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Colour changes of greenlip abalone (*Haliotis laevigata* Donovan) fed fresh macroalgae and dried algal supplement

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ABSTRACT

Abalone colour is an important market trait in the seafood industry. Two experiments were conducted over 93 days to test the effect of diet on the colour of the foot and shell of 1-year old greenlip abalone *Haliotis laevigata*. In Experiment 1, a commercial control diet and two species of fresh macroalgae (*Gracilaria cliftonii* and *Ulva* sp.) were used and each macroalgae species was either non-enriched or enriched with nutrients in the culture media. The shell of abalone fed the commercial diet and fresh *Ulva* sp. was green but abalone fed fresh *G. cliftonii* developed a brown shell. The fresh *G. cliftonii* increased shell colour purity while fresh *Ulva* sp. increased shell brightness. Feeding abalone with either fresh *Ulva* or fresh *G. cliftonii* produced yellowish foot. Nutrient enrichment of algae did not significantly affect the pigment contents in both macroalgae and abalone, and had minimal impact on the colour of shell and foot. With the exception of zeaxanthin, the pigment contents were significantly lower in fresh *G. cliftonii* than in fresh *Ulva* sp. Moreover, β-carotene was the main pigment in abalone fed both species of fresh macroalgae. In Experiment 2, the inclusion of dietary dried algae affected abalone colour. Three diets including a commercial control diet, a diet containing 3% dried *Spirulina* sp. and a diet containing 10% dried *Ulva* sp. were used. The shell of abalone fed dried *Spirulina* sp. was yellow-brown with higher colour purity while the shell remained light green in abalone fed dried *Ulva* sp. or the commercial control diet. The colour of abalone foot became bright yellow when abalone fed dried *Ulva* sp. Abalone fed dried algae contained β-carotene as the principal pigment. This study indicates that fresh macroalgae and dried algae supplementation in feed can change the colour of abalone foot and shell. Feed effect on shell colour was far more than on tissue colour. Feeding abalone with fresh *G. cliftonii* contributes to the formation of brown colour on the shell.

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

1. Introduction

Abalone (*Haliotis* spp.) are valuable species in both wild fisheries and aquaculture worldwide. As a marketing characteristic, abalone colour is an important trait that can be used to appeal to consumers and influence price (Oakes and Ponte, 1996; Freeman, 2001). Specifically, shell colour is a characteristic for some ethnical consumer groups (Brown et al., 2008), while lighter foot pigmentation commonly commands a higher price (Freeman, 2001). In the wild, abalone consume a variety of micro- and macroalgae species (Viera et al., 2005), and the colour of shell and foot may be affected by the diet. In addition, some abalone are named based on the shell colour such as green abalone *Haliotis fulgens*, red abalone *Haliotis rufescenslip* (Oakes and Ponte, 1996), black abalone *Haliotis cracherodii* and white abalone *Haliotis sorenseni* (Mottet, 1978) or based on the colour of lip and foot such as blackfoot *Haliotis iris*, blacklip *Haliotis ruber*, and greenlip *Haliotis laevigata* (Brown, 1995; Allen et al., 2006). It has been reported that the colour of abalone meat and shell can be influenced by diet manipulation (Brown et al., 2008). On farm, blackfoot abalone *H. iris* fed formulated diets exhibited a paler foot while others had distinct darkening of the foot by feeding *Gracilaria* spp. (Allen et al., 2006). In another case, the shell of juvenile *Haliotis asinina* fed the formulated diets was bluish green while it was retained the brown colour in those fed seaweed *Gracilariopsis bailinae* (Bautista-Teruel and Millamena, 1999) and Japanese abalone *Haliotis discus hannai* fed diets containing *Porphyra* powder and *Spirulina* produced a yellow-red and orange shell, which is similar to wild abalone (Lim and Lee, 2003). These colour changes may potentially affect the product acceptance by consumers, or at least provide a point of product differentiation between cultured and wild abalone. Therefore, it may be possible to manipulate and match the colour of farmed and wild abalone or produce colour based on market demand by dietary manipulation.

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Seaweed such as *Gracilaria* sp. and *Ulva* sp. contain significant levels of protein, carbohydrate, fibre, mineral and amino acid which are essential for abalone growth (Mercer et al., 1993; Fleurence, 1999; Viera et al., 2005; Fleurence et al., 2012). In addition, those macroalgae contain a variety of pigments such as β-carotene, chlorophyll, zeaxanthin, β-cryptoxanthin and phycoerythrin (Norziah and Ching, 2000; Schubert et al., 2006; Fleurence et al., 2012) which have been used as colourants in the food industry (Fleurence et al., 2012). Although the change of shell colour has been reported in some abalone species fed fresh algae (Leighton, 1961; Sakai, 1962; Olsen, 1968; Gallardo et al., 2003), little effort has been made to improve abalone colour in an aquaculture situation using a rigorous experimental design.

Nutrient enrichment of macroalgae can increase the contents of protein and lipids in some macroalgal species (Boarder and Shpigel, 2001; Liu and Dong, 2001; Viera et al., 2011). Nutrient enrichment may also lead to a visually perceivable colour or shade change in macroalgae (Bansemer et al., 2016). In the formulation of artificial diets for aquatic animals, macronutrients such as protein and lipid have been well studied, but the impact of macroalgae after nutrient enrichment on the change of abalone shell colour and tissue pigments has rarely been considered. Microalgae contain important pigments such as chlorophylls *a*, *b* and *c*, β-carotene, phycocyanin, xanthophylls, phycoerythrin and phycobiliproteins (Spolaore et al., 2006). Specifically, *Spirulina* sp. is a genus of blue-green algae that is rich in carotenoids such as β-carotene, zeaxanthin, myxoxanthophyll, echinenone and cryptoxanthin (Miki et al., 1985; Belay et al., 1996; El-Baky et al., 2003) and has been used as a source of carotenoid pigments for rainbow trout, *Oncorhynchus mykiss*, red tilapia, *Oreochromis niloticus* and black tiger prawn, *Penaeus monodon* (Choubert, 1979; Matsuno et al., 1980; Boonyaratpalin and Unprasert, 1989; Liao et al., 1993). However, there is little information on the supplementation of dietary microalgae to manipulate the colour of shell and foot in abalone.

