour alone and species identification should be based on colouration of other parts. The lip colour of wild greenlip abalone feeding on natural algae is typically green (Mottet, 1978; Brown, 1995; Department of Fisheries, 2011). However, cultured greenlip abalone fed formulated diet exhibit a milky lip, light green shell and darker foot (Hoang et al., 2016). The discolouration of abalone may be due to formulated diets lacking pigments in natural diets (Bautista-Teruel and Millamena, 1999; Qi et al., 2010; Hoang et al., 2016). In addition, abalone colour is relevant to market sale, and colour changes may also affect product price and attraction to customers (Brown et al., 2008).

Macroalgae such as brown algae, *Laminaria* spp. and *Undaria pinnatifida* or red algae, *Corallina elungata* and *Jania rubens*, *Gracilaria conferta* contain carotenoids which are widely used or currently being investigated in the food and feed industry as colour enhancers (Christaki et al., 2013). In aquaculture, there has been considerable interest to change or enhance the colour of some aquatic animals including abalone through feeding or adding macroalgae in the diet

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(Leighton, 1961; Sakai, 1962; Leighton and Boolootian, 1963; Olsen, 1968; Oakes and Ponte, 1996; Bautista-Teruel and Millamena, 1999; Gallardo et al., 2003; Shpigel et al., 2005). Recently, Qi et al. (2010) and Ju et al. (2015) reported that the shell colour of Pacific abalone *Haliotis discus hannai* fed kelp *Laminaria japonica* was green, but it was purple or dark-brown when fed red macroalgae *Gracilaria lemaneiformis* or Pacific dulse *Palmaria mollis*. The purple or dark-brown shell of this species is preferred by Asian markets due to similarity to wild-caught abalone. Greenlip abalone produce a brown shell and a light yellow foot when fed live *Gracilaria clifftoni*, while exhibit a light green shell and light yellow foot when fed live *Ulva* sp. (Hoang et al., 2016).

Partial inclusion of dried micro- or macroalgae meal in formulated feed is a promising way to not only gain nutritional benefits for aquatic animals including abalone, but also potentially alter or improve their colour (Choubert, 1979; Lim and Lee, 2003; Wassef et al., 2005; Valente et al., 2006; Ergün et al., 2009; Soler-Vila et al., 2009; Cyrus et al., 2013; Bansemer et al., 2014; O'Mahoney et al., 2014; Ragaza et al., 2015; Valente et al., 2015; Hoang et al., 2016; Valente et al., 2016). Lim and Lee (2003) reported that the shell of *H. discus hannai* fed a diet containing 2% *Porphyra* became yellow-red and orange, which is similar to the shell colour of wild abalone. Our recent study shows that the shell of abalone fed 3% dried *Spirulina* sp. was yellow-brown while the shell and foot of greenlip abalone fed 10% dried *Ulva* sp. became light green and bright yellow in 92 days (Hoang et al., 2016). Therefore, the present study was carried out to further explore the levels of dried *Ulva* sp. meal and also to investigate the potential of *G. cliftonii* meal inclusion in the formulated diet on the colour of greenlip abalone. The aim of study was to evaluate the effects of green or red dried macroalgae meal at different inclusion levels on colour of lip, foot, shell and pigmentation of greenlip abalone.

2. Materials and methods

2.1. Experimental animal and system

2.1.1. Abalone source

Greenlip abalone were obtained from South Australian Mariculture (Boston Point, Port Lincoln, SA, Australia) and were then kept at the South Australian Research and Development Institute (SARDI) Aquatic Sciences Centre in 170 L holding tanks in a flow-through, UV-treated, seawater system. The animals were fed ad libitum daily on a commercial diet - Abgrow premium abalone in 5-mm chip made by Eyre Peninsula Aquafeed Pty Ltd. (EPA), Lonsdale, SA, Australia.

2.1.2. System structure

The design of the experimental system was previously described in Stone et al. (2013). Briefly, the system was comprised of 32 blue plastic culture tanks (Nally IH305, Viscount Plastics Pty Ltd.; 39.2 × 28.8 × 11.0 cm). Water level was set a depth of 2.5 cm using a standpipe providing a water volume of 2.8 L in each tank. The water flow was kept at 300 mL min^{-1} and flow-through UV-treated water was supplied to tanks from a saltwater system. Photoperiod was controlled at 12 h light (low intensity with fluorescent lighting at 3.4 lx) and 12 h dark. The water temperature was maintained at 22 ± 1 °C throughout the experimental period using an immersion heater (240 V, 3 kW; Austin & Cridland, Carlton, NSW, Australia).

2.2. Experimental design and diets

2.2.1. Diet types

The animals were fed one of eight diets. A basal diet (0% dried macroalgae meal inclusion) and six diets which consisted of either of two species of macroalgae (*Ulva* sp. and *Gracilaria cliftonii*) at three inclusion levels (5%, 10% and 20%) formulated into the basal diet by reducing solvent extracted soybean meal, wheat flour and de-hulled lupins levels. A commercial diet (EPA Abgrow premium 5 mm chip; Eyre Peninsula Aquafeed Pty Ltd., Lonsdale, SA, Australia) which is typically fed to greenlip abalone in Australia was also included in this study as a control for comparison. The basal diet was EPA mash. The proximate composition, amino acid and pigment contents of the experimental diets are presented in Table 1.

