

**THE EFFECTS OF DIET AND WATER TEMPERATURE ON THE COLOUR OF
AUSTRALIAN GREENLIP ABALONE (*Haliotis laevigata* Donovan)**



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Abstract

The Greenlip abalone *Haliotis laevis* is a commercially important species for aquaculture in Australia. It is mainly farmed in land-based systems using formulated feeds but the colour of farmed abalone fed a formulated diet is different compared to their wild counterparts. Wild greenlip abalone fed algae typically have a green lip, yellow foot and a variety of shell colours, whereas cultured abalone fed a formulated diet have a milky lip, brown foot and light green shell. The colour of abalone, along with texture, size and taste, is an important trait affecting marketability and consumer preference. The loss of abalone's natural colour due to the introduction of a formulated feed suggests a need for pigment supplementation via the inclusion of macroalgae, commercial synthetic pigments or other compounds to abalone feed. This thesis evaluates the effects of diet and water temperature on the colour of greenlip abalone shell, foot and lip, and pigment deposition in whole tissue. This thesis includes four data chapters (2, 3, 4 and 5).

Chapter 2 addresses colour change of the foot and shell in abalone fed various fresh macroalgae. The 1-year-old abalone fed either fresh *Ulva* sp. or fresh *Gracilaria cliftonii* developed a yellow coloured foot. Abalone developed a brown shell when fed fresh *G. cliftonii*, but exhibited a light green shell when fed fresh *Ulva* sp. or a commercial diet. Although chlorophyll *a* and zeaxanthin were the dominant pigments found in fresh *Ulva* sp. and fresh *G. cliftonii*, respectively, β -carotene was the main pigment in the whole tissue of abalone when fed either species of fresh macroalgae. Nutrient (nitrogen) enrichment of the macroalgae did not significantly affect pigment content in both macroalgae and the whole abalone tissue, and had little impact on shell and foot colour. The shell colour of abalone fed 3% dried *Spirulina* sp. was yellow-brown, while the foot was bright yellow when 10% of enriched dried *Ulva* sp. meal was included in the diet. Chlorophyll *a* was the main pigment in the diets of dried *Spirulina* sp. and dried *Ulva* sp., but the whole tissue of abalone fed those dried algae contained β -carotene as the principal pigment.

In Chapter 3, abalone fed >5% dried *G. cliftonii* meal developed a red/brown colour on the shell and the increased inclusion of algal meal resulted in a darker brown shell. Additionally, when abalone were fed >10% *G. cliftonii* meal, the lip became green. However, abalone exhibited a light green shell, milky lip and brown/darker foot when fed a diet with up to 20% *Ulva* sp. meal, similar to those fed the control or commercial diet. Although zeaxanthin was the major pigment in all the diets containing dried *G. cliftonii*, and chlorophyll *a* was the principal pigment in the diet containing >10% *Ulva* sp., β -carotene was the dominant pigment in the tissue of abalone fed these diets and its content increased significantly with the level of macroalgae inclusion.

In Chapter 4, the foot colour of 3-year-old abalone was significantly influenced by diet and water temperature, while the lip and shell colour were less affected. The foot of abalone fed enriched fresh *Ulva* was light gold in colour, whereas abalone fed the 30% *Ulva* sp. meal or the commercial diet had a dark brown foot. Abalone cultured at 22 °C had a darker brown foot than those at 26 °C. The content of β -carotene was significantly higher in abalone fed enriched fresh *Ulva* sp., than those fed other diets. Abalone cultured at 26 °C had a significantly lower amount of β -carotene than those at 22 °C.

In Chapter 5, the supplementation of the commercial synthetic carotenoid, astaxanthin (0.05, 0.1 and 0.2 g kg⁻¹), and a free amino acid, tyrosine (10, 15 and 20 g kg⁻¹), in the formulated diet, did not significantly affect the colour and pigment deposition in the whole abalone tissue.

Overall, the results of this project indicate that the shell, foot and lip colour of greenlip abalone can be modified by feeding fresh red algae or dried red algae supplements in the formulated diet. Water temperature had a significant influence on foot colour only. β -carotene was the predominant pigment in the whole tissue of abalone fed fresh or dried macroalgae inclusion in formulated diet. Greenlip abalone were unable to utilise synthetic astaxanthin or the free form of the amino acid, tyrosine. This thesis expands our knowledge on the factors

that regulate colour change in abalone and provides practical approaches to change abalone colour through diet and water temperature manipulation.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Hoang Hai Thanh

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Chapter 1

General Introduction

1.1. Abalone aquaculture status in the world and Australia

Abalone (*Haliotis* spp.) are gastropod molluscs in the family Haliotidae and are one of the most highly valued seafoods in many parts of the world. Recently, wild abalone populations have seriously declined due to over-exploitation, illegal harvesting, disease outbreak and habitat degradation (Cook, 2014). A decrease in fishing, together with a high global demand for abalone, has provided opportunities to establish aquaculture farms to supply the market (Cook, 2014). This has resulted in a significant increase in the number of abalone farms and overall production in the world (Cook, 2014). During the period 2002 – 2010, abalone production on farms increased by more than 7.5 times, and worldwide production reached over 65,000 mt in 2010 (Cook, 2014). From 2010 to 2013, farmed abalone production increased by 158% and reached 103,464 mt (Cook, 2014). In order to sustain production at this level and continue to succeed in the future, abalone farms need to overcome a number of challenges, including high production costs, environmental pollution, disease outbreak, and high feed costs (Nicolas et al., 2002; Hooper et al., 2007; Stone et al., 2013; Cook, 2014; Lange et al., 2014; Stone et al., 2014; Bansemer et al., 2015; Duong et al., 2016). The loss of natural colour in farmed abalone fed formulated diet has received more attention as it is linked to product quality and identity and is also an important trait affecting consumer decision and market acceptability (Lim and Lee, 2003; Qi et al., 2010; Ju et al., 2015; Hoang et al., 2016; 2017).

Australia has been one of the leading abalone producers in the world, from both fisheries and aquaculture (Cook, 2014). Abalone was the highest valued species in 2013-14, constituted 81% of total wild-caught mollusc production, despite a 10% (\$16 million) decrease in its production value to \$138 million (ABARES, 2015). Commercial fishing for abalone began in Australia in 1963 and experienced a period of fast growth (Mottet, 1978). This has since declined, especially over the last decade, mainly due to disease outbreak,

which has provided an opportunity for the development of abalone aquaculture (Cook, 2014; Stone et al., 2014). Abalone farming in Australia is mainly conducted in the coastal regions of Victoria, South Australia, Tasmania and Western Australia (Hooper et al., 2007). The value of abalone aquaculture in Australia has increased from <\$1 million in 1998 - 1999 to over \$34 million in 2013 (Stone et al., 2014). There are three commercial abalone species in Australia, greenlip *Haliotis laevis*, blacklip *Haliotis rubra*, and Roe's *Haliotis roei* (Mottet, 1978).

Greenlip abalone *Haliotis laevis* is widely cultured in Australia due to its fast growth, white flesh, and high market price (Mottet, 1978; Hone and Fleming, 1998; Freeman, 2001). Wild greenlip abalone live at depths of 5 - 40 m in moderate to rough-water areas. They prefer smooth rock substrate and will remain stationary in the same location for a period of months, migrating only if the habitat becomes unsuitable to live. This species almost entirely relies on drift algae in the wild and commonly grows to 13 - 14 cm in 3-4 years (Mottet, 1978). The farming of greenlip abalone has developed in South Australia, Victoria and Tasmania since the early 1990's and is now recognised as a significant contributor to the state economy (Hone and Fleming, 1998; Stone et al., 2014). However, similar to some farmed haliotid species, the colour of cultured greenlip abalone fed formulated diets is inferior to their wild counterparts, which may be discriminated by consumers or lower market prices (Oakes and Ponte, 1996; Brown et al., 2008; Hoang et al., 2016; 2017).

1.2. Abalone colour

In an intact abalone the most obvious pigments visible are located along the epipodium and extended to the edges of the pedal sole (also known as lip). Other pigments are on the shell and foot of the abalone (Olley and Thrower, 1977). The common names of different species are often derived from colour of these body parts. For example, *Haliotis laevis*, *Haliotis rubra* and *Haliotis conicopora* are named after the green, back and brown colour on

the lip, respectively, while others are named after their shell colour such as red abalone *Haliotis rufescens* or green abalone *Haliotis fulgens* (Olley and Thrower, 1977; Mottet, 1978; Department of Fisheries., 2011).

Aside from flesh texture and size, the colour of abalone is an important trait for assessing product quality and is directly associated with the product price (Oakes and Ponte, 1996; Brown et al., 2008). Species with a light foot colour are generally considered to be of better quality and usually command a higher price. The dark or more pigmented species are graded low and require more trimming, washing or bleaching prior to sale to mask colour distinctions (Oakes and Ponte, 1996; Brown et al., 2008). According to Qi et al. (2010), the shell colour of *Haliotis discus hannai* fed red algae *Gracilaria lemaneiformis* is purple and has a higher market value in north China as it is similar to the shell colour of wild-caught abalone.

The colour of abalone is also a characteristic for species identification and taxonomy. As mentioned above, a number of abalone species are named after the colour of shell or other body parts (Mottet, 1978; Brown et al., 2008). Red abalone *Haliotis rufescens*, black abalone *Haliotis cracherodii* and green abalone *Haliotis fulgens* have derived their names from the shell colour, while blackfoot abalone *Haliotis iris*, or blacklip abalone *Haliotis rubra* and greenlip abalone *Haliotis laevigata* are named after their foot or lip colour (Mottet, 1978; Department of Fisheries, 2011). Since the colour, shape and sculpture of the shell and other body parts are commonly used to distinguish between species, the loss of natural colour in abalone fed formulated diets may also affect species identification.