The aim of this study was to understand the effects of dietary algae on the colour change of abalone. Specifically, we first investigated the effects of two species of fresh macroalgae and nutrient enrichment in the algal culture media on abalone colour, and then we further examined the effect of dried algae supplementation in the diet on abalone colour.

2. Materials and methods

2.1. Experimental animal and system

One-year old greenlip abalone (0.80 ± 0.01 g and 17.97 ± 0.04 mm shell length) were purchased from Kangaroo Island Abalone Pty Ltd. (Smith Bay, SA, Australia). Abalone were fed with a commercial diet (Eyre Peninsula Aquafeed Pty Ltd., Lonsdale, SA, Australia) prior to the trial. Upon arrival at the South Australian Research and Development Institute Aquatic Science Centre at West Beach, South Australia, the abalone were acclimated in a 180-L tank provided in a flow-through seawater system at ambient water temperature (22 ± 1 °C) for 12 days prior to the experiment.

The experiment was conducted at a photoperiod of 12 h low light (3.4 lx) and 12 h dark. The seawater flowed through a UV treatment system (model 025120-2.120 W, Emperor Aquatics, Pottstown, PA, USA) comprising a sump tank, an intermediate tank, a header tank (780 L) and twenty eight 12.5 L tanks (39 × 29 × 11 cm). Water temperature was controlled at 22 ± 1 °C) throughout the 93-day experiment using a chiller (2.2 KW, Daeil Cooler Co., Ltd., Busan, Korea) and an immersion heater (3 KW, Austin & Cridland, Carlton, Australia). Each tank was provided with flow-through water from the reservoir by gravity at 300 mL min^{-1}. Water was 3-cm deep in each tank using a standpipe with a screen (0.8 mm mesh size) on the outlet.

2.2. Experimental design and feeding

In Experiment 1, one commercial control diet and two species of fresh macroalgae (*Ulva* sp. and *Gracilaria cliftonii*) collected from intertidal sand-flats at the outer harbour, Gulf St Vincent, SA, Australia were used and each species was fed either as it was or following enrichment in a modified Guillard's f/2 nutrient medium (Guillard and Ryther, 1962; Guillard, 1975). This resulted in five dietary treatments: 1) non-enriched fresh *Ulva* sp.; 2) enriched fresh *Ulva* sp.; 3) non-enriched fresh *G. cliftonii*; and 4) enriched fresh *G. cliftonii*; and 5) a commercial control diet.

In Experiment 2, three diets were used: 1) a commercial diet as the control (Eyre Peninsular Aquafeeds, Lonsdale SA); 2) the commercial diet was supplemented with 10% enriched dried *Ulva* sp.; and 3) the commercial diet supplemented with 3% dried *Spirulina* sp. by weight. The enriched dried *Ulva* sp. was chosen as it yielded superior growth in greenlip abalone fed this diet compared to non-enriched fresh *Ulva* sp. (Bansemer et al., 2016). The commercial diet contained 34% crude protein, 4.8% crude lipid and 15.5 MJ kg^{-1} gross energy.

Twenty animals were stocked per tank in four replicates for each diet. Abalone were fed to apparent satiation with a daily ration of 14% body weight in Experiment 1 or 4.5% body weight in Experiment 2. The rations were adjusted based on the biomass at stocking and the biomass increment was determined every 30 days by bulk weighing the abalone in each tank. Feed was delivered once daily at 16:00 h and tanks were cleaned daily at 08:30 h the next morning.

2.3. Specimen sampling and analyses

Prior to each experiment, 20 abalone were initially sampled for colour analysis then stored at −80 °C for carotenoid analysis. At the end of each experiment, five abalone from each tank were collected, weighed, measured and photographed and then frozen at −80 °C prior to carotenoid analysis. To capture the digital image of abalone, a light table was made with two natural white colour bulbs mounted on two sides of a table and a digital camera (Canon IXUS 230HS) was placed on an adjustable arm between the two lights. The camera was set up at 25 cm above the specimen and each digital image was captured together with a reference colour card (X-Rite; Colourchecker®). Digital images were analysed using Gimp2 software. The mean of red, green and blue (RGB) values was converted to the hue, saturation and brightness (HSB) values, respectively.

The HSB values represented colour properties corresponding to red, green, and blue. All possible colours were specified as hue (i.e. colour purity), saturation (i.e. colour intensity) and brightness as visualized in a reversed cone shape model (Yasir and Qin, 2010). Hue was expressed as a number indicating the degrees around the cone with red at zero degree, 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.

For pigment extraction, all samples were thawed at the room temperature and then freeze-dried for 48 h until a constant weight was reached. Whole abalone (without gut and shell) and macroalgae were separately ground into fine powder before extraction. About 0.3 g accurately weighed sample was extracted sequentially three times with 10 mL ethanol-hexane (1:1, v/v) until the residue turned colourless. Each extraction was followed by centrifugation 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 extractions were 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 (solvent A) and acetone (solvent B). The flow rate was 1 mL min^{-1} with a 5 μL injection. The wavelengths of detection were

set at 450 nm for 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), 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 chlorophyll *a* was 0.001 μg g^{-1} and for carotenoid pigments (β-carotene, β-cryptoxanthin and zeaxanthin) was 0.0003 μg g^{-1}.