2.2.2. Diet preparation

For the preparation of dried *G. cliftonii* meal, live *G. cliftonii* was collected from Gulf St. Vincent, SA, Australia, and cultured in a 4000-L tank under ambient sunlight. Live *G. cliftonii* was enriched with 8 L of modified F2 nutrient media in order to improve the protein content from 12.9 to 38.1% (Bansemer et al., 2016a). Enriched *G. cliftonii* was harvested and sun-dried for about 4 h and then oven-dried at 45 °C for 72 h. Dried *G. cliftonii* was homogenised into a fine powder (300 μm) using a blender (model HGBTWT53, Waring Commercial, Torrington, CT, USA) and stored at −20 °C until the diets were made. Enriched *Ulva* sp. meal was supplied from Venus Shell Systems (Narrawallee, NSW, Australia).

Diets were formulated to contain 35% crude protein, 5% crude lipid and a gross energy content of 17.5 MJ kg^{-1}, based on the nutritional requirements for greenlip abalone (Stone et al., 2013; Bansemer et al., 2016a). All ingredients were weighed out and mixed in a Hobart mixer (Hobart Corp., Troy, OH, USA) for 5 min. Water (~30%) was added to the diet mash and mixed for further 3 min. The diets were made using a TR110 pasta machine (Machine Per Pasta SRL, Molina Di Malo, VI, Italy). All diets were produced with a size dimension of 5 × 5 × 2 mm. Diets were then oven-dried at 45 °C for 48 h and frozen at −20 °C.

2.3. Stocking and feeding

Twenty greenlip abalone (2.89 ± 0.01 g; shell length 22.41 ± 0.06 mm SL) were randomly stocked into each of the 32 tanks (20 abalone $tank^{-1}$); four replicate tanks were allocated to each treatment. The experiment ran for 92 days. Feeding was performed daily at 16:00 h and abalone were fed to excess at 4% biomass day^{-1} in line with Buss et al. (2015). Tanks were cleaned between 08:30 h and 09:30 h on the following day at which point uneaten feed was collected by pouring the entire tank contents through a fine mesh screen (500 μm). The uneaten feed then was weighed and oven-dried at 105 °C for 16 h. The difference between the amount of uneaten feed and feed delivered was used to calculate daily feed consumption. For corrected feed consumption, leaching loss was taken into account by immersing experimental diets in water at 22 °C for 16.5 h without animals, then collected through a fine mesh net (500 μm), and dried to constant weight.

2.4. Specimen collection and colour analyses

At the beginning of the experiment, 20 abalone were photographed for image analysis then stored at −80 °C for initial pigment analyses. At the end of the experiment, four abalone from each tank were collected, weighed, measured, photographed and then frozen at −80 °C for final pigment analyses.

2.4.1. Photographic image analysis

The colour of the shell, foot and lip was assessed by analysing photographic images of abalone. To capture the image, a white-mat surfaced light table with two natural white colour bulbs (NEC, Adelaide, Australia, 18 W with natural white colour) mounted on two sides of a table (75 × 75 cm) and a digital camera [Canon PowerShot G16, 12.1 megapixel (backside illuminated) 1/1.7″ CMOS sensor)] placed on an adjustable arm between the two sides of the light was used (Hoang et al., 2016). The camera was set up at 25 cm above the specimen. A reference colour card (X-rite; colour checker passport, manufactured by the

Table 1
Proximate composition, amino acids, minerals and pigment content experimental diets.

Macroalgal species		*Ulva* sp.				*G. cliftonii*		
Inclusion level (%)	Comm[a,b]	0*	5	10	20	5	10	20
Proximate composition[a] (g 100 g^{-1} diet as fed)								
Moisture	12.20	12.70	11.90	12.00	11.90	12.01	11.80	11.30
Crude protein	29.70	34.60	35.00	34.80	34.70	35.10	35.00	35.20
Lipid	4.20	5.10	5.40	5.20	5.10	5.40	5.40	5.50
Gross energy (MJ kg^{-1})	16.94	17.54	17.58	17.34	17.00	17.45	17.24	16.86
Ash	5.80	4.80	5.90	6.90	8.80	6.50	8.00	10.90
Carbohydrate	48.10	42.80	41.80	41.10	39.50	40.90	39.80	37.10
Amino acids[a] (g 100 g^{-1} diet as fed)								
Alanine	1.10	1.38	1.45	1.56	1.69	1.47	1.42	1.56
Aspartic acid	2.66	3.36	3.46	3.75	3.78	3.62	3.42	3.46
Arginine	1.66	2.54	2.62	2.65	2.74	2.59	2.56	2.73
Glutamic acid	5.10	5.83	5.89	5.76	5.93	5.94	5.70	5.55
Glycine	1.20	1.60	1.65	1.63	1.73	1.65	1.63	1.65
Histidine	0.56	0.85	0.83	0.81	0.81	0.84	0.83	0.85
Isoleucine	1.03	1.46	1.48	1.40	1.41	1.50	1.47	1.47
Leucine	1.95	2.51	2.53	2.58	2.57	2.60	2.51	2.53
Lysine	1.80	1.97	1.99	2.09	2.19	2.18	1.99	1.89
Methionine	0.37	0.55	0.63	0.46	0.49	0.44	0.56	0.46
Phenylalanine	1.28	1.57	1.58	1.66	1.67	1.71	1.57	1.70
Proline	2.43	2.35	2.35	2.02	1.93	1.98	2.25	1.91
Serine	1.14	1.63	1.64	1.53	1.57	1.53	1.67	1.65
Threonine	0.90	1.36	1.39	1.24	1.30	1.25	1.39	1.33
Tyrosine	0.85	1.10	1.29	1.15	1.13	1.20	1.09	1.23
Valine	1.36	1.65	1.68	1.85	1.89	1.84	1.68	1.68
Diet pigments								
Astaxanthin (μg g^{-1})	–	–	1.47	2.12	3.02	–	–	–
β-carotene (μg g^{-1})	1.23	1.00	1.48	3.13	5.68	1.65	1.85	3.62
Chlorophyll *a* (μg g^{-1})	–	–	–	3.06	6.64	–	–	–
β-cryptoxanthin (μg g^{-1})	–	–	–	0.53	1.09	–	–	–
Zeaxanthin (μg g^{-1})	0.60	0.52	0.57	0.66	1.04	2.32	4.51	8.63