Cultured abalone usually lack the same pigmentation as in the wild. On farms, blackfoot abalone *H. iris* fed formulated diets have a paler foot than in the wild, but the foot colour can be darkened by feeding the animals with *Gracilaria* spp. (Allen et al., 2006). In another case, the shells of juvenile *Haliotis asinina* are blue-green when fed a formulated

diet, but when fed seaweed *Gracilariopsis bailinae* the shells become a darker shade of brown (Bautista-Teruel and Millamena, 1999). It has also been observed that wild greenlip abalone consuming macroalgae typically have a green lip and a light yellow foot, this is however, difficult to maintain in land-based farms where abalone are fed formulated diets (Department of Fisheries, 2011; Bansemer et al., 2014; Mayfield et al., 2014; Hoang et al., 2016). Due to inadequate pigments in the diet, farmed greenlip abalone usually have a milky lip, light green shell and darker foot (Hoang et al., 2016). Since colour is used to grade the quality of product and identify species, there have been attempts to preserve the natural colour of abalone in an aquaculture environment (Lim and Lee, 2003; Qi et al., 2010; Ju et al., 2015). However, little research has been conducted in terms of maintaining or manipulating the colour of greenlip abalone in a farming situation.

1.3. Relationship between diet and abalone colour

The colour of wild abalone is directly related to diet. This is due to most abalone species being unable to biosynthesize carotenoids *de novo* (Shahidi and Brown, 1998; Maoka et al., 2010; Maoka, 2011). Since algae constitute a major portion of the diet of wild abalone (Shepherd, 1973), it is logical to assume that the colour of haliotids is related to pigments or colour precursors present in algae. Therefore, it is necessary to investigate the kinds of micro/macroalgae species that may enhance or alter the colour of cultured abalone.

1.3.1. Dietary algae in abalone

In the wild, macroalgae is the main food source of the *Haliotis* species. Juvenile abalone consume a variety of macroalgae species such as *Ulva* spp., *Gracilaria* spp., and *Sargassum* spp. (Viera et al., 2005). In China, Korea, South Africa and Chile, macroalgae are widely used to feed farmed abalone, not only because of its relatively high abundance and low cost, but also due to evident improved health, feeding activity and marketability (Bansemer et al., 2014). Herbivorous gastropods can consume seaweed at a rate close to 35%

of their body weight per day (Viera et al., 2005). A variety of seaweeds such as *Ecklonia maxima*, *Laminaria japonica*, *Gracilaria lemaneiformis*, *Sargassum pallidum*, *Ulva rigida*, *Ulva lactuca* and *Gracilaria gracilis* have previously been cultured to feed abalone (Shpigel et al., 1999; Demetropoulos and Langdon, 2004; Taylor and Tsvetnenko, 2004; Alcantara and Noro, 2006; Qi et al., 2010; Bansemer et al., 2014). For example, brown algae such as *Laminaria sinclairii*, *Macrocystis pyrifera*, *Nereocystis luetkeana*, *Egregia menziesii* and *Ecklonia maxima* have been used to feed *H. rufescens*, *H. fulgens* and *H. midae* (Guest et al., 2008). According to Dunstan et al. (1996), food preference can vary among abalone habitats and with seasonal availability of seaweeds. For example, Southern Californian abalone *Haliotis corrugata* and *Haliotis fulgens* prefer consuming brown and red algae such as *Macrocystis* sp., *Dictyopteris* sp., *Gelidium* sp., *Plocamium* sp. and *Gigartina* sp. but they were shown to avoid eating *Egregia* sp. (Tutschulte and Connell, 1988). However, Leighton (1966) reports the same species, showing a preference for *Egregia* sp. In other cases, red algae such as *Plocamium* sp. *Pterocladia capillacea* and *Asparagopsis armata* are preferred by wild *H. rubra*, *H. laevigata* and *H. roei* even though some brown algae such as *Phyllospora comosa*, *Sargassum* sp. are also consumed (Shepherd 1973; Guest et al., 2008).

Abalone colour change is associated with macroalgae in the diet (Leighton, 1961; Sakai, 1962; Leighton and Boolootian, 1963; Olsen, 1968; Gallardo et al., 2003; Qi et al., 2010; Ju et al., 2015). The shell of Pacific abalone *Haliotis discus hannai* is dark brown when fed Pacific dulse *Palmaria mollis*, green when fed kelp *Laminaria japonica* and becomes purple when fed red algae *Gracilaria lemaneiformis* (Qi et al., 2010; Ju et al., 2015). Similarly, the tropical abalone *Haliotis asinina* has a brownish shell when fed red algae *Gracilariopsis bailinae* (Gallardo et al., 2003). Furthermore, it appears that certain shell colours are related to types of algae fed the same abalone species or specific abalone species consume same diet. For example, when feeding on brown algae *Macrocystis* sp., the shell of

H. cracherodii is dark-blue, whereas the shell of *H. rufescens* is brick-red and the shell of *H. corrugata* ranges from dull-green to reddish-brown (Chew, 1973). It is also observed that when *H. discus hannai* are fed brown algae, green algae and diatoms, the shell colour is bluish-green, but when fed red algae the shell becomes brown (Sakai, 1962). According to Leighton (1961), *H. rufescens* had a red shell colour when fed red macroalgae, but became white, cream or pale green when the animal was fed with brown and green macroalgae. Thus, it is hypothesised that greenlip abalone colour can be changed in farming conditions by varying the pigment composition in the diet.

Nutrient enrichment is a way to increase the protein content of macroalgae fed to abalone (Shpigel et al., 1999; Boarder and Shpigel, 2001; Viera et al., 2011; Bansemer et al., 2014, 2016). The protein content of enriched *Ulva rigida* and *Hypnea spinella* are about 17.16% and 19.89% higher than without enrichment (Viera et al., 2011). Similarly, nitrogen enrichment also increased the protein level of *Ulva* sp. and *G. cliftonii* by 25.3% and 25.2%, respectively (Bansemer et al, 2016). It has been reported that enriched macroalgae 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). Nutrient enrichment may also lead to a visual colour change in macroalgae (Bansemer et al, 2016a). Despite having positive effects on the growth of abalone, the impact of macroalgae after nutrient enrichment on the change of abalone colour and tissue pigments has rarely been experimentally tested.

1.3.2. Supplementation of dried micro/macroalgae as natural pigments in abalone feed

Even though macroalgae is a part of abalone's natural diet, due to conservation restrictions and seasonal availability, it is not always accessible as a food source to land-based farms (Dunstan et al., 1996; Bansemer et al., 2014). In contrast, a formulated diet offers a convenient supply as well as a number of other advantages including, an enhanced

nutritional profile and lower costs for farm management (Kirkendale et al., 2010). Typically, the growth rate of abalone fed a formulated diet is higher than those fed live macroalgae. For instance, a formulated diet has been shown to sustain a higher growth rate in juvenile *H. rufescens* (Garcia-Esquivel and Felbeck, 2009) and 1-year-old *H. laevigata* (Bansemer et al., 2016a). However, due to insufficient pigments in the formulated diet, the colour of farmed abalone fades, manifested as a pale foot and loss of natural colour on the shell and lip (Allen et al., 2006; Bautista-Teruel and Millamena, 1999; Lim and Lee, 2003; Bansemer et al., 2014). The inclusion of dried micro- or macroalgae meal in aquafeeds has been an effective way to achieve natural colour or manipulate colour in a number of aquatic species (Choubert, 1979; Lim and Lee, 2003; Bansemer et al., 2014; Hoang et al., 2016; 2017; Valente et al., 2016). In fish, such as Nile tilapia *Oreochromis niloticus*, muscle and skin colour is important for consumers and marketability. The addition of 5 - 10 % of green macroalgae *Ulva* spp. meal can increase the total skin carotenoid content which may increase consumer acceptance of tilapia fillets and benefit the aquaculture industry (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. Therefore, it is hypothesised that the inclusion of dried macroalgae meal in commercial abalone diets will result in colour changes to match the wild population.

Microalgae are also widely used in aquaculture as live food for bivalves, larvae, juvenile crustaceans, fish and abalone in aquaculture (Spolaore et al, 2006). Many species of microalgae play important roles in enhancing the colour of high valued species in aquaculture. For example, *Haematococcus* sp. has a high content of astaxanthin and its supplementation to the diet of salmonids has successfully changed fish flesh colour (Sommer et al.,1991; Choubert and Heinrich, 1993; Borowitzka, 1997). *Chlorella vulgaris* has also been recommended as a pigmentation enhancer in rainbow trout *Oncorhynchus mykiss*,

gilthead seabream *Sparus aurata*, koi carps *Cyprinus carpio* and goldfish *Carassius auratus* (Gouveia et al., 1996, 2002, 2003). In the blue-green algae genus, *Spirulina*, cells are rich in carotenoids such as β -carotene, zeaxanthin, myxoxanthophyll, echinenone and cryptoxanthin (Miki et al., 1986; Belay et al., 1996). A commercially available powder of *Spirulina maxima* contains 648 mg 100 g⁻¹ of carotenoids, including 15% β -carotene, 11-13% echinenone, 25% zeaxanthin and 13 - 17% myxoxanthophyll (Miki et al., 1986). *Spirulina* sp. has been used as a source of carotenoid in the diet of rainbow trout *Oncorhynchus mykiss*, Red tilapia *Oreochromis niloticus*, Black tiger prawn *Penaeus monodon* and pond-reared *Fenneropenaeus indicus* (Choubert, 1979; Boonyaratpalin and Unprasert, 1989; Liao et al, 1993; Regunathan and Wesley, 2006). Recently, the inclusion of 7.5% *Spirulina platensis* has also been recommended to achieve a desirable flesh colour in cultured rainbow trout *Oncorhynchus mykiss* (Teimouri et al., 2013). The colour of Japanese ornamental koi carp *Cyprinus carpio* can also be modified by feeding them a diet including 75.0 g kg⁻¹ *S. platensis* (Sun et al., 2012). However, there is little information on the use of microalgae as a supplement in the diet of abalone to enhance or alter body colour.

1.3.3. Use of synthetic carotenoids as dietary supplements

The coloration of many aquatic species, including abalone, is often related to carotenoids, other pigments and precursor chemicals and compounds in their food (Shahidi et al. 1998). The use of micro/macroalgae as a natural source of pigments to enhance or alter the colour of aquatic animals has been well documented, but the recent attention has been directed towards using commercially synthesised pigments as food additives due to their low cost, ease of access and chemical stability (SajjadMirzaee et al., 2012; Teimouri and Keramat Amirkolaie, 2015).