2.4. Statistical analysis

The data were analysed using SPSS (version 22) and the level of significance was set at $P < 0.05$. Three tests were used to examine the effect of diets on shell and foot colour and pigment contents of abalone. In Experiment 1, a two-way ANOVA was used to analyse the effect of macroalgae type and nutrient enrichment on shell and foot colour hue, saturation and brightness and pigment contents in the abalone tissue and diets. When the interaction between the macroalgae type and nutrient enrichment was not significant, the main effect was considered and comparisons were done using the post-hoc Tukey's HSD multiple comparison procedure. When significant interactions between the macroalgae type and nutrient enrichment were observed, pairwise comparisons were used to determine significant differences between treatment combinations. One-factor ANOVA and a Dunnet's test were then used to determine if the pigment level of the tissue or the colour of the shell and foot of abalone fed the enriched or non-enriched fresh *G. cliftonii* or *Ulva* sp. diets differed from abalone fed the commercial control diet. In Experiment 2, one-way ANOVA was used to examine the effects of diet type (commercial diet, commercial diet + 10% *Ulva* sp., commercial diet + 3% *Spirulina* sp.) on shell and foot colour hue, saturation and brightness and the pigment contents in the whole abalone tissue and the diet.

3. Results

3.1. Effects of fresh macroalgae on abalone colour in experiment 1

3.1.1. Shell and foot colour

We saw a difference in the abalone shell colour between treatments by week 6. At the end of 93 days, the hue of abalone shell was significantly affected by macroalgae type (two-factor ANOVA; $P < 0.001$; Table 1), but not by nutrient enrichment ($P = 0.984$). Abalone fed fresh *G. cliftonii* showed significantly lower values in shell hue than those fed fresh *Ulva* sp. ($P < 0.001$). In addition, abalone fed fresh *G. cliftonii* developed brown colour on the shell while those fed fresh *Ulva* sp. exhibited green colour on the shell (Fig. 2). Furthermore, the degree of shell hue was not significantly affected by the interaction between macroalgae type and nutrient enrichment ($P = 0.196$). Shell colour saturation of abalone fed fresh *G. cliftonii* was significantly higher than those fed fresh *Ulva* sp. ($P < 0.001$; Table 1). Nutrient enrichment did not affect shell colour saturation ($P = 0.827$; Table 1). There was no interaction between macroalgae type and nutrient enrichment effects on shell colour saturation ($P = 0.764$). Shell colour brightness was significantly affected by the type of macroalgae ($P < 0.001$; Fig. 1). Typically, abalone fed fresh *Ulva* sp. had a brighter shell ($P < 0.001$). Abalone fed enriched fresh *G. cliftonii* significantly increased shell colour brightness ($P = 0.03$), but no interaction was observed between the effects of macroalgae type and nutrient enrichment on shell brightness ($P = 0.646$).

In comparison with the commercial control diet, the shell hue of abalone was significantly higher than those fed any of the fresh macroalgae diets (one-factor ANOVA; $P < 0.001$; Dunnet's test; Table 2). Shell saturation of abalone fed the commercial control diet was significantly lower than those fed fresh *G. cliftonii* ($P < 0.001$; Table 2). No significant difference was detected between the shell saturation of abalone fed the commercial control diet and fresh *Ulva* sp. diet ($P > 0.05$). Shell brightness of abalone fed the commercial control diet was significantly lower than those fed fresh *Ulva* sp. ($P < 0.001$; Table 2) but not of those fed fresh *G. cliftonii* ($P > 0.05$). The shell colour of abalone fed the commercial control diet was also light green.

Neither macroalgae type ($P > 0.260$ Table 1) nor nutrient enrichment ($P > 0.291$) affected foot hue and brightness. The type of macroalgae significantly influenced foot saturation ($P = 0.031$; Table 1), but nutrition enrichment did not ($P = 0.423$). Moreover, there was a significant interaction between the type of macroalgae and enrichment on foot colour saturation ($P < 0.001$). The interaction was due to a significant increase in foot saturation for abalone fed non-enriched *Ulva* sp. compared to enriched *Ulva* sp., whereas foot saturation in abalone fed non-enriched *G. cliftonii* decreased significantly compared to those fed enriched *G. cliftonii*. Abalone fed non-enriched *G. cliftonii* had significantly higher foot saturation than those fed non-enriched *Ulva* sp. ($P < 0.001$) and

Table 1
Two-factor ANOVA results for shell and foot colour of abalone and the pigment contents in abalone and diets.

Items	Non-enriched macroalgae		Enriched macroalgae		Two-factor ANOVA (*P* value)		
	Ulva sp.	*G. cliftonii*	*Ulva* sp.	*G. cliftonii*	Algae type (A)	Enrichment (B)	A × B
Macroalgae pigments							
β-carotene (μg g^{-1})	1.27 ± 0.08	0.12 ± 0.03	1.40 ± 0.16	0.11 ± 0.04	<0.001	0.540	0.469
Chlorophyll *a* (μg g^{-1})	4.68 ± 0.52	1.29 ± 0.08	5.26 ± 0.45	1.24 ± 0.21	<0.001	0.482	0.409
β-cryptoxanthin (μg g^{-1})	0.53 ± 0.09	0.20 ± 0.01	0.63 ± 0.12	0.18 ± 0.08	0.002	0.655	0.459
Zeaxanthin (μg g^{-1})	0.87 ± 0.05	2.72 ± 0.23	1.07 ± 0.18	2.98 ± 0.13	<0.001	0.196	0.875
Whole abalone body pigments							
β-carotene (μg g^{-1})	5.40 ± 1.00	2.82 ± 0.23	5.49 ± 0.26	2.59 ± 0.18	<0.001	0.901	0.767
Chlorophyll *a* (μg g^{-1})	1.72 ± 0.09	0.16 ± 0.04	2.34 ± 0.65	0.24 ± 0.06	<0.001	0.312	0.424
β-cryptoxanthin (μg g^{-1})	0.22 ± 0.06	0.23 ± 0.09	0.44 ± 0.03	0.38 ± 0.13	0.793	0.051	0.728
Zeaxanthin (μg g^{-1})	0.30 ± 0.09	0.40 ± 0.10	0.24 ± 0.03	0.78 ± 0.27	0.054	0.285	0.167
Colour components of shell							
Hue (degree)	54.47 ± 2.21	16.62 ± 1.99	57.75 ± 3.62	13.44 ± 1.57	<0.001	0.984	0.196
Saturation (%)	43.91 ± 1.56	70.43 ± 1.43	43.18 ± 1.61	70.54 ± 0.86	<0.001	0.827	0.764
Brightness (%)	41.93 ± 1.43	29.09 ± 1.52	45.05 ± 1.43	33.51 ± 1.25	<0.001	0.009	0.646
Colour components of foot							
Hue (degree)	36.72 ± 0.33	34.53 ± 1.01	35.40 ± 0.51	36.02 ± 0.77	0.267	0.905	0.050
Saturation (%)	59.04 ± 1.03	68.02 ± 2.16	63.49 ± 1.46	61.14 ± 1.12	0.031	0.423	<0.001
Brightness (%)	51.01 ± 0.67	51.05 ± 1.24	52.30 ± 0.68	51.66 ± 0.88	0.736	0.291	0.704