*The control of non-macroalgae inclusion was shared by both *Ulva* sp. and *G. cliftonii* treatments.
– Not detectable.
[a] Reported by Bansemer et al. (2016b).
[b] Comm. = Commercial diet.

Munsell Colour Services laboratory, Rochester Institute of Technology, 54 Lomb Memorial Drive, Rochester, NY 14623) was placed next to the specimens. The image was analysed with Gimp2 software which is available at www.gimp.org. The means of red, green and blue (RGB) values were converted to hue, saturation and brightness (HSB) values, respectively with an online software program (www.colorizer.org at http://colorizer.org/).

All possible colour combinations were specified as hue (i.e. colour purity), percent of saturation (i.e. colour intensity) and brightness. The HSB model can be visualized as an upside-down cone for illustrating the relationship of colour hue, saturation and brightness (Yasir and Qin, 2009). Hue was expressed as a number indicating the degrees around the cone with red at zero, green at 120, and blue at 240°. Colour saturation ranged from 0% (no saturation) to 100% (full saturation). Brightness ranged from 0% (black) to 100% (white), but both hue and saturation become meaningless at 0% brightness.

2.4.2. Pigment extraction and analysis

The methods of pigment extraction and analysis were previously described in Hoang et al. (2016). Briefly, all samples including experimental diets and the whole tissue of abalone without gut were thawed at the room temperature and then freeze-dried for 48 h until a constant weight. All samples were separately ground into fine powder before pigment extraction. About 0.35 g was accurately weighed and was sequentially extracted three times with 10 mL ethanol-hexane (1:1, v/v) until the residue became colourless. Each extraction was centrifuged at 16,000*g* for 5 min and then transferred to 2 mL HPLC vials to dry completely under a stream of pure nitrogen gas. The dried extract was then dissolved in 200 μL heptane and acetone (1:1, v/v) and vortexed for 20 s before analysis on HPLC (Shimadzu UFLC, Kyoto, Japan).

The HPLC was equipped with the Waters Symmetry 300™ analytical C18 column (5 μm, 3.9 × 150 mm). Solvents included 80% acetonitrile and 20% water and acetone. The flow rate was 1 mL min^{-1} with a 5 μL injection. The wavelengths of detection were set at 450 nm for astaxanthin, zeaxanthin, β-carotene and β-cryptoxanthin, and 630 nm for chlorophyll *a*. The calibration curves were developed from known concentrations of zeaxanthin (Fluka, 14,681), astaxanthin (Sigma, SML0982), β-carotene (Sigma, C4582), β-cryptoxanthin (Sigma, C6368), and chlorophyll *a* (Sigma, C6144), respectively. Pigment quantification was performed by the Shimadzu software (LabSolutions v1.25). The detection limit for HPLC was 0.3 μg/mL. The retention time was 4.7 min for astaxanthin, 6.1 min for zeaxanthin, 9.9 min for β-cryptoxanthin, 12.1 min for β-carotene and 10.2 min for chlorophyll *a*.

2.5. Statistical analysis

The data were analysed using SPSS (version 22) and the significant level was set at $P < 0.05$. Two-way ANOVA was used to determine the interactive effects between macroalgal meal species (*Ulva* sp. meal and *G. cliftonii* meal) and dietary inclusion level (0, 5, 10 and 20%) on colour components (hue, saturation and brightness) and pigment contents in abalone tissue. The basal diet (0% macroalgal meal) was as the control for both macroalgal species. When no significant interactions between dried macroalgal meal species and inclusion level was observed, post-hoc test was used to detect significant differences between treatment means (one-factor ANOVA; Tukey's HSD test), whereas differences in level of inclusion are compared within *Ulva* sp. meal or *G.*

cliftonii meal (one-factor ANOVA; Tukey's HSD test) when significant interactions between those two factors was observed. Two-tailed *t*-test was used to determine differences between abalone fed the basal diet and those fed the commercial diet.