Synthetic carotenoids such as astaxanthin and canthaxanthin have chemically identical structures to the natural compounds found in aquatic animals and are manufactured at an

industrial scale for pigment supplementation into aquaculture feeds (Higuera-Ciapara et al., 2006). The basic process of carotenoid synthesis is to use crude mineral oil to produce intermediates such as acetone, acetylene and aldehyde for further pigment synthesis. In most cases, the precursor for carotenoid synthesis is β -ionone (Hertrampf and Piedad-Pascual, 2012). Among the synthesised carotenoids, astaxanthin is the most efficient product in terms of colour change, providing a desirable reddish-orange colour in crustaceans, salmonids and other farmed aquatic animals that do not have access to a natural source of carotenoids (Higuera-Ciapara et al., 2006; Kurnia et al., 2015).

Astaxanthin is a pigment belonging to the family of xanthophylls, which are oxygenated derivatives of carotenoids from lycopene in plants. Synthetic astaxanthin is an identical molecule to that produced in living organisms, consisting of a mixture 1:2:1 of isomers (3S, 3S'), (3R, 3S'), and (3R, 3R), respectively (Higuera-Ciapara et al., 2006; Fig.1). However, synthetic astaxanthin is not esterified, unlike when it is found naturally in algae (Higuera-Ciapara et al., 2006). The production of synthetic astaxanthin began at a large scale in the 1990s and practically fulfilled the demand for pigment supplementation requirements. It is currently available under the commercial brand name Carophyll Pink™ (Higuera-Ciapara et al., 2006). The use of synthetic astaxanthin in aquaculture has an economical advantage to compete with the natural product (Higuera-Ciapara et al., 2006). Previous studies have demonstrated equal or superior pigmentation when using the synthetic form compared with the natural astaxanthin. For example, Pan and Chien (2009) reported that supplementation of the synthetic (Carophyll® Pink 8% AX: 100% free form, DSM Nutrition) and natural astaxanthin (*Haematococcus pluvialis*), in the diet, showed similar effects on skin and fin coloration in Red devil *Cichlasoma citrinellum*. Synthetic astaxanthin (Carophyll Pink) is more effective at enhancing red pigments of both red and white coloured carp *Cyprinus carpio* compared to those fed microalgae, *Spirulina* sp. or *Chlorella* sp. (Kim et al., 2008).

According to Sommer et al. (1991) and Choubert and Heinrich (1993), Rainbow trout *Oncorhynchus mykiss*, fed synthetic astaxanthin had higher levels of total carotenoids in their flesh than those fed green algae, *Haematococcus pluvialis*. Despite the wide use of synthetic carotenoids for improving pigments in fish, prawns and other aquatic species, there is still a lack of information regarding the effects on molluscan species like greenlip abalone.

Dietary supplementation of synthetic carotenoids has desirable effects on pigmentation in aquacultured fish and crustacean species, but the optimal levels are species-specific (Tlusty and Hyland, 2005; Niu et al., 2009; Yasir and Qin, 2010). The optimum dietary level of synthetic astaxanthin is between 0.05 - 0.2 mg kg⁻¹ for the giant tiger shrimp *Penaeus monodon*, 0.1 - 0.2 mg kg⁻¹ for the Pacific white shrimp *Litopenaeus vannamei* and 0.22 mg kg⁻¹ for the American clawed lobster *Homarus americanus* (Tlusty and Hyland, 2005; Niu et al., 2009; Wade et al., 2015a,b). Similarly, the recommended level of synthetic astxanthin in fish diets is also species specific. The suggested inclusion levels in the diet, range from 0.04 - 0.1 g kg⁻¹ for salmon feed, 0.05 - 0.1 g kg⁻¹ for trout, 0.02 - 0.03 g kg⁻¹ for gilthead sea bream and 0.05 - 0.1 g kg⁻¹ for gold fish (Diler and Dilek, 2002). Therefore, it is proposed that the inclusion of a synthetic carotenoid in the diet will affect the colour of abalone.

1.3.4. Supplementation of dietary tyrosine and its impact on melanin content in tissues

Melanins, along with carotenoids and tetrapyrroles can be attributed to mollusc colouration (Fox, 1966; Williams, 2016). The pigments found in gastropod molluscs, are commonly found in the epidermis, but also can be found elsewhere (Fox, 1983). Specifically, the pigment melanin is found in cephalopod mollusc ink, the siphonal tip of bivalves, and in the black lines along the mantle edge of *Haliotis tuberculata* (Williams, 2016). The green and black pigments on the lip and foot tissue of the greenlip abalone, blacklip abalone and blackfoot paua are also associated with melanin production (Chew, 1973). Although melanin

may play an important role in the formation of the green lip colour of *H. laevigata*, until now no published attempt has been made to explore the relationship between dietary melanin supplementation and green pigment of lip and its content found in tissues.

Melanin is likely to be biosynthesized and contributes to the green or black colour in abalone (Chew, 1973). The molecular pathway for the synthesis of melanin is associated with tyrosine since several studies have shown that tyrosine plays an important role in the regulation and production of melanin in molluscs (Fig.2; Fox, 1979; Williams, 2016). For example, tyrosinase genes, precursor of melanin production, may contribute to the formation blue pearls or shell pigmentation in the pearl oyster *Pinctada fucata*, the black-lipped pearl oyster *Pinctada margaritifera* and the scallop *Mizuhopecten yessoensis* as it have been identified in the mantle tissue of these animals (Miyashita and Takagi, 2011; Miyamoto et al., 2013; Lemer et al., 2015; Sun et al., 2015). Therefore, it is proposed that the addition of tyrosine into the diet, may affect colour synthesis in cultured abalone, especially in the development of the green lip pigments.

1.4. Relationship between water temperature abalone colour

Abalone are classified as thermoconformers, meaning their body temperature varies with the surrounding environment (Prosser, 1991). Therefore, abalone cannot maintain a constant body temperature as ambient temperatures fluctuate. Abalone survival, feed intake, respiration, growth, digestive physiology, gastrointestinal evacuation time and digestive enzyme activity are all significantly affected by water temperature (Harris et al, 2005; Schaefer et al., 2013; Stone et al., 2013; Currie et al., 2015; Bansemer et al., 2015; Duong et al., 2016; Bansemer et al., 2016a,b). Generally, within the optimal temperature range, feed intake, somatic growth and metabolism increase directly with temperature, whereas outside the range of optimal temperature, these functions are adversely affected (Duong et al., 2016). Temperature changes of one or two degrees have been shown to have significant

repercussions on metabolism (Van Barneveld, 2008). Although in the laboratory, the optimal water temperature for the growth of greenlip abalone is 22 °C in Australia, abalone in the land-based farm system are subject to seasonal temperature variation from below 10 °C, in Tasmania, during winter to above 24 °C, in South Australia, during summer (Stone et al., 2013; Bansemer et al., 2015). Thus, it is hypothesised that abalone body colour intensity will be also influenced by water temperature. since it governs feed and pigment intake, absorption, digestion, biosynthesis and deposition in tissues (Stone et al., 2013; Lange et al., 2014; Bansemer et al., 2015).

The effect of water temperature on abalone colour is rarely studied, but there are several reports on other aquatic animals. For example, the flesh of Arctic charr *Salvelinus alpinus* fed a commercial diet at 10 °C had a more intense orange colour than those at 15 °C (Gines et al., 2004). Arctic charr fed six diets containing 0 to 192 mg astaxanthin kg⁻¹ diet at 8 °C had significantly higher pigmentation than those at 12 °C (Olsen and Mortensen, 1997). According to Storebakken et al. (1986), the extent of pigmentation in Atlantic salmon *Salmo salar*, rainbow trout *Salmo gairdneri* and sea trout *Salmo trutta*, varies due to geographical location, presumably due to differences in water temperature. Lin et al. (2009) also reported a change of skin colour in juvenile seahorses *Hippocampus erectus* when reared at three different temperatures (23, 26 and 29 °C). Thus, it is again hypothesised that abalone colour changes could be related to ambient water temperatures.

1.5. Metabolism of carotenoid pigments in abalone

After carotenoid intake from food, the processes of assimilation, metabolism and storage of carotenoids in invertebrates can be classified into four groups. 1) The animals generally eject the carotenoids in an undigested form via faeces; 2) the animals assimilate and store carotenoids in the body with an unchanged chemical form; 3) the animals assimilate and convert the carotenoids to other pigment forms; and 4) animals assimilate and completely

catabolise the ingested carotenoids (Fox 1979; Shahidi and Brown, 1998). A number of carotenoids have been found in gastropods such as β -carotene, echinenone, β -cryptoxanthin and zeaxanthin in *Patella depressa* and *P. vulgata* and astaxanthin from *Flabellinopsis iodinea* (Shahidi and Brown, 1998). For example, pigments identified in Pacific abalone *Haliotis discus hannai*, include α -carotene, fucoxanthin and isofucoxanthin (Shahidi and Brown, 1998). Common pigments found in the shell of Japanese abalone *Haliotis discus hannai* are chlorophyll *a*, *b* and *c*, β -carotene, lutein, violaxanthin, neoxanthin and pheophytin *a*, while some other pigments, such as, pheophytin *a*, are found in the viscera, β -carotene in muscle and carotenes and pheophytin *a* in the testis and ovary (Tajima et al., 1980a, b). Zeaxanthin is also found in the muscle of *H. discus hannai* (Maoka et al. 1986). Although a number of pigments have been identified, the relationship between supplemented pigments in the diet and the content of pigments in the whole body tissue is poorly understood.

1.6. Measurement of abalone colour

In order to evaluate colour change, assessment should reflect the visual perception of colour to the human eye. In the human eye, colour is a brain reaction to a specific visual stimulus described using three broad bands corresponding to red, green, and blue (RGB) (Yasir and Qin 2009). Hue, saturation and brightness (HSB) are values assigned represent the colour properties corresponding to red, green, and blue. All possible colour properties are specified as hue (i.e., colour purity), saturation (i.e., colour intensity) and brightness as visualised in a reversed cone shape model (Yasir and Qin, 2010. Fig. 2). Hue is expressed as a number indicating the degrees around the cone with red at zero degrees, green at 120 degrees, and blue at 240 degrees. Colour saturation ranges from 0% (no saturation) to 100% (full saturation) and brightness ranges from 0% (black) to 100% (white). However, both hue and saturation become meaningless at 0% brightness. This method of colour measurement has previously been used to examine the colour change in false clownfish *Amphiprion*

ocellaris when subject varying light intensity, dietary carotenoids and background (Yasir and Qin, 2009 a, b; 2010). In this study, the HSB model has also been used to analyse visual colour in abalone based on photographic images.