A significance level of $P < 0.05$ was used for all statistical tests. Where significant main effects were detected, post-hoc tests were used to determine differences between means (one-factor ANOVA; Tukey's HSD; $P < 0.05$). For the variable with a significant interaction, the main effect was not considered and the comparisons were made using pairwise comparisons to examine the dependent relationship between the two independent factors (algae type and nutrient enrichment).

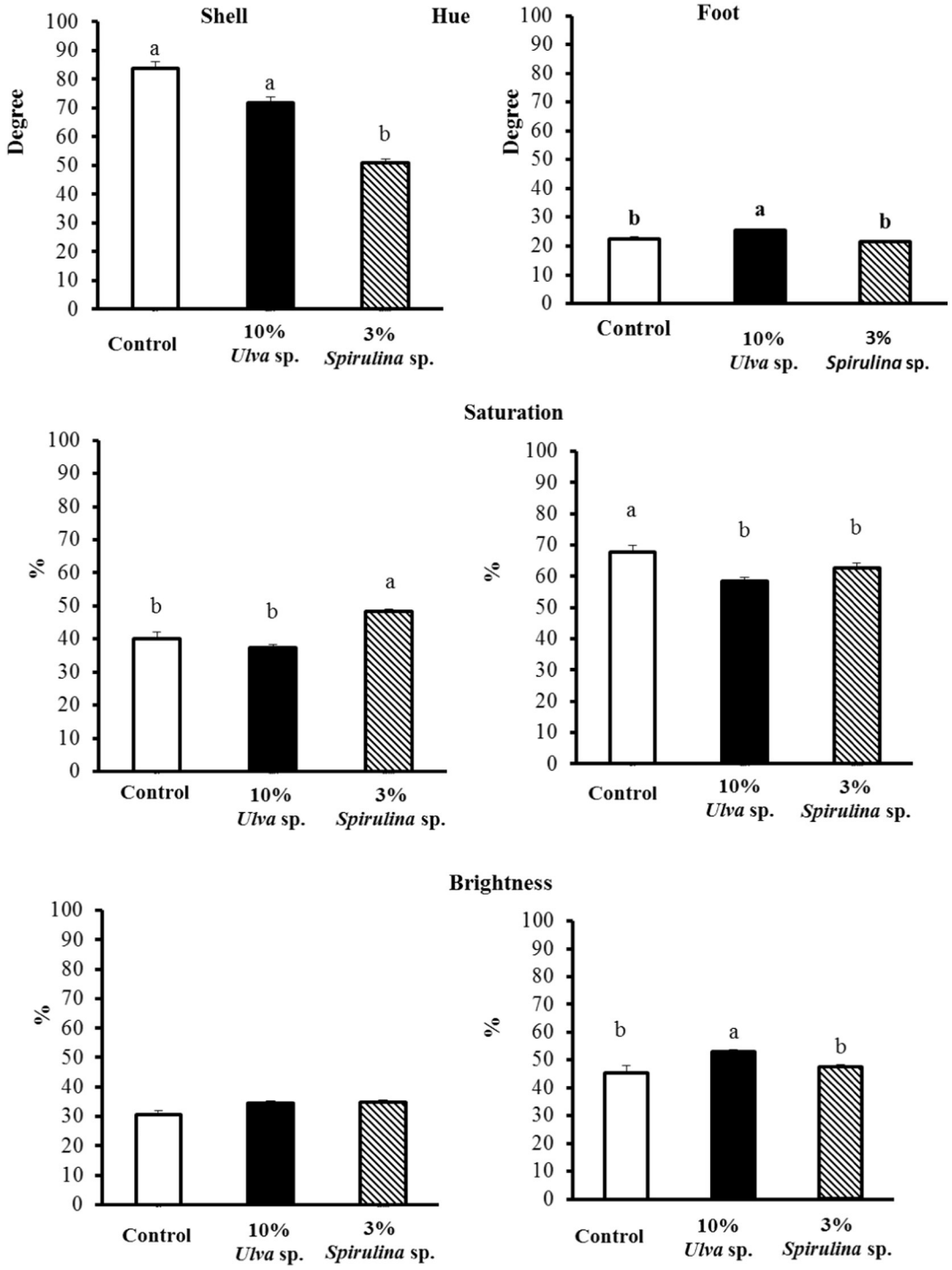

Fig. 1. Hue, saturation and brightness values of shell (left) and foot (right) in abalone fed dried micro and macroalgae in Experiment 2.

enriched *Ulva* sp. ($P = 0.037$). However, abalone fed enriched *G. cliftonii* had similar foot saturation to those fed un-enriched *Ulva* sp. ($P = 0.328$) and enriched *Ulva* sp. ($P = 0.274$). Greenlip abalone foot was light yellow when fed fresh *Ulva* sp. or fresh *G. cliftonii*.