3. Results

3.1. Shell colour properties

3.1.1. Hue

Shell hue was significantly affected by macroalgae meal species (two-factor ANOVA; $P < 0.001$; Table 2; Fig. 1), inclusion level ($P < 0.001$) and the interaction between these two factors ($P < 0.001$). The significant interaction was due to the change in shell hue for abalone fed different levels of *G. cliftonii* inclusion compared to abalone fed the basal diet. In contrast, abalone fed *Ulva* sp. inclusion had similar shell hue to those fed the basal diet. The shell of abalone fed 20% *G. cliftonii* meal was significantly more brown-red in colouration than those fed 5% *G. cliftonii* ($P = 0.037$), but was not significantly different from those fed 10% *G. cliftonii* ($P = 0.423$). There was also no significant difference of hue value between abalone fed 5% and 10% *G. cliftonii* meal inclusion level ($P = 0.195$). The shell colour of abalone was visually different between dietary treatments after one week of feeding. The shell of abalone fed the *G. cliftonii* meal turned brown-red while the shell of abalone fed *Ulva* sp. remained light green (Fig. 1). There was no statistically significant difference in the shell hue values between abalone fed the commercial diet and the basal diet (Two-tailed *t*-tests; $P = 0.639$).

3.1.2. Saturation

The shell saturation of abalone was significantly affected by the macroalgae meal species (two-factor ANOVA; $P < 0.001$; Table 2), inclusion level ($P = 0.026$) and their interaction ($P < 0.001$). The interaction was caused by the significant increase in shell saturation of abalone fed graded levels of *G. cliftonii* meal compared to those fed the basal diet. In comparison, abalone fed all levels of *Ulva* sp. meal had similar shell saturation to those fed the basal diet ($P > 0.05$). The shell saturation of abalone fed 10% *G. cliftonii* meal was significantly greater than those fed 5% ($P = 0.039$), whereas similar shell saturation values were observed in abalone fed 10% and 20% *G. cliftonii* ($P = 0.970$). Abalone fed the commercial diet had similar shell saturation values as those fed the basal diet (Two-tailed *t*-tests; $P = 0.398$).

3.1.3. Brightness

Macroalgae meal species (two-factor ANOVA; $P < 0.001$; Table 2), inclusion level ($P < 0.001$) and the interaction between these two factors ($P < 0.001$) significantly affected the shell brightness. The significant interaction was due to the significant low value of shell brightness of abalone fed 5 and 10% *G. cliftonii* meal relative to abalone fed the basal diet, whereas, shell brightness of abalone fed 5 and 10% *Ulva* sp. meal was similar to abalone fed the basal diet. In addition, abalone fed 20% *G. cliftonii* or 20% *Ulva* sp. inclusion level had significantly reduced shell brightness compared to abalone fed the basal diet. Among the *Ulva* sp. treatments, only the inclusion of 20% *Ulva* sp. significantly reduced shell brightness compared to the diet containing 5% ($P = 0.006$) and 10% ($P = 0.047$) meal inclusion. Among *G. cliftonii* diets, shell brightness of abalone fed 20% *G. cliftonii* was significantly lower than those fed 10% *G. cliftonii* ($P = 0.002$), but not those fed 5% *G. cliftonii* ($P = 0.113$). There was no significant difference in shell brightness between 5 and 10% *G. cliftonii* inclusion treatments ($P = 0.128$). The shell brightness of abalone fed the commercial diet was similar to those fed the basal diet (Two-tailed *t*-tests; $P = 0.051$).

3.2. Lip and foot colour properties

3.2.1. Lip colour properties

Lip hue was significantly affected by macroalgae meal species (two-factor ANOVA; $P < 0.001$; Table 2; Fig. 3), inclusion level ($P < 0.001$) and their interaction ($P = 0.001$). The interaction was due to the significant change of lip hue value in abalone fed graded levels of *G. cliftonii* meal compared to those fed the basal diet, whereas, lip hue in abalone fed all levels of *Ulva* sp. meal was similar to those fed the basal diet. Lip hue of abalone fed *G. cliftonii* meal was significantly greener with increasing inclusion level, while the lip hue value was similar among abalone fed different *Ulva* sp. meal inclusion levels. Abalone fed 20% *G. cliftonii* meal had significantly greener lip than those fed 5% *G. cliftonii* ($P < 0.001$), but not to those fed 10% *G. cliftonii* ($P = 0.489$). There was also a significantly greener lip in abalone fed 10% *G. cliftonii* compared to those fed 5% *G. cliftonii* ($P = 0.002$).

Table 2
Whole tissue pigment contents and colour components of shell, foot and lip of greenlip abalone tissue, *Haliotis laevigata* fed graded levels of dried macroalgae meal inclusion.