1.7. Overall study objectives

The main objective of this study was to gain a better understanding on the impacts of diet and water temperature on the colour change of the shell, lip and foot and the pigment deposition in the whole tissue of greenlip abalone. Overall, this study is an attempt to develop management protocol to control colour change in land-based culture systems and meet the expectations of seafood consumers in terms of abalone colour. Specifically, the study objectives are:

- 1) to understand the possible factors which contribute to the colour change of abalone and identify the knowledge gaps that need to be addressed;
- 2) to identify the potential fresh macroalgae species (with and without nutrient enrichment) or dried micro/macroalgae meals which can be used to enhance or manipulate the colour of abalone;
- 3) to determine the type and appropriate levels of dried macroalgae meal as a supplement in the formulated diet to improve or alter abalone colour;
- 4) to understand the impact of water temperature and diet on the colour of greenlip abalone; and
- 5) to evaluate the potential of using the commercial synthetic carotenoid, astaxanthin, and the amino acid, tyrosine, as feed additives to change the colour of abalone.

To address the first objective, the general introduction in the first chapter of this thesis is presented as a literature review, followed by four experimental trials to address the remaining objectives. In brief:

- 1) Trial 1: Colour changes of greenlip abalone (*Haliotis laevis* Donovan) fed fresh macroalgae and dried algal supplement. This experimental trial was designed to address objective two. Two fresh macroalgae species, red macroalgae *Gracilaria cliftonii* and green macroalgae *Ulva* sp., with and without nutrient enrichment, were used to feed 1-year-old greenlip abalone. Inclusions of 3% dried *Spirulina* and 10% dried *Ulva* sp. meal, in a formulated diet, were also tested. The colour components (i.e. hue, saturation and brightness) of shell, lip and foot and the pigments in the whole abalone tissue were measured. The results are presented in Chapter 2.
- 2) Trial 2: Colour changes of greenlip abalone (*Haliotis laevis* Donovan) fed formulated diets containing graded levels of dried macroalgae meal. This trial was conducted to identify potential macroalgal species and their optimum inclusion levels to change abalone pigmentation as outlined in objective three. Two species of dried macroalgae (*Ulva* sp. and *G. cliftonii*) were included at four levels (0% basal diet, 5%, 10% and 20%) in the formulated diet and fed to 1-year-old greenlip abalone for 92 days. The results are presented in Chapter 3.
- 3) Trial 3: Effects of diet and water temperature on the colour of greenlip abalone (*Haliotis laevis* Donovan). To gain a better understanding on the impacts of diet to abalone colour at different water temperatures, three diets including enriched fresh *Ulva* sp., a commercial diet and a formulated feed containing 30% of *Ulva* sp. in the commercial diet were used to feed 3-year-old greenlip abalone at either 22 or 26 °C for 38 days. The results address objective four and are presented in Chapter 4.
- 4) Trial 4: Dietary supplementation of the commercial synthetic astaxanthin or tyrosine had limited impact on the colour of greenlip abalone (*Haliotis laevis* Donovan).

Donovan). This trial was carried out to address objective five. One-year-old greenlip abalone were fed a control diet (0% pigment addition), three levels of astaxanthin (0.05, 0.1 and 0.2 g kg⁻¹) or three levels of tyrosine (10, 15 and 20 g kg⁻¹) in formulated diets. The experiment was conducted in tanks with four replicates of each treatment over 72 days. The results were presented in Chapter 5.

1.8. Thesis outline

This thesis is presented in six chapters. Chapter 1 is the general introduction, providing background information regarding abalone aquaculture in Australia and in the world, updates current research progress in the field of abalone body colour, and identifies the knowledge gaps that need to be addressed. It introduces the research objectives and presents the thesis organisation. Current literature suggests that the colour of abalone can be improved or manipulated by dietary algae such as fresh algae or dried algae meal, but little is known about the specific effects on greenlip abalone. Although water temperature can significantly affect survival, feed intake and growth rate of abalone, it is not clear if temperature affects pigment deposition and abalone colour. Four data chapters (2, 3, 4 and 5) are organised as follows.

Chapter 2 investigates the colour changes of greenlip abalone (*Haliotis laevis*) fed fresh macroalgae and dried algal supplements. In the first experiment, a commercial control diet and two species of fresh enriched and non-enriched macroalgae (*Gracilaria cliftonii* and *Ulva* sp.) were used to feed 1-year-old greenlip abalone for 93 days. 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. The foot was light yellow in abalone fed both fresh macroalgae species. In the second experiment, the shell of abalone fed 3% dried *Spirulina* sp. was yellow-brown with higher colour purity, while the shell remained light green in abalone fed 10% dried *Ulva* sp. or the commercial control diet. Those experiments indicate that fresh

macroalgae and dried algae supplementation in formulated feed can change abalone foot and shell colour. The results from this study were used to design the consequent experimental trial in Chapter 3.

In Chapter 3, the inclusion of dried macroalgae meal into the formulated diet was examined as a pigment source for greenlip abalone. Since the previous chapter revealed that fresh macroalgae could change the shell and foot colour, greenlip abalone were fed, exclusively, a formulated diet with dried macroalgae inclusion in laboratory conditions. Seven formulated diets including a basal diet (0% diet), three inclusion levels (5, 10 and 20%) of enriched dried *Ulva* sp. meal or dried *G. cliftonii* meal were used to feed 1-year-old greenlip abalone for 92 days. This study indicated that the inclusion of red macroalgae *G. cliftonii* in the diet significantly influenced abalone colour. Specifically, the inclusion of $\geq 10\%$ *G. cliftonii* intensifies the green colour of the lip, while the inclusion of $\geq 5\%$ *G. cliftonii* can produce a red/brown shell. The next experiment was then conducted to further investigate the potential inclusion of up to 30% *Ulva* sp. meal, which is approximately the same amount of fresh *Ulva* sp. consumed daily by abalone.

Chapter 4 was conducted to investigate the relationship between the diet and water temperature on abalone colour. The colour components of the shell, lip and foot and pigment content in whole tissue of 3-year-old abalone, fed three different diets, including a commercial diet, fresh *Ulva* sp. and 30% of *Ulva* sp. supplemented in a commercial diet, at two water temperatures, 22 or 26 °C, for 38 days, were measured. The foot of abalone fed enriched fresh *Ulva* sp. was light gold, whereas abalone fed the diet containing 30% *Ulva* sp. meal and the commercial diet had a dark brown foot at 22 °C. Abalone at 26 °C had paler foot than those at 22 °C. This study indicates that both water temperature and diet can affect foot colour and the β -carotene content in the whole abalone tissue.

The first three experimental trials focussed on investigating fresh and dried algae as natural sources of pigment for greenlip abalone. However, synthetic pigments are also widely used for colour enhancement in aquaculture. For that reason, colour change of greenlip abalone were fed a commercial synthetic carotenoid or an amino acid was investigated. The details are presented in the next chapter.

In Chapter 5, a trial was carried out to evaluate the effects of commercial additives on shell, foot and lip colour and melanin content in the lip and whole tissue of greenlip abalone. One-year-old greenlip abalone were fed with a control diet (0% pigment addition), three levels of a commercial carotenoid, astaxanthin, (0.05, 0.1 and 0.2 g kg⁻¹) and three levels of the amino acid, tyrosine, (10, 15 and 20 g kg⁻¹) in formulated diets for 72 days. Unexpectedly, neither supplement in the diet significantly influenced the colour components (hue, saturation and brightness) of any body parts or the pigment contents in the whole abalone tissue, compared to the control diet. Melanin content in the abalone lip was also not significantly affected by dietary tyrosine. This study indicates that greenlip abalone had little capacity to absorb and metabolise the commercial synthetic astaxanthin or free tyrosine to have any effect on body colour.

Finally, Chapter 6 are a general discussion where all major research findings are summarised and discussed. Recommendations are also provided to the greenlip abalone industry to improve colour in aquaculture and an indication of future research direction is provided.

1.9. Publications

1.9.1. Co-authorship of chapters

The manuscripts (here referred to as Chapters 2, 3, 4, and 5) have either been published (Chapter 2 and 3) or submitted (Chapters 4) or will be submitted (Chapter 5) to peer-reviewed journals. Although I am the principal contributor for each manuscript, some people who made

substantial contributions including experimental design, data analysis and interpretation are listed as co-authors. My principal supervisor, Professor Jian Qin and two co-supervisors, Associate Professor James O. Harris and Associate Professor David A.J. Stone are co-authors of all 4 manuscripts, due to major contributions to experimental design, sample analysis and the review of each manuscript. All manuscripts are also co-authored by Mr. Duong N. Duong due to his contribution towards running the experiments and assisting in sample collection, analysis and reviewing manuscripts. Dr. Matthew S. Bansemer is also listed as a co-author for Chapters 2 and 3 due to his contribution towards preparing, running experiments and reviewing the manuscripts.

1.9.2. Thesis publications

Chapter 2: Hoang, T.H., Qin, J.G., Stone, D.A., Harris, J.O., Duong, D.N., Bansemer, M.S., 2016. Colour changes of greenlip abalone (*Haliotis laevis* Donovan) fed fresh macroalgae and dried algal supplement. *Aquaculture* 456, 16-23.

Chapter 3: Hoang, T.H., Qin, J.G., Stone, D.A., Harris, J.O., Duong, D.N., Bansemer, M.S., 2017. Colour changes of greenlip abalone (*Haliotis laevis* Donovan) fed formulated diets containing graded levels of dried macroalgae meal. *Aquaculture* 468, 278-285.

Chapter 4: Hoang, T.H., Qin, J.G., Harris, J.O., Duong, D.N., Stone, D.A., 2016. The effects of diet and water temperature on the pigment deposition in whole tissue and colour of greenlip abalone (*Haliotis laevis* Donovan). Submitted to *Aquaculture*.

Chapter 5: Hoang, T.H., Qin, J.G., Harris, J.O., Duong, D.N., Stone, D.A., 2016. Dietary supplementation of synthetic astaxanthin and tyrosine had limited impact on the colour of greenlip abalone (*Haliotis laevis* Donovan). In preparation.