Foot hue ($P < 0.001$) and foot brightness ($P = 0.002$) were significantly lower in abalone fed the commercial control diet than other diets ($P < 0.001$; Table 2). Foot saturation of abalone fed the commercial control diet was significantly higher than those fed non-enriched fresh *Ulva* sp. and enriched fresh *G. cliftonii* ($P < 0.001$; Table 2).

3.1.2. Pigment contents in the diets and in abalone

The content of all four pigments were significantly affected by the type of macroalgae: β-carotene (two-factor ANOVA; $P < 0.001$; Table 1), chlorophyll *a* ($P < 0.001$), β-cryptoxanthin ($P = 0.002$) and zeaxanthin ($P < 0.001$). However, neither nutrient enrichment ($P > 0.05$) nor the interaction between the type of macroalgae and nutrient enrichment ($P > 0.05$) affected those pigment contents. The contents of β-carotene ($P < 0.001$) and chlorophyll *a* ($P < 0.001$) in fresh *Ulva* sp. were significantly higher than in fresh *G. cliftonii*.

Table 2
One-factor ANOVA results for the shell and foot colour of abalone and pigment contents in abalone and diets ($n = 4$).

Items		Non-enriched macroalgae		Enriched macroalgae		ANOVA (P value)
	Commercial control diet	*Ulva* sp.	*G. cliftonii.*	*Ulva* sp.	*G. cliftonii*	
Dietary pigments						
β-carotene (μg g^{-1})	0.10 ± 0.05^a	1.27 ± 0.08^b	0.12 ± 0.03^a	1.40 ± 0.16^b	0.11 ± 0.04^a	<0.001
Chlorophyll *a* (μg g^{-1})	$<0.001^a$	4.68 ± 0.52^b	1.29 ± 0.08^a	5.26 ± 0.45^b	1.24 ± 0.21^a	<0.001
β-cryptoxanthin (μg g^{-1})	$<0.0003^a$	0.53 ± 0.09^b	0.20 ± 0.01^a	0.63 ± 0.12^b	0.18 ± 0.08^a	0.001
Zeaxanthin (μg g^{-1})	0.07 ± 0.03^a	0.87 ± 0.05^b	2.72 ± 0.23^b	1.07 ± 0.18^b	2.98 ± 0.13^b	<0.001
Whole abalone body pigments						
β-carotene (μg g^{-1})	0.05 ± 0.02^a	5.40 ± 1.00^b	2.82 ± 0.23^b	5.49 ± 0.26^b	2.59 ± 0.18^b	<0.001
Chlorophyll *a* (μg g^{-1})	$<0.001^a$	1.72 ± 0.09^b	0.16 ± 0.04^a	2.34 ± 0.65^b	0.24 ± 0.06^a	<0.001
β-cryptoxanthin (μg g^{-1})	$<0.0003^a$	0.22 ± 0.06^a	0.23 ± 0.09^a	0.44 ± 0.03^b	0.38 ± 0.13^b	0.011
Zeaxanthin (μg g^{-1})	0.06 ± 0.02^a	0.30 ± 0.09^a	0.40 ± 0.10^a	0.24 ± 0.03^a	0.78 ± 0.27^b	0.021
Colour component of shell						
Hue (degree)	83.33 ± 6.19^a	54.47 ± 2.21^b	16.62 ± 1.99^b	57.75 ± 3.62^b	13.44 ± 1.57^b	<0.001
Saturation (%)	40.21 ± 1.90^a	43.91 ± 1.56^a	70.43 ± 1.43^b	43.18 ± 1.61^a	70.54 ± 0.86^b	<0.001
Brightness (%)	30.50 ± 1.44^a	41.93 ± 1.43^b	29.09 ± 1.52^a	45.05 ± 1.43^b	33.51 ± 1.25^a	<0.001
Colour component of foot						
Hue (degree)	22.51 ± 0.84^a	36.72 ± 0.33^b	34.53 ± 1.01^b	35.40 ± 0.51^b	36.02 ± 0.77^b	<0.001
Saturation (%)	67.78 ± 2.16^a	59.04 ± 1.03^b	68.02 ± 2.16^a	63.49 ± 1.46^a	61.14 ± 1.12^b	<0.001
Brightness (%)	45.21 ± 2.37^a	51.01 ± 0.67^b	51.05 ± 1.24^b	52.30 ± 0.68^b	51.66 ± 0.88^b	0.002

Abalone fed the commercial diets, and used as a control and compared to abalone fed fresh macroalgae ($n = 4$; one-factor ANOVA; Dunnett's post-hoc test). ^{a,b} values without a common superscript compared to the control diet are significantly different. A significance level of $P < 0.05$ was used.

However, the content of zeaxanthin was significantly lower in fresh *Ulva* sp. ($P < 0.001$). The contents of β-cryptoxanthin in enriched fresh *Ulva* sp. was significantly higher than in non-enriched ($P = 0.028$) and enriched *G. cliftonii* ($P = 0.021$), but the contents of β-cryptoxanthin in non-enriched *Ulva* sp. was not different from that in *G. cliftonii* with or without enrichment ($P > 0.05$).

The commercial control diets exhibited significantly lower content of β-carotene than fresh *Ulva* sp. (one-factor ANOVA; $P < 0.001$; Table 2). The content of zeaxanthin was significantly higher in fresh macroalgae than in the commercial control diet ($P < 0.001$). Chlorophyll *a* and β-cryptoxanthin were not detected from the commercial control diet.