	Ulva sp. meal				*G. cliftonii* meal				ANOVA (*P* value)		
Macroalgal species									Species	Inclusion level	
Inclusion level (%)	0[a]	5	10	20	0	5	10	20	(A)	(%) (B)	A × B
Colour components of shell											
Hue (degree)	113.2 ± 6.42	124.7 ± 12.3	115.5 ± 11.8	124.0 ± 14.1	113.2 ± 6.42	45.3 ± 3.77	28.0 ± 5.29	17.3 ± 3.01	<0.001	<0.001	<0.001
Saturation (%)	22.4 ± 4.45	23.2 ± 1.56	17.5 ± 1.77	20.1 ± 5.35	22.4 ± 4.45	34.0 ± 1.61	46.8 ± 1.06	46.6 ± 1.86	<0.001	0.026	<0.001
Brightness (%)	43.5 ± 1.10	43.9 ± 0.61	42.5 ± 1.41	38.9 ± 1.98	43.5 ± 1.10	32.6 ± 1.41	35.4 ± 1.03	29.7 ± 1.22	<0.001	<0.001	<0.001
Colour components of lip											
Hue (degree)	37.9 ± 2.02	42.2 ± 1.65	41.6 ± 1.60	40.6 ± 2.04	37.9 ± 2.02	44.2 ± 1.21	52.5 ± 1.63	54.2 ± 2.06	<0.001	<0.001	0.001
Saturation (%)	29.3 ± 4.32	23.6 ± 1.26	25.9 ± 1.70	28.5 ± 2.27	29.3 ± 4.32	36.6 ± 1.84	38.6 ± 3.23	51.6 ± 1.95	<0.001	0.001	0.001
Brightness (%)	52.9 ± 0.89	52.1 ± 1.52	51.2 ± 1.46	50.8 ± 1.68	52.9 ± 0.89	50.1 ± 1.30	48.8 ± 0.99	44.2 ± 1.00	0.002	<0.001	0.065
Colour components of foot											
Hue (degree)	18.8 ± 1.30	18.3 ± 1.76	17.7 ± 1.27	20.7 ± 1.29	18.8 ± 1.30	18.3 ± 1.54	17.5 ± 1.35	19.3 ± 1.24	0.675	0.366	0.944
Saturation (%)	48.7 ± 2.63	50.7 ± 1.75	54.3 ± 1.14	55.1 ± 1.52	48.7 ± 2.63	52.4 ± 1.48	54.9 ± 1.17	55.5 ± 1.61	0.602	0.001	0.967
Brightness (%)	53.6 ± 1.44	51.8 ± 1.89	49.7 ± 1.22	54.6 ± 2.20	53.6 ± 1.44	53.0 ± 1.52	51.2 ± 1.26	48.4 ± 1.54	0.453	0.229	0.058
Whole abalone body pigments											
β-carotene ($\mu g\ g^{-1}$)	1.25 ± 0.13	6.30 ± 1.19	6.67 ± 1.04	7.28 ± 0.21	1.25 ± 0.13	4.65 ± 1.07	6.38 ± 0.66	7.26 ± 0.57	0.363	<0.001	0.472
Chlorophyll *a* ($\mu g\ g^{-1}$)	–	–	2.31 ± 0.09	3.32 ± 0.12	–	–	–	–	<0.001	<0.001	<0.001

The significance level of $P < 0.05$ was used for all statistical tests. Post-hoc tests were used to determine differences between means (two-factor ANOVA; Student Newman-Keuls). For the variable with a significant interaction, differences in level of inclusion are compared within each macroalgae species using pairwise comparisons.
– Not detectable.
[a] The control of non-macroalgae inclusion was shared by both *Ulva* sp. and *G. cliftonii* treatments.

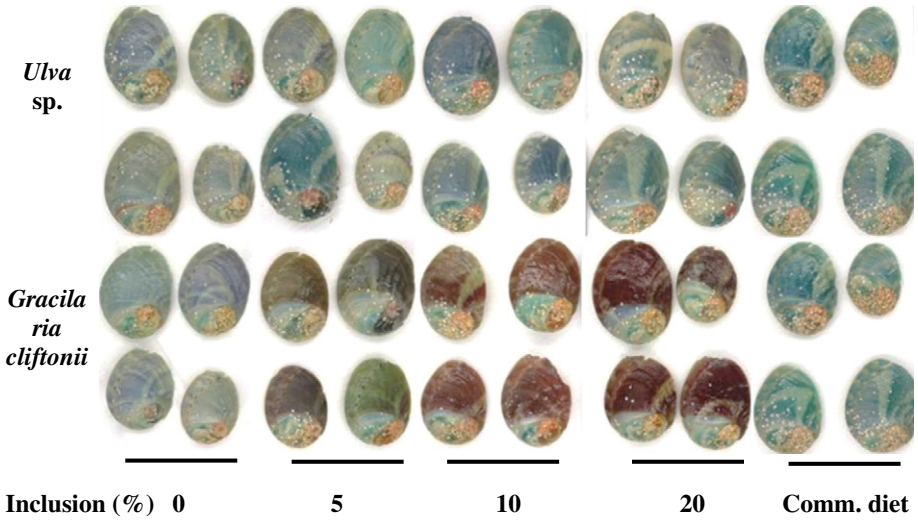

Fig. 1. Shell colour of greenlip abalone, *Haliotis laevigata* fed graded levels of macroalgae inclusion and the commercial diet from left to right with two columns of shells per treatment.