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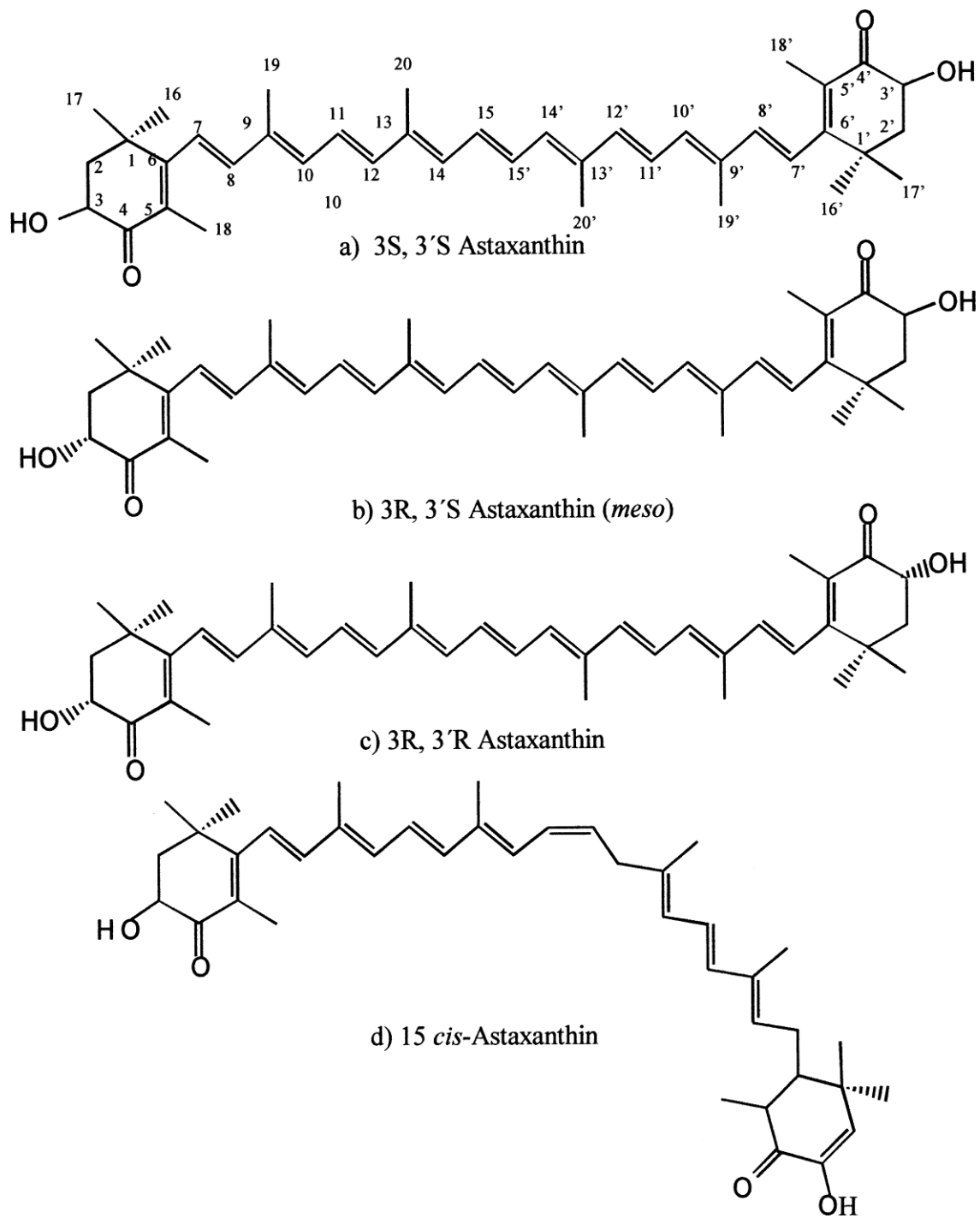


Fig. 1. Chemical structures of synthetic astaxanthin (Higuera-Ciapara et al., 2006)

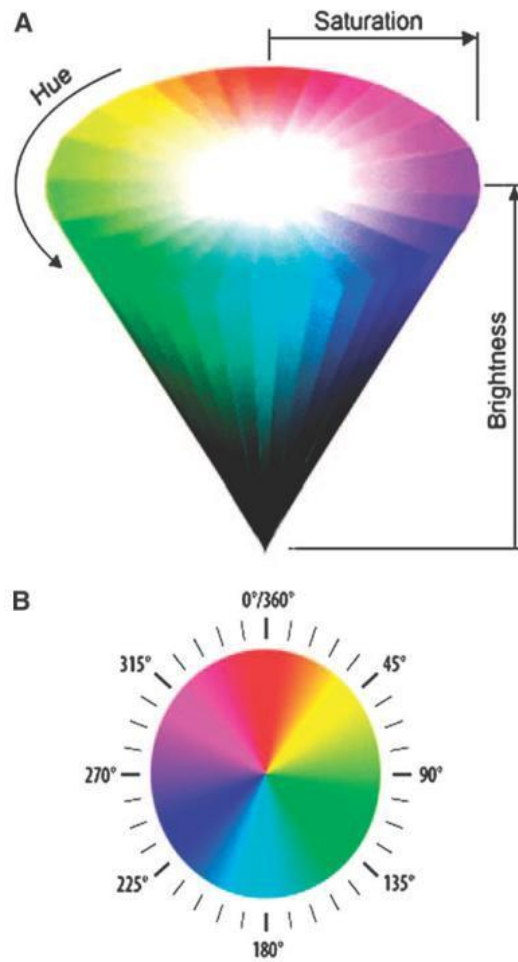


Fig.2. The hue-saturation-brightness (HSB) model to illustrate the relationship of hue, saturation, and brightness as a cone (Yasir and Qin 2009)

Chapter 2

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

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2.1. 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 laevis*. 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.

Key words: abalone, *Ulva* sp., *Gracilaria cliftonii*, *Spirulina* sp., Enrichment, Macroalgae.

2.2. 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 and 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 rufescens* (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 rubra*, and greenlip *Haliotis laevigata* (Brown, 1995 and 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.

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 and 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 and 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 and 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 and 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 and 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 and 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.

2.3. Materials and methods

2.3.1. 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.

2.3.2. Experimental design, stocking and feeding

Experimental design

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 and 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; 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. All diets in the second experiment were manufactured by Eyre Peninsular Aquafeeds, Lonsdale SA. The procedure to add 10%

enriched dried algae was to replace similar proportion of the EPA diet mash from the commercial diet by above algae powders. These algal powders and diet mash were mixed with the present of 30% water. The diets were then manufactured to produce a 5-mm flat sinking pellet.

Stocking and feeding

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.

2.3.3. Specimen sampling and analyses

Image analysis

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 degrees, and blue at 240 degrees. 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.

Pigment analysis

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 μ L heptane and acetone (1:1, v/v) and vortexed for 20 secs before analysis on HPLC (Shimadzu UFLC, Kyoto, Japan). The HPLC was equipped with the Waters Symmetry 300TM analytical C18 column (5 μ m, 3.9 \times 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 μ L 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, 14681), 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 0.3µg/mL. The retention time was 6.1 mins for zeaxanthin, 9.9 mins for β-cryptoxanthin, 12.1 mins for β-carotene and 10.2 mins for chlorophyll *a*.

2.3.4. *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 Dunnett'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.

2.4. Results

Water quality parameters were maintained at levels appropriate for greenlip abalone growth throughout the study: water temperature (21.9 ± 0.4), dissolved oxygen ($97 \pm 0.4\%$), pH (8.2 ± 0.1) and salinity (35 ± 1.0). The overall mortality of abalone during the study was 0.76%, and was not affected by the diet (Bansemmer et al., 2016a)

2.4.1. Effects of fresh macroalgae on abalone colour in Experiment 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 macroalage 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 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).

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*. 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 macroalage 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.

2.4.2. Effects of dried algae supplementation on abalone colour in Experiment 2

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 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 ($P < 0.001$).

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.

2.5. 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 Boolootian, 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 Boolootian, 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 red macroalgae 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 *Ulva 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, Gerasimenko et al. 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, 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; Cyrus et al., 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. cliftonii* 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* sp., 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 *Dunaliella 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; 1980b) 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 *Ulva 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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Table 1. Two-factor ANOVA results for shell and foot colour of abalone and the pigment contents in abalone and diets ($n = 4$).

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

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

Items	Commercial control diet	Non-enriched macroalgae		Enriched macroalgae		ANOVA (P value)
		<i>Ulva</i> sp.	<i>G. cliftonii</i> .	<i>Ulva</i> sp.	<i>G. cliftonii</i>	
Dietary pigments						
β -carotene ($\mu\text{g g}^{-1}$)	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 <i>a</i> ($\mu\text{g g}^{-1}$)	-	4.68 ± 0.52^b	1.29 ± 0.08^a	5.26 ± 0.45^b	1.24 ± 0.21^a	< 0.001
β -cryptoxanthin ($\mu\text{g g}^{-1}$)	-	0.53 ± 0.09^b	0.20 ± 0.01^a	0.63 ± 0.12^b	0.18 ± 0.08^a	0.001
Zeaxanthin ($\mu\text{g g}^{-1}$)	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 ($\mu\text{g g}^{-1}$)	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 <i>a</i> ($\mu\text{g g}^{-1}$)	-	1.72 ± 0.09^b	0.16 ± 0.04^a	2.34 ± 0.65^b	0.24 ± 0.06^a	< 0.001
β -cryptoxanthin ($\mu\text{g g}^{-1}$)	-	0.22 ± 0.06^a	0.23 ± 0.09^a	0.44 ± 0.03^b	0.38 ± 0.13^b	0.011
Zeaxanthin ($\mu\text{g g}^{-1}$)	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. Denoted “-“ as not detectable.

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

Different superscripts mean significant difference ($P < 0.05$). Denoted “-“ as not detectable.