The contents of β-carotene (two-factor ANOVA; $P < 0.001$; Table 1) and chlorophyll *a* ($P < 0.001$) in abalone fed fresh macroalgae were significantly influenced by the type of macroalgae but β-cryptoxanthin ($P = 0.793$) and zeaxanthin ($P = 0.054$) were not. Nutrient enrichment ($P > 0.05$) and the interaction between the type of macroalgae and nutrient enrichment ($P > 0.05$) had no apparent influence on pigment contents of abalone. The content of β-carotene in abalone fed fresh *Ulva* sp. was significantly higher than those fed fresh *G. cliftonii* ($P < 0.001$; Table 1). The content of chlorophyll *a* in abalone fed fresh *Ulva* sp. was also significantly higher than in those fed fresh *G. cliftonii* ($P < 0.001$). No significant differences were found in the contents of β-cryptoxanthin ($P = 0.793$) and zeaxanthin ($P = 0.054$) among abalone fed different macroalgae types.

Abalone fed the commercial control diet exhibited significantly lower content of β-carotene than those fed fresh macroalgae (one-factor ANOVA; $P < 0.001$; Table 2) and the content of zeaxanthin in abalone fed the commercial control diet group was significantly lower than those fed enriched *G. cliftonii*. Chlorophyll *a* and β-cryptoxanthin were not detected from abalone fed the commercial control diet.

3.2. Effects of dried algae supplementation on abalone colour in experiment 2

3.2.1. Shell and foot colour

The shell hue was significantly lower (one-factor ANOVA; $P < 0.001$; Fig. 2) while the shell saturation was significantly higher ($P < 0.001$) in

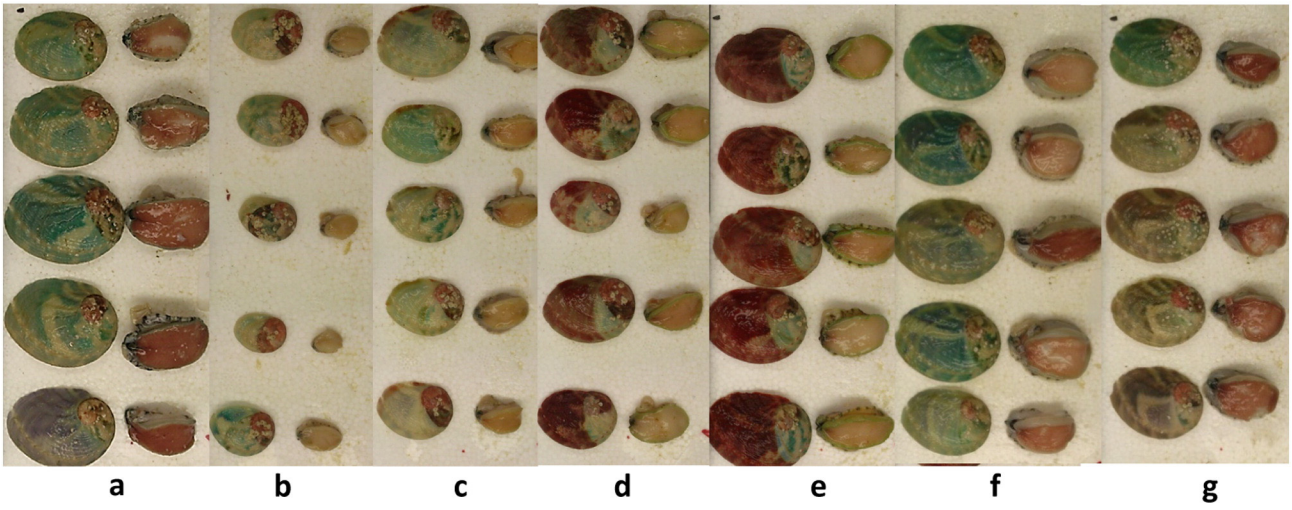

Fig. 2. Shell and foot of greenlip abalone fed (a) commercial control diet, (b) fresh non-enriched *Ulva* sp., (c) fresh enriched *Ulva* sp., (d) fresh non-enriched *G. cliftonii*, (e) fresh enriched *G. cliftonii*, (f) dried *Ulva* sp. and (g) dried *Spirulina* sp.

abalone fed dried *Spirulina* sp. in the supplemented diet than those fed the other two diets, resulting in a yellow-brown shell. All colour components of the shell were not significantly different between abalone fed the diet supplemented with dried *Ulva* sp. and the control diet ($P > 0.05$). Shell brightness was not significantly affected by the inclusion of dried micro or macroalgae ($P = 0.087$).

For foot colour, abalone fed the diet supplemented with dried *Ulva* sp. showed significantly higher hue (one-factor ANOVA; $P = 0.001$; Fig. 2) and brightness ($P = 0.007$) than those fed the other diets, resulting in a yellow foot. Both dried *Ulva* sp. ($P < 0.001$) and *Spirulina* sp. ($P = 0.001$) supplementations in feed significantly reduced foot colour saturation. No significant differences in hue ($P = 0.065$) or brightness ($P = 0.336$) were found between abalone fed the dried *Spirulina* sp. supplemented diet and those fed the control diet, except for colour saturation ($P < 0.001$).

3.2.2. Pigment contents of diets and abalone fed dried algae supplementation

The results for the pigment contents of the diets used in experiment 2 are displayed in Table 3. No significant differences were found in the content of β-carotene for all diets ($P = 0.094$). The contents of chlorophyll *a* ($P = 0.001$) and zeaxanthin ($P = 0.032$) were significantly higher in the *Spirulina* sp. diet than others. Chlorophyll *a* and β-cryptoxanthin were not detected in the control diet.

The pigment contents in abalone tissue are displayed in Table 3. The β-carotene ($P = 0.003$) and zeaxanthin ($P = 0.003$) contents were significantly lower in abalone fed the control diet than those fed dried macro and microalgae supplemented diets. The amount of β-carotene was not significantly different between abalone fed dried *Ulva* sp. and dried *Spirulina* sp. ($P > 0.05$), whereas the content of zeaxanthin in abalone fed dried *Spirulina* sp. was significantly higher than those fed *Ulva* sp. ($P = 0.003$) and control diet ($P = 0.003$). Chlorophyll *a* and β-cryptoxanthin were not detected in abalone fed diets containing dried micro or macroalgae.