Lip saturation was significantly influenced by macroalgae meal species (two-factor ANOVA; $P < 0.001$; Table 2), inclusion level ($P = 0.001$) and the interaction between these two factors ($P = 0.001$). The interaction was due to the significant increase lip saturation in abalone fed graded levels of *G. cliftonii* meal relative to abalone fed the basal diet, while the lip saturation of abalone fed *Ulva* sp. inclusions was similar to that of abalone fed the basal diet. Lip saturation of abalone fed *G. cliftonii* meal significantly increased with the increasing inclusion level, but was similar among the *Ulva* sp. treatments. Lip saturation of abalone fed 20% *G. cliftonii* meal was significantly higher than those fed 10% *G. cliftonii* ($P = 0.002$) and 5% ($P < 0.001$). There was no significant difference in lip saturation between abalone fed 5% and 10% *G. cliftonii* ($P = 0.611$).

Lip brightness was significantly affected by macroalgae meal species (two-factor ANOVA; $P = 0.002$; Table 2) and inclusion level ($P < 0.001$), but not by their interaction ($P = 0.065$). Abalone fed inclusion of *Ulva* sp. meal had significantly brighter lip than those fed *G. cliftonii* meal ($P = 0.002$). Lip brightness of abalone fed 20% macroalgae meal inclusion was the lowest and significant from the rest ($P < 0.05$). Lip brightness was similar among abalone fed the basal diet, 5% and 10% macroalgae inclusion ($P > 0.05$).

Abalone fed the commercial diet had similar lip hue, lip saturation and lip brightness values with those fed the basal diet (Two-tailed T-Tests; $P > 0.05$).

3.2.2. Foot colour properties

The macroalgae meal species (two-factor ANOVA; $P = 0.675$; Table 2; Fig. 2), inclusion level ($P = 0.366$) and their interaction ($P = 0.966$) did not significantly influence foot hue. Foot saturation was significantly affected by inclusion level (two-factor ANOVA; $P = 0.001$; Table 2), but not by macroalgae meal species ($P = 0.602$) or their interactions ($P = 0.967$). Foot saturation of abalone fed 10 and 20% dried macroalgae meal inclusion was significantly higher than those fed the basal diet ($P = 0.001$). Abalone fed 5%, 10% and 20% levels of macroalgal meal inclusion had similar foot saturation ($P > 0.05$). There was no significant effect of macroalgae meal species (two-factor ANOVA; $P = 0.453$; Table 2), inclusion level ($P = 0.229$), or their interaction ($P = 0.058$) on foot brightness. There were no significant differences in foot colour properties between abalone fed the commercial diet and the basal diet (Two-tailed T-Tests; $P > 0.05$).

3.3. Pigment contents in abalone tissue

The content of β-carotene in abalone tissue was only significantly influenced by the dietary inclusion level of macroalgal meal (two-factor ANOVA; $P < 0.001$; Table 2). Abalone fed both macroalgal meals at all inclusion level had significantly higher amounts of β-carotene than those fed the basal diet ($P < 0.05$). Abalone fed either of the level of 20% macroalgal inclusion had significantly higher amounts of β-carotene

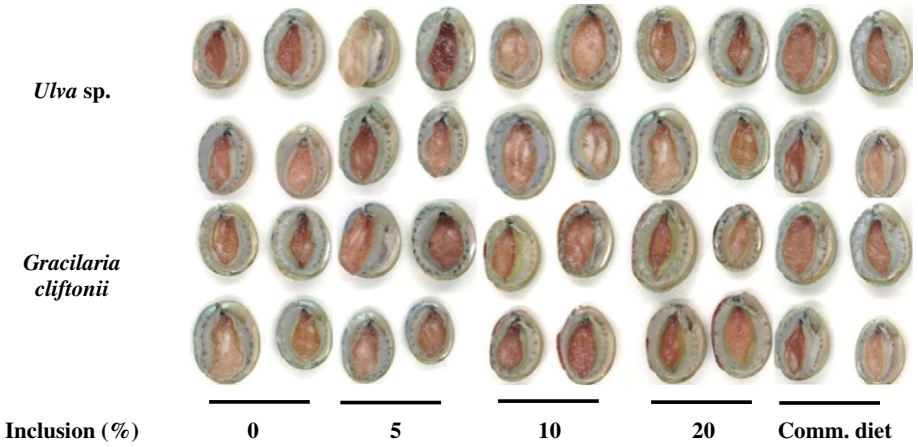

Fig. 2. Foot colour of greenlip abalone, *Haliotis laevigata* fed graded levels of macroalgae inclusion and the commercial diet from left to right with two columns of shells per treatment.

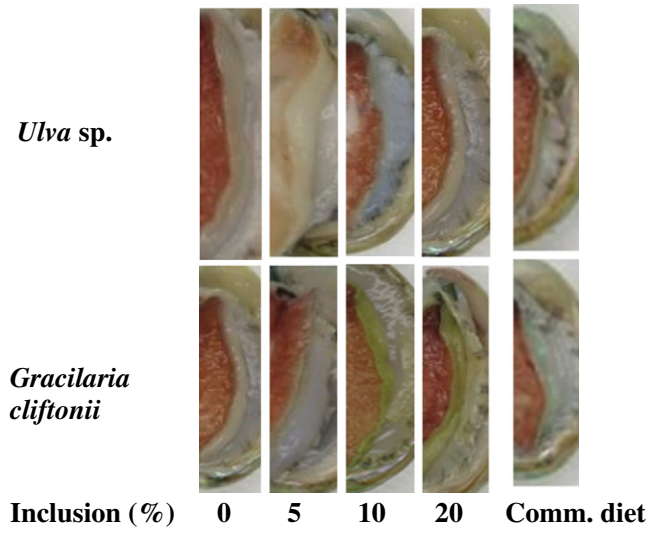

Fig. 3. Lip colour of greenlip abalone, *Haliotis laevigata* fed graded levels of macroalgae inclusion and the commercial diet.