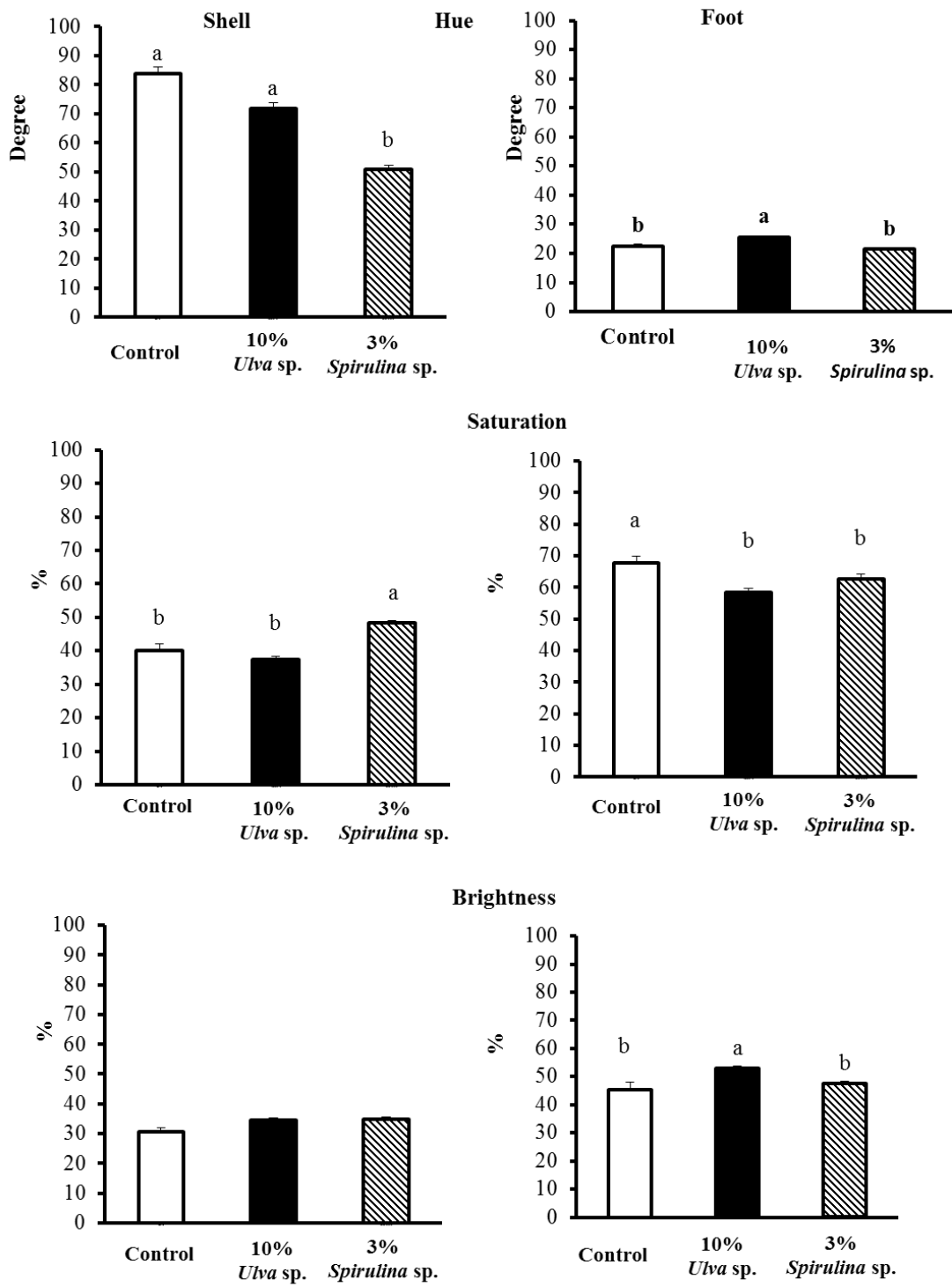


Fig. 1: Hue, saturation and brightness values of shell (left) and foot (right) in abalone fed dried micro and macroalgae in experiment 2.

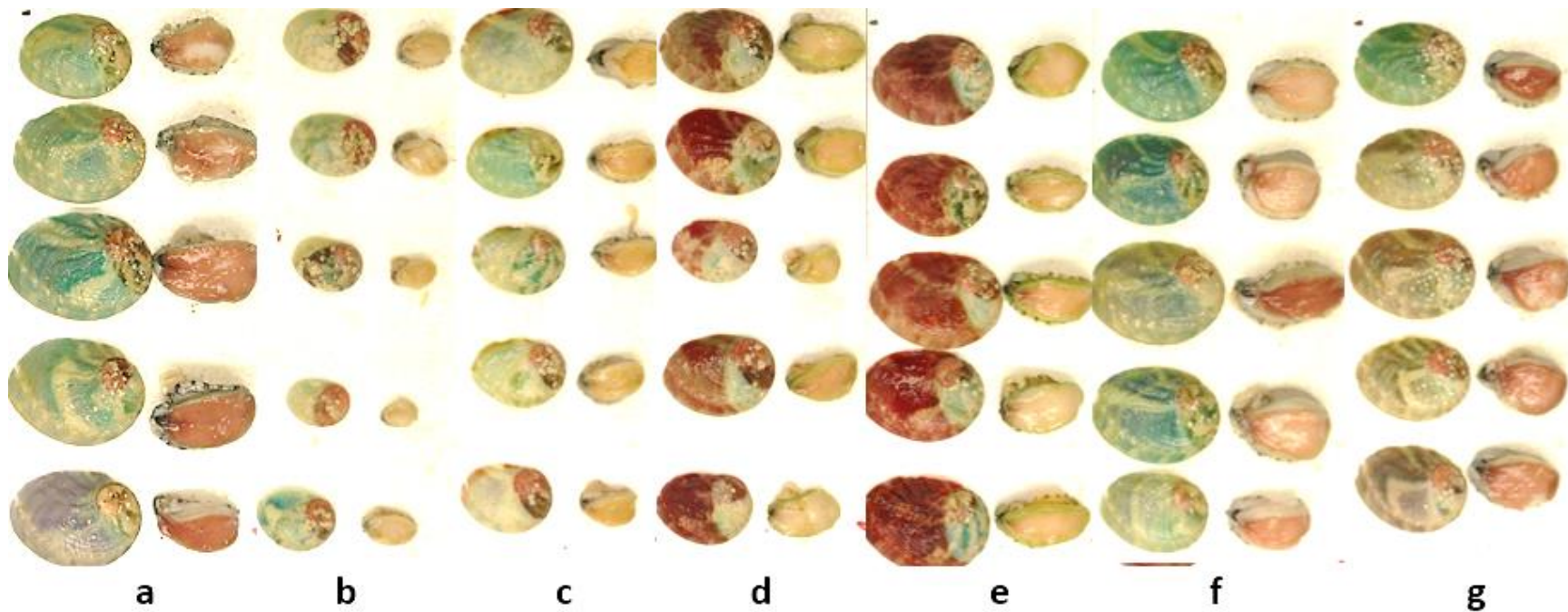


Fig. 2: Shell and foot of greenlip abalone fed (a) commercial control diet, (b) fresh non-enriched *Ulva sp.*, (c) fresh enriched *Ulva sp.*, (d) fresh non-enriched *G. cliftonii*, (e) fresh enriched *G. cliftonii*, (f) dried *Ulva sp.* and (g) dried *Spirulina sp.*

Chapter 3

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

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3.1. 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 laevis*. 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 red/brown 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 $\geq 10\%$ macroalgal meal was significantly higher than those fed the basal diet. Abalone developed a green lip when fed $\geq 10\%$ 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 $\geq 5\%$ *G. cliftonii* can produce a red/brown shell and the inclusion of $\geq 10\%$ *G. cliftonii* intensifies the green colour of the lip. The red/brown 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, food and lip colour.

Keywords: Greenlip abalone; *Gracilaria cliftonii*; *Ulva* sp.; macroalgae; pigment

3.2. 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 (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. clifftonii* 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.

3.3. Materials and methods

3.3.1. Experimental animal and system

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.

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).

3.3.2. Experimental design, stocking and feeding

Experimental design and diets

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 in the basal diet. 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.

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% of the total ingredient by weight), fish oil, sodium alginate and calcium sulphate were then added to the diet mash and mixed for further 5 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.

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.

3.3.3. Specimen sampling and analyses

The methods for image analysis were fully described in section 2.3.3 Chapter 2

The methods of pigment extraction and analysis were previously described in section 2.3.3 Chapter 2. Astaxanthin (Sigma SML0982), apart of zeaxanthin, β-cryptoxanthin, β-carotene and chlorophyll *a*, was added in this chapter and its retention time was 4.7 mins.

3.3.4. 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.

3.4. Results

Water quality parameters were maintained at levels optimal for greenlip abalone growth: water temperature 21.9 ± 0.3 °C, dissolved oxygen 94 ± 4 , 85% saturation or 6.8 ± 0.3 mg L⁻¹, pH 8.15 ± 0.05 and salinity 36 ± 1 ppt. The survival was 94.37% (Bansemer et al., 2016b).

3.4.1. Shell colour properties

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 red/brown 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; $P = 0.639$).

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; $P = 0.398$).

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; $P = 0.051$).

3.4.2. Lip colour properties

Hue

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* ($P = 0.002$).

Saturation

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* ($P = 0.611$).

Brightness

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; $P > 0.05$).

3.4.3. 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; $P > 0.05$).

3.4.4. 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 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 $\geq 10\%$ *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.

3.5. Discussion

The modification of lip, shell and foot colour by feeding macroalgae depends on both abalone species and macroalgal types (Leighton, 1961; Leighton and Boolootian, 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 Boolootian, 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 also show that the shell of abalone fed the inclusion of dried *G. cliftonii* meal turned red/brown and the higher the level of *G. cliftonii* inclusion, the more red/brown shell became. 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 $\geq 10\%$ 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 (3S)-phoenicoxanthin, 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 Perrot, 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 red/brown 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 red/brown 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 red/brown shell induced by feeding *G. cliftonii* may be an ideal characteristic for growers to establish product identity in these markets.

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. cliftonii* can be used to produce a shell colour marker in abalone for ranching or stock enhancement applications.

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

Macroalgal species	Comm ^{1,2}	<i>Ulva</i> sp.				<i>G. cliftonii</i>		
		0*	5	10	20	5	10	20
Proximate composition ¹ (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 ¹ (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 <i>a</i> (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

¹ Reported by Bansemer (2016b). ² Comm. = Commercial diet. * The control of non-macroalgae inclusion was shared by both *Ulva* sp. and *G. cliftonii* treatment. Denoted “-” as not detectable

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

Macroalgal species	<i>Ulva</i> sp. meal				<i>G. cliftonii</i> meal				ANOVA (<i>P</i> value)			
	Inclusion level (%)	0*	5	10	20	0	5	10	20	Species (A)	Inclusion level (%) (B)	A × B
Whole abalone body pigments												
β-carotene (µg 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 <i>a</i> (µg g ⁻¹)	-	-	2.31 ± 0.09	3.32 ± 0.12	-	-	-	-	< 0.001	< 0.001	< 0.001	
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 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	
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	

A 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; Post-hoc tests). For the variable with a significant interaction, differences in level of inclusion are compared within each macroalgae species using pairwise comparisons Denoted “-“ as not detectable. * The control of non-macroalgae inclusion was shared by both *Ulva* sp. and *G. cliftonii* treatments.