4. Discussion

As colour and appearance of seafood are important traits that influence the buyers' decision on purchasing seafood, along with nutritional value, the body and flesh colour of aquaculture animals have drawn increasing attention from seafood researchers. Carotenoids are important for the development of yellow, orange, and red colours on the skin, shell and exoskeleton of some aquatic animals such as fish and crustacean (Shahidi and Brown, 1998). In this study, the shell colour of abalone was altered by feeding different types of fresh and dried macroalgae. Abalone developed a brown shell when fed fresh *G. cliftonii*, but had a green shell similar to that fed the commercial control diet when the animals fed either fresh or dried *Ulva* sp. These results agree with previous studies that the colour of abalone shell depends on dietary pigments in algae (Leighton, 1961; Leighton and Booloottian, 1963; Gallardo et al., 2003). The *H. discus hannai* and *H. sorenseni* exhibited bluish-green and green-white shell, respectively, when fed green algae *Ulva pertusa* and *Enteromorpha linza* (Sakai, 1962; Olsen, 1968). However, brown and red shell were observed when red algae such as *Pachymenia* sp., *Rhodoglossum pulcherum*, *Carpopeltis affinis* or *Gelidium* sp. were fed to the same abalone (Sakai, 1962; Olsen, 1968). Abalone fed red algae showed a red shell in *H. rufescens*, brown-red shell in *H. corrugata*, reddish-brown shell in *H. cracherodii* and brownish shell in *H. asinina* (Leighton, 1961; Leighton and Booloottian, 1963; Olsen, 1968; Gallardo et al., 2003). Depending on abalone species, abalone shell becomes dark red or brown after feeding red algae (Mottet, 1978). Similarly, greenlip abalone showed a brown shell after consuming green brown algae in the present study. However, the shell colour of greenlip abalone fed the control diet was light green which was also reported in other species. For example, *H. asinina* fed *Gracilaria bailinae* produced a brown shell but the shell was blue-green when fed a formulated diet (Gallardo et al., 2003).

Macroalgae are not only the preferred feed of some abalone but are also a pigment source for colour enhancement in other aquatic animals (Shpigel et al., 2005; Viera et al., 2005; Qi et al., 2010). Green macroalgae such as *Ulva lactuca* mainly contain chlorophyll, β-carotene, lutein, violoxanthin, neoxanthin and zeaxanthin (Chandini et al., 2008; El-Baky et al., 2008) whereas red macroalgae such as *Gracilaria gracilis*, *Gracilaria textorii* and *Gracilariopsis lemaneiformis* predominantly contain zeaxanthin, α- and β-carotene and lutein (Schubert et al., 2006; Chandini et al., 2008). As chlorophyll is usually the main pigment in green algae (Shahidi and Brown, 1998; Chandini et al., 2008), it was predominate in *Ulva* sp. and also existed in abalone tissues in this study. Chlorophyll *a*, chlorophyll *b*, β-carotene, lutein, violaxanthin and neoxanthin are the main pigments in *U. pertusa* and in the shell of Japanese abalone *H. discus hannai* fed this green alga (Tajima et al., 1980a). Similarly, zeaxanthin was the major pigment in the red algae *G. cliftonii* and abalone fed this red alga contained zeaxanthin in the tissue, but the zeaxanthin in the abalone shell was not quantified in this study. Liu et al. (2009) suggest that the shell colour of Pacific abalone is subject to genetic control and dietary modification. In the present study, the shell colour of greenlip abalone was affected by diets, but the mechanism by which the shell changes colour in abalone fed different algae warrants further investigation. Additionally, the amount of colour deposition on the shell of wild abalone may depend on the seasonal change of pigment composition in macroalgae as both the contents of chlorophyll and carotenoid in macroalgae vary with seasons (Gerasimenko et al., 2011, 2014).

Enrichment of macroalgae with a high nitrogen medium has been used to increase the protein content in algae as animal feed in aquaculture (Shpigel et al., 1999; Viera et al., 2005). Boarder and Shpigel (2001) reported that the enrichment of wild *U. rigida* increased the protein content in algae from 11.4% to 32.2% by dry weight. The enriched macroalgae have improved the growth of *H. tuberculata* and *H. discus hannai* (Shpigel et al., 1999), *H. roei* (Boarder and Shpigel, 2001) and *H. tuberculata coccinea* (Viera et al., 2005; Viera et al., 2011). In the present study,

Table 3
One-factor ANOVA results for pigment contents in diets and in abalone fed dried algae supplementary diets ($n = 4$).

Items	Commercial control diet	*Ulva* sp.	*Spirulina* sp.	ANOVA (*P* value)
Dietary pigments				
β-carotene (μg g^{-1})	0.10 ± 0.05	0.20 ± 0.09	0.38 ± 0.09	0.094
Chlorophyll *a* (μg g^{-1})	<0.001	0.48 ± 0.15^{a}	1.54 ± 0.22^{b}	0.001
β-cryptoxanthin (μg g^{-1})	<0.0003	0.02 ± 0.01^{b}	0.01 ± 0.00^{ab}	0.030
Zeaxanthin (μg g^{-1})	0.07 ± 0.03^{a}	0.13 ± 0.08^{a}	0.35 ± 0.06^{b}	0.032
Whole abalone body pigments				
β-carotene (μg g^{-1})	0.05 ± 0.02^{a}	0.15 ± 0.03^{b}	0.22 ± 0.02^{b}	0.003
Chlorophyll *a* (μg g^{-1})	<0.001	0.00 ± 0.00	0.00 ± 0.00	
β-cryptoxanthin (μg g^{-1})	<0.0003	0.00 ± 0.00	0.00 ± 0.00	
Zeaxanthin (μg g^{-1})	0.06 ± 0.02^{a}	0.11 ± 0.01^{b}	0.17 ± 0.01^{c}	0.003

Different superscripts mean significant difference ($P < 0.05$). Chlorophyll *a* content less than 0.001 μg g^{-1} and carotenoid less than 0.0003 μg g^{-1} as not detectable.

nitrogen enrichment increased the protein level in fresh *Ulva* sp. and fresh *G. cliftonii* by 25.3% and 25.2%, respectively (Bansemer et al., 2016). In addition, the colour of enriched macroalgae was darkened and had a higher content of pigments than those without nutrient enrichment. However, nutrient-enriched algae did not significantly affect abalone colour.