than those fed the diets with lower inclusion levels ($P < 0.05$). No significant difference in the content of β-carotene was found between abalone fed the basal diet and 5% macroalgae inclusion ($P = 0.266$). Among graded diets with different levels of algal meal, the content of β-carotene was similar between 5 and 10% algal meal inclusions ($P = 0.078$). Furthermore, the β-carotene content of abalone fed 20% *G. cliftonii* was significantly higher than those fed the basal diet ($P < 0.001$) or 5% *G. cliftonii* ($P = 0.021$), but not different from those fed 10% *G. cliftonii* ($P = 0.410$). Inclusion of *Ulva* sp. significantly increased β-carotene in abalone tissue compared to the basal diet ($P < 0.001$). However, abalone fed 5%, 10% and 20% of *Ulva* sp. inclusion had a similar content of β-carotene ($P > 0.05$).

Chlorophyll *a* was found in abalone tissue fed the diets of ≥10% *Ulva* sp. while abalone fed 20% *Ulva* sp. had significantly higher chlorophyll *a* than those fed 10% *Ulva* sp. (two-factor ANOVA; $P < 0.001$; Table 2). Astaxanthin, β-cryptoxanthin and zeaxanthin were not detected in the tissue of any abalone despite the presence of pigments in the diets (Table 1).

Abalone fed the commercial diet had a similar content of β-carotene as those fed the basal diet (Two-tailed *t*-tests; $P = 0.123$). Chlorophyll *a*, astaxanthin, β-cryptoxanthin and zeaxanthin were not detected in the tissue of abalone fed the commercial diet or basal diet.

4. Discussion

The modification of lip, shell and foot colour by feeding macroalgae depends on both abalone species and macroalgal types (Leighton, 1961; Leighton and Booloottian, 1963; Bautista-Teruel and Millamena, 1999; Gallardo et al., 2003; Allen et al., 2006). Abalone develop a red to brown shell when they fed on red macroalgae. For example, feeding red macroalgae such as *Gigartina spinose*, *Plocamium pacificum*, *Gigartina canaliculata*, *Gelidium purpurascens* and *Gracilariopsis bailinae* resulted in red shell in red abalone, *H. rufescens*, a reddish-brown shell colour in black abalone, *H. cracherodii*, and brownish shell colour in the tropical abalone, *H. asinina* (Leighton, 1961; Leighton and Booloottian, 1963; Bautista-Teruel and Millamena, 1999; Gallardo et al., 2003). Similarly, feeding live red macroalgae *Gracilaria cliftonii* to greenlip abalone resulted in brown colour of the shell in our previous study (Hoang et al., 2016). Results from the current study show that the shell of abalone fed the inclusion of dried *G. cliftonii* meal was brown-red and the colour intensity depended on the level of algal meal inclusion. However, feeding Pacific abalone, *H. discus hannai*, red abalone, *H. rufescens*, tropical abalone *H. asinina* or greenlip abalone, *H. laevigata* with green or brown macroalgae such as *Undaria pinnatifida*, *Ulva* sp. or formulated diets promoted green on the shell (Sakai, 1962; Olsen, 1968; Bautista-Teruel and Millamena, 1999; Gallardo et al., 2003; Hoang et al., 2016). Our data are consistent with previously studies in that the shell of abalone fed dried *Ulva* sp. meal inclusion or formulated diet remained green.

Wild greenlip abalone consuming algae have a green lip (Department of Fisheries, 2011; Mayfield et al., 2014), but cultured greenlip fed formulated diet exhibited milky lip colour in our previous study (Hoang et al., 2016). In the same study, feeding live *Ulva* sp. or 10% dried *Ulva* sp. meal inclusion did not cause a significant lip colour change compared to the formulated diet (Hoang et al., 2016). However, in the present study the inclusion of ≥10% of dried *G. cliftonii* meal enhanced the green colour on the lip, which is similar to the lip colour of the wild abalone. Most green pigments in wild greenlip abalone are located on the lip (Chew, 1973; Mottet, 1978; Brown, 1995). The green pigment of the lip is presumably associated with melanin since it is a common pigment in the epidermis of vertebrates and invertebrates, including gastropod molluscs (Fox, 1983; Fox, 1979). Melanins are almost certainly responsible for the dark and blue pigmentation of many molluscan shells, the ink of cephalopod molluscs and dark pigments in the integument (notably the mantle) of many gastropods (Comfort, 1951; Palumbo, 2003; Miyashita and Takagi, 2011; Miyamoto et al., 2013; Lemer et al., 2015; Sun et al., 2015). Chew (1973) and Olley and Thrower (1977) reported that although yellow and green pigments of the epidermis of *H. iris*, *H. rubra* and *H. laevigata* were not identified, all pigments gave the infrared spectra of certain melanins, and the different pigments were different polymers of melanin. The nitrogen content of the isolated pigments varied from 2% to 5%, indicating a mixture of indole and catechol melanins (Chew, 1973; Olley and Thrower, 1977). In the green ormer abalone, *Haliotis tuberculata*, melanin in the epidermal cells of the foot epithelium gives this skin its appearance and characteristic black colour (Bravo et al., 2001). Although the molecular pathway for the synthesis of melanin in mollusca is not well understood, tyrosinase enzymes have been shown to be important in the regulation and production of melanin in molluscs (Williams, 2016). In a range of other molluscs, tyrosine is a very important precursor in the eumelanin pathway, and its end products produce a range of colouration including, black, brown, yellow, red and green (Miyashita and Takagi, 2011; Miyamoto et al., 2013; Lemer et al., 2015; Williams, 2016). Since, there are no published studies to date showing a correlation between diet and melanin-based pigmentation in the abalone lip, further research into this aspect of abalone metabolism is required, particularly, on the tyrosine and the eumelanin pathway.