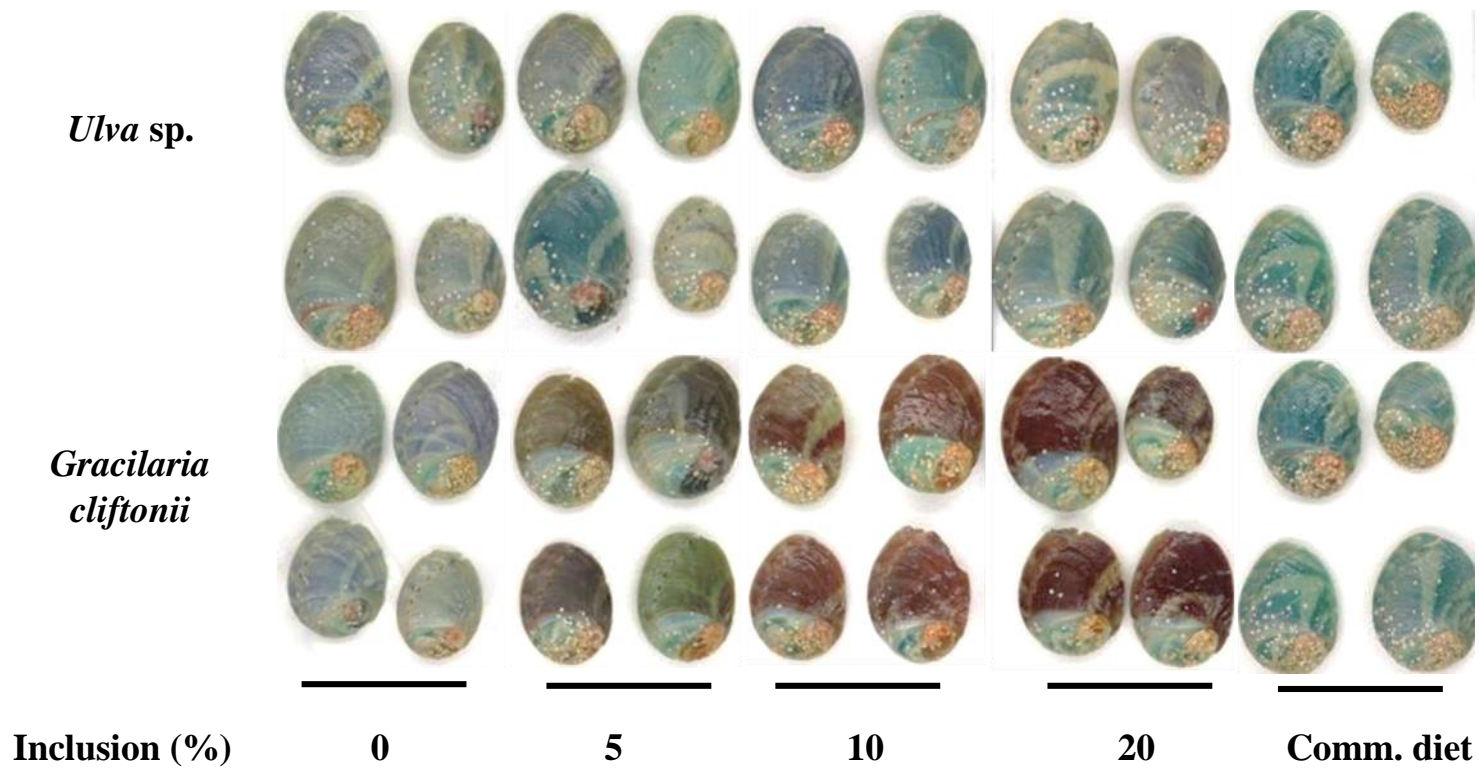


Fig.1 Shell colour of greenlip abalone, *Haliotis laevis* fed graded levels of macroalgae inclusion and the commercial diet from left to right with two columns of shells per treatment.

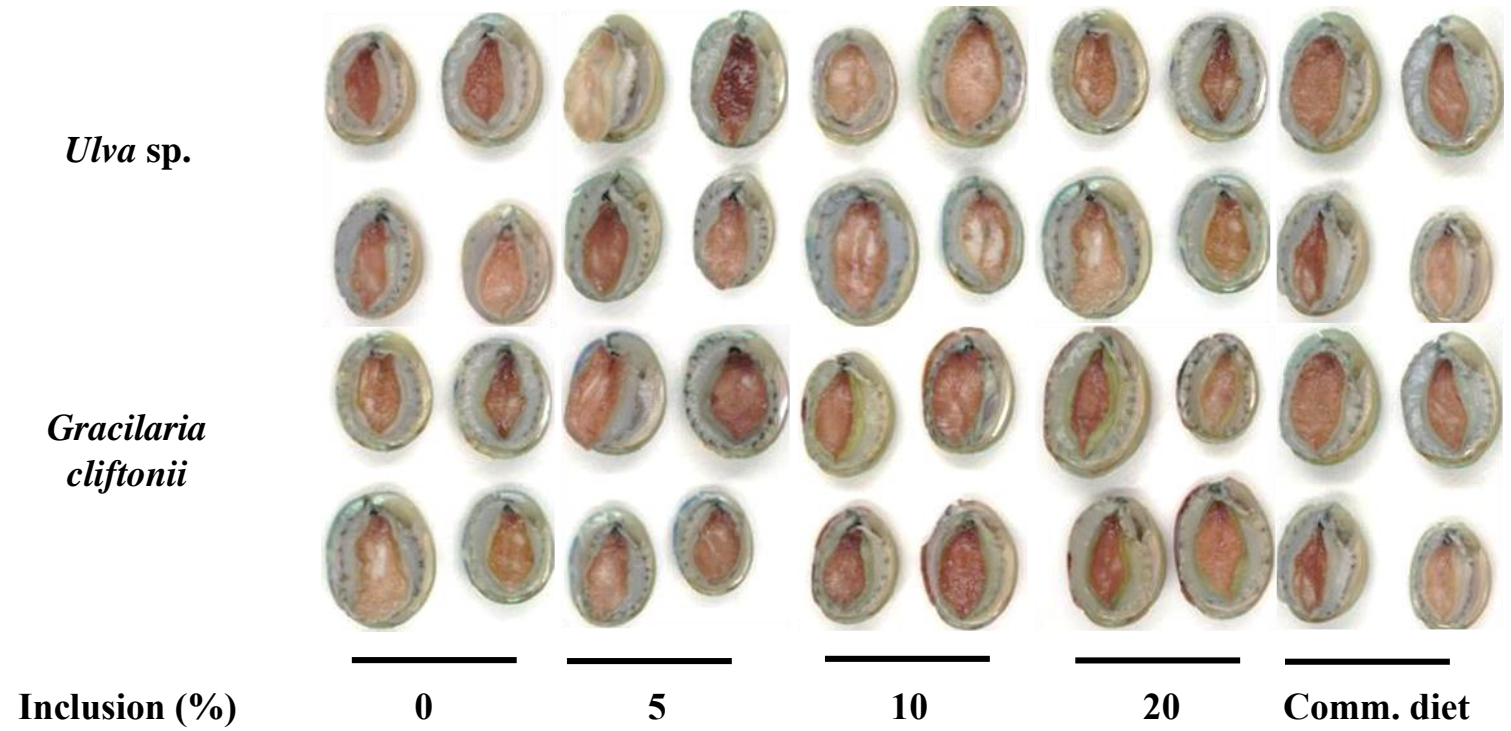


Fig.2 Foot colour of greenlip abalone, *Haliotis laevigata* fed graded levels of macroalgae inclusion and the commercial diet from left to right with two columns of shells per treatment.

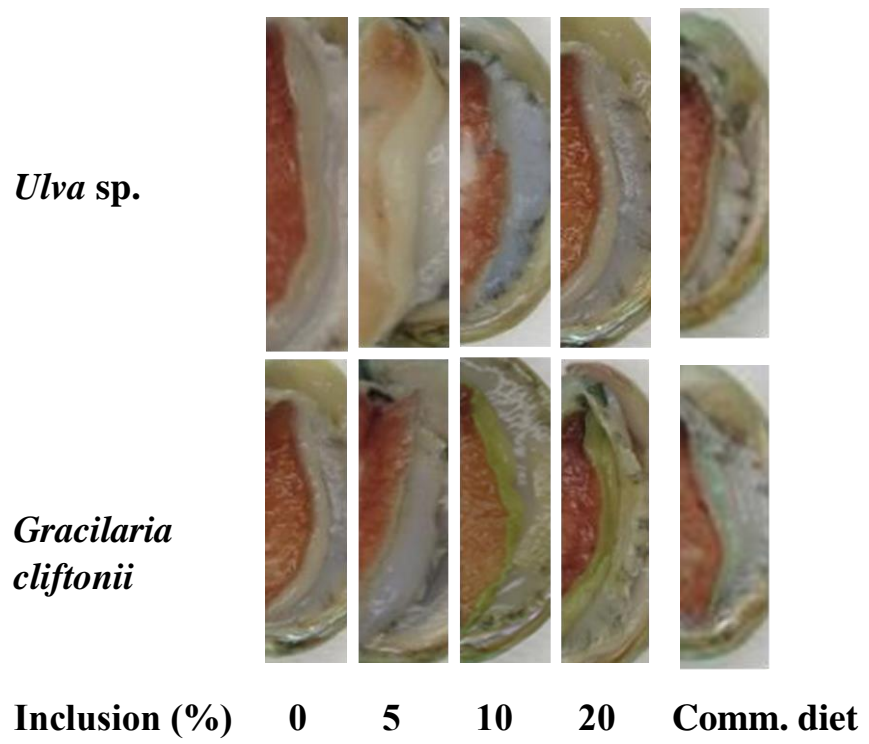


Fig.3 Lip colour of greenlip abalone, *Haliotis laevigata* fed graded levels of macroalgae inclusion and the commercial diet.