Microalgae are recognised as an excellent source of food pigment (Dufossé et al., 2005; Spolaore et al., 2006). Recently, there has been an increasing interest of using microalgae for colour enhancement in the food industry, pharmaceuticals, cosmetics and animal feed (Dufossé et al., 2005). Early studies showed that the blue pigment in *Spirulina* spp. such as phycocyanin and carotenoids such as beta carotene, astaxanthin, luteine, zeaxanthin and cryptoxanthin can affect the body colour of various animals upon food consumption (Liao et al., 1993; Boonyaratpalin and Unprasert, 1989; Belay et al., 1996; Saleha et al., 2011; Vasudhevan and James, 2011; Ghaeni et al., 2014). In addition, the level of *Spirulina* sp. inclusion in the diet as a pigment additive is species-dependent (Mori et al., 1987; Okada et al., 1991; Liao et al., 1993; Teimouri et al., 2013a,b). Results from the present study demonstrated that the abalone shell became yellow-brown by including 3% *Spirulina* sp. in the diet compared with the blue-green shell of abalone fed the control diet. These results are in accordance with Lim and Lee (2003) who reported that the shell of abalone *H. discus hannai* fed 2% *Spirulina* sp. became orange, a similar colour to the abalone in wild. Despite the change of shell colour, the addition of dietary *Spirulina* sp. did not change the colour of abalone foot in the present study.

The importance of *Ulva* sp. inclusion as a colour enhancer in animal feed has recently been demonstrated in some studies (Xu and Hirata, 1990; Xu and Hirata, 1991; Cyrus et al., 2013, 2014). In abalone, although feeding fresh *Ulva* sp. resulted in a blue-green shell in some abalone species (Sakai, 1962; Olsen, 1968), the inclusion of *Ulva* sp. in the diet to manipulate abalone colour is rarely done. The present study showed that abalone fed 10% *Ulva* sp. added more yellow pigment on the foot, but no significant change on shell colour was detected in comparison with abalone fed the control diet in experiment 2. Similarly, abalone foot also gained yellow pigment when fed fresh *Ulva* sp. or fresh *G. cliftoniii* in Experiment 1. As β-carotene and zeaxanthin were the major pigment in the foot of abalone fed fresh algae or the diet with 10% dried *Ulva*, it is likely that those carotenoid pigments are attributable to the yellow foot in abalone. In other aquatic animals, sea urchin gained yellow-orange pigment in the gonad after feeding dried algae *Duneliella salina* (Robinson et al., 2002) and fish gained yellow-orange colour on the body after feeding zeaxanthin pigment in the diet (Gupta et al., 2007).

The red macroalgae *Gracilaria* spp. are rich in zeaxanthin, accounting for 59.9% to 78.6% of the total carotenoids (Schubert et al., 2006) whereas in *Ulva* sp. chlorophyll *a* and *b* account for 30.9% and 14.9% of the total pigments, respectively (El-Baky et al., 2008). Similarly, in the current study zeaxanthin was the most abundant pigment in *G. cliftonii*, while chlorophyll *a* was most abundant in fresh *Ulva* sp. However, β-carotene was the dominant pigment in greenlip abalone fed fresh macroalgae. This finding is in agreement with the report on *H. discus hannai* fed *U. pertusa* by Tajima et al. (1980a,b) that the β-carotene was the main pigment in the muscle whereas chlorophyll *a* and β-carotene were commonly detected in the shell of abalone fed *U. pertusa* and the content of those pigments in the shell was higher than in the dietary macroalgae. Our results also show that only a small amount of chlorophyll *a* and zeaxanthin was detected in abalone, but the content of both pigments were high in macroalgae. In a recent study, Maoka (2011) found that marine animals can accumulate carotenoids from food and convert carotenoids into other pigments through metabolic pathways. Specifically, in largemouth bass *Micropterus salmoides*, no astaxanthin was found in the tissue even though its diet contained a high amount of astaxanthin and zeaxanthin (Yamashita et al., 1996). In other studies, lutein can be converted to astaxanthin in yellowtail kingfish (Miki et al., 1985) and zeaxanthin can be converted to astaxanthin in fancy red carp (Matsuno et al., 1979). In comparison, the ability of greenlip abalone to convert chlorophyll *a* and zeaxanthin in macroalgae to their tissue is limited, and the metabolic pathway of pigmentation in greenlip abalone needs further investigation.

In conclusion, feeding fresh *Ulva* sp. and fresh *G. clintonii* and the addition of 3% dried *Spirulina* sp. and 10% dried *Ulva* sp. in abalone diet can influence the colour of shell and foot. Abalone developed a brown shell when fed fresh *G. cliftonii*, and grew a green shell when fed fresh *Ulva* sp. or the commercial control diet. The shell of abalone fed the control diet supplemented with dried *Spirulina* sp. at 3% was yellow, but was green in abalone fed the control diet supplemented with 10% dried *Ulva* sp. or the control diet. Abalone foot became yellow when fed a diet with dried *Ulva* sp. inclusion. Although nutrient enrichment improved the protein content of macroalgae, it had little impact on the colour of abalone shell and foot. Abalone fed fresh *G. cliftonii* displayed a brown shell and this species of red algae can be potentially used as a brown colour enhancer on the shell of farmed abalone.

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