Carotenoids, apart from melanin, are the chief contributor to the development of yellow, orange, and red colours on the integument, gonad and eggs in molluscs (Fox, 1966). However, because carotenoids are synthesised only in plants, animals have to obtain carotenoids from the diet (Shahidi and Brown, 1998; Maoka, 2011). In some edible clams, their muscle exhibits bright orange or yellow colour due to the presence of carotenoids, which originate from microalgae as the major food source (Maoka et al., 2010). In gastropods, the major carotenoids from the reddish-orange muscle tissue of the spindle shell, *Fusinus perplexus* are (3*S*)-phoenicoxanihin, 4, 4′-dihydroxypirardixanthin, and canthaxanthin (Matsuno et al., 1984). Particularly, sea urchin *Paracentrotus lividus* fed natural algae diet, yellowish-orange colour in gonad is principally due to the pigment of echinone, which is synthesised from β-carotene in algae (Griffiths and Perrott, 1976). Since red algae constitute a major portion of the diet of greenlip (Shepherd, 1973), it is logical to assume that the green lip colour is derived from red algae pigments. However, natural colour pigments are the products of complex biosynthetic pathways and the mechanisms by which the compounds or pigments in the abalone are biosynthesised may be difficult to unravel. Further research on biochemical pathways that lead to the green lip colour could be useful in developing a diet with specific pigment supplement.

In the present study, although chlorophylls *a*, astaxanthin, zeaxanthin, β-cryptoxanthin and β-carotene were detected in the diet with *Ulva* sp. or *G. cliftonii* inclusion, β-carotene was the major pigment in the tissue of abalone fed those diets. Thus, it is possible that after abalone ingest carotenoids in the food, some carotenoids are directly deposited in the tissue while others are bioconverted into other forms of pigments (Maoka, 2011; Maoka et al., 2011).

Analyses of pigments extractable from the shell of abalone, Tajima et al. (1980) detected porphyrins chlorophylls *a* and *b* and pheophytin *a*, β-carotene and lutein from the shells of *H. discus hannai* fed green macroalgae *Ulva pertusa*. However, we did not find any measureable pigments (β-carotene, chlorophyll *a*, astaxanthin, β-cryptoxanthin and zeaxanthin) in the shell of greenlip abalone in the current study. Our results are in accordance with Comfort (1951) and Ju et al. (2015) in that the carotenoids do not appear in the shell of abalone. According to Fox (1979) and Wilbur (2014), the shell of abalone contains bilichromes which were reported to have arisen from the consumption of red algae containing the related red bilichrome phycoerythrin. Ju et al. (2015) reported that dark-brown shell of the Pacific abalone fed the red Pacific dulse *Palmaria mollis* were consistent with the presences of biliverdin and cysteine-biliverdin. Since the cause of brown-red shell colouration in greenlip abalone fed *G. cliftonii* remains unclear, future research is required to elucidate the biochemical pathways.

Shell marking is an important tool for the identification of abalone for ranching or stock enhancement programs (Gallardo et al., 2003; Dixon et al., 2006; Kube et al., 2007). Some shell marking methods included tagging with adhesives or the use of tags attached to the respiratory pores may result in irritation to the abalone, fouling or tag shedding, and may negatively affect growth (Prince et al., 1988; Gallardo et al., 2003; Haddon et al., 2008). There is a need to develop harmless shell marking methods that may be used en masse (McShane et al., 1988; Gallardo et al., 2003; Kube et al., 2007; Haddon et al., 2008). The brown-red colouration imparted on the shell of greenlip abalone by feeding *G. cliftonii* is a harmless marking method, and may have application in future ranching or stock enhancement programs. In addition, the majority of farmed abalone are sold in shell live in numerous domestic and international markets (Oakes and Ponte, 1996). The brown-red shell induced by feeding *G. cliftonii* may be an ideal characteristic for growers to establish product identity in these markets.

5. Conclusion

The dietary inclusion of the red macroalgae *G. cliftonii* meal is a useful tool to manipulate the shell and lip colour of greenlip abalone to improve market acceptance. It may also serve as a tool for producers to manipulate shell colour in order to develop a consistent product between sites. The dietary inclusion of *G. cliftonni* can be used to produce a shell colour marker in abalone for ranching or stock enhancement applications.

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