Chapter 4

Effects of diet and water temperature on colour of greenlip abalone

(*Haliotis laevis* Donovan)

Submitted as

Hoang, T.H., Qin, J.G., Harris, J.O., Duong, D.N., Stone, D.A., 2016. The effects of diet and water temperature on the pigment deposition in whole tissue and colour of greenlip abalone (*Haliotis laevis* Donovan). Submitted to Aquaculture.

4.1. Abstract

In Australia, commercially farmed greenlip abalone *Haliotis laevis*, display a faint lip colour in comparison to wild abalone. As diet and environmental conditions can influence the colour of aquatic animals, the effects of diet and water temperature on the colour of shell, foot and lip pigment deposition in greenlip abalone was evaluated. Three experimental diets, enriched fresh *Ulva* sp., a commercial diet and a feed containing 30% of *Ulva* sp. in the commercial diet were used to feed 3-year-old greenlip abalone (26.8 g, 57.9 mm) at either 22 or 26 °C for 38 days. The shell colour saturation of abalone fed 30% enriched dried *Ulva* sp. meal was similar to those fed the commercial diet, but was significantly enhanced in comparison with those fed enriched fresh *Ulva* sp. The shell colour was brighter at 22 °C than at 26 °C. The lip colour saturation was highest in abalone fed enriched fresh *Ulva* sp. and was significantly higher than those fed 30% enriched dried *Ulva* sp. meal. Abalone cultured at 22 °C had significantly higher lip colour saturation than those at 26 °C. The foot of abalone fed enriched fresh *Ulva* sp. was light golden, whereas abalone fed 30% *Ulva* sp. meal or the commercial diet had a brown foot. Foot brightness was similar between abalone fed the commercial diet and the enriched fresh *Ulva* sp., but was significantly higher than those fed the diet containing 30% enriched dried *Ulva* sp. Abalone had a darker brown foot at 22 °C compared to those at 26 °C. The content of β -carotene was significantly higher in abalone fed enriched fresh *Ulva* sp., than those fed the other diets. Abalone had a significantly higher amount of β -carotene at 22 °C compared to those at 26 °C. This study indicates that both water temperature and diet can affect foot colour and the β -carotene content in the whole abalone tissue.

4.2. Introduction

Greenlip abalone *Haliotis laevis* is one of the main species for commercial aquaculture in Australia (Stone et al., 2013). Previous studies have shown that greenlip

abalone lose their natural colour under artificial culture conditions (Hoang et al., 2016; 2017). Wild greenlip abalone consuming macroalgae typically have a green lip, yellow foot and a variety of shell colours, whereas cultured abalone fed the commercial formulated diets exhibited a milky lip, brown foot and light green shell (Hoang et al., 2016; 2017). The advantage for using formulated diets in abalone farming is faster animal growth rates, however differences in pigments between wild algal diets and formulated feeds may result in colour loss of farmed abalone (Bautista-Teruel and Millamena, 1999; Ju et al., 2015). The altered colour may influence market acceptability as greenlip abalone with a milky lip and darker foot fetch a lower price (Freeman, 2001; Hoang et al., 2017). Feeding certain fresh macroalgae or a supplementation of dried macroalgae meals in formulated diets are ways to provide both nutrients and pigments to grow better and healthier abalone (Bautista-Teruel and Millamena, 1999; Lim and Lee, 2003; Qi et al., 2010; Ju et al., 2015; Bansemer et al., 2016; Hoang et al., 2016, 2017). In land-based culture systems, *H. laevigata* fed formulated diets normally need up to years to reach market size, and it is essential to maintain the natural colour or a desirable colour to meet consumers' requirements.

Among potential pathways of colour development in molluscs such as: indoles (melanins and indigoids), tetrapyrroles (porphyrins and bilichromes), the colour of abalone is attributed by pigments/carotenoids in diets and particularly in macroalgae (Olsen, 1968; Lim and Lee, 2003; Liu et al., 2009; Qi et al., 2010; Ju et al., 2015; Hoang et al., 2016, 2017). Green macroalgae such as *Ulva* sp., a secondary dietary preference of wild greenlip abalone, contains a variety of pigments including chlorophyll, β -carotene, lutein, violoxanthin, neoxanthin and zeaxanthin that may change the colour of abalone tissue (Chandini et al., 2008; El-Baky et al., 2008). Recently, Hoang et al. (2016) found that 1-year-old greenlip abalone fed fresh *Ulva* sp. produced a light yellow foot colour, similar to wild abalone. However, an inclusion of 20% enriched dried *Ulva* sp. meal in the formulated diet did not

affect the colour of 1-year-old greenlip abalone (Hoang et al., 2017). A possible reason for this is that the inclusion of 20% enriched dried *Ulva* sp. meal may have been too low to induce colour change. Therefore, inclusion of 30% *Ulva* sp. in formulated diets is worthy of further investigation, which may provide similar amounts of major pigments contents found in a diet of sole fresh *Ulva* sp.

Water temperature is an important environmental factor, not only governing physiological activities but also affecting the colour of animals (Olsen and Mortensen, 1997; Gines et al., 2004; Harries et al., 2005; Stone et al., 2013; Morash and Alter, 2015). For example, the flesh of Arctic charr *Salvelinus alpinus*, fed a commercial diet at 10 °C, had a more intense orange colouration than those fed at 15 °C (Gines et al., 2004). Arctic charr fed six diets ranging from 0 - 192 mg kg⁻¹ astaxanthin, at 8 °C, had significantly more pigmentation compared to those at 12 °C (Olsen and Mortensen, 1997). According to Storebakken et al. (1986), the extent of pigmentation varied according to geographical location in yearlings of Atlantic salmon *Salmo salar*, rainbow trout *Salmo gairdneri* and sea trout *Salmo trutta* due to temperature differences between regions. Lin et al. (2009) also reported that skin colour change in 10-day-old juvenile seahorses *Hippocampus erectus* differed significantly among three different temperatures, 23, 26 and 29 °C. In abalone, the influence of water temperature on growth, survival, food intake, metabolism, and reproduction are well documented (Moss, 1998; Grubert and Ritar, 2004; Harris et al., 2005; Stone et al., 2013, 2014; Lange et al., 2014; Bansemmer et al., 2015), but there are no published reports of the effect of temperature on abalone colour. Thus, it is possible that temperature will effect abalone colour as it governs dietary pigment intake, absorption and digestion.

Most studies on abalone colour have focused on feeding abalone with various pigment sources such as fresh macroalgae, synthetic pigments or algae pigments in formulated diets

(Olsen, 1968; ; Horiguchi et al., 1987; Lim and Lee, 2003; Liu et al., 2009; Canales-Gómez et al., 2010; Qi et al., 2010; Ju et al., 2015; Hoang et al., 2016, 2017). However, little is known about environment effects such as water temperature on pigment deposition in abalone. The production of Australian greenlip abalone occurs predominantly in land-based systems, where animals experience large seasonal fluctuations in water temperature and may suffer high mortality during high summer water temperature (Stone et al., 2013). It is further hypothesised that feeding enriched fresh *Ulva* sp. or an inclusion of 30% enriched dried *Ulva* sp. in the diet, will not only improve the survival rate of 3-year-old greenlip abalone at high temperature (Lange et al., 2014; Stone et al., 2014), but also assist in retaining their natural colour.

The aim of this study was to evaluate the effects of diet and water temperature on the colour change of abalone lip, foot and shell and pigment deposition in whole abalone tissue at 22 °C (a preferable growth temperature) in comparison to 26 °C (a suboptimal water temperature).

4.3. Materials and methods

4.3.1. Experimental animal and system

Three-year-old greenlip abalone were kept at the South Australian Research and Development Institute (SARDI) Aquatic Sciences Centre in holding tanks with a flow-through, UV-treated, seawater system. The animals were fed a commercial Abgrow premium abalone food (Eyre Peninsula Aquafeed Pty Ltd (EPA), Lonsdale, SA, Australia) *ad libitum* daily prior to the experiment and had not been used previously for any other feeding experiments.

The experimental system used is previously described in Stone et al. (2013). Briefly, the system was comprised of blue plastic culture tanks (Nally IH305, Viscount Plastics Pty

Ltd; 39.2 × 28.8 × 11.0 cm). Water level was set at 5 cm deep using a standpipe providing a water volume of 5.4 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 low intensity with fluorescent lighting at 3.4 lx and 12 h dark. Air temperature was maintained at 20 ± 1 °C. Water temperature was controlled at 22 or 26 °C using 3 kW immersion heaters (240V, JQ20, Austin and Cridland, Carlton, NSW, Australia). The experiment lasted 38 days and abalone were fed respective diets.

4.3.2. Experimental design, stocking and feeding

Experimental design and diets

Three experimental diets including a control diet (the commercial diet - EPA Abgrow premium diet), fresh enriched *Ulva* sp. and a formulated diet with 30% enriched dried *Ulva* sp. meal were used to feed 3-year-old abalone (26.8 g, 57.9 mm shell length) at 22 or 26 °C. *Ulva* sp. was collected from Gulf St. Vincent, SA, Australia, and cultured in a 4000-L tank under ambient sunlight. *Ulva* sp. was enriched with 8 L of modified F2 nutrient media in seawater every 15 days (Lange et al., 2014).

To produce enriched *Ulva* sp. meal, fresh enriched *Ulva* sp. was sun-dried for about 8 h and then oven-dried at 60 °C for 48 h. Enriched dried *Ulva* sp. 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.

To produce a diet of 30% enriched dried *Ulva* sp., 30% of the EPA diet mash from the commercial diet was removed and replaced with enriched dried *Ulva* sp. meal. The inclusion level was calculated to meet the same amount of fresh *Ulva* sp. that would be consumed daily by 3-year-old abalone (Stone et al. 2014). 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 3 min. The diets were made using a TR110 pasta machine (Machine Per Pasta SRL, Molina Di Malo, VI, Italy) into 5 mm flat sinking pellets. Diets were then oven-dried at 45 °C for 48 h and frozen at -20 °C. The proximate and pigment compositions of experimental diets are presented in Table 1.

Stocking and feeding

Greenlip abalone (26.8 ± 14.7 g; shell length 57.9 ± 11.6 mm) were screened from larger populations, weighed, measured, and randomly stocked (12 abalone tank⁻¹) into each of the 18 tanks; three replicate tanks were allocated to each treatment. The water temperature was slowly adjusted from ambient (23 °C) to the required treatment water temperatures (22 and 26 °C) over a period of seven days. Tank water temperature was then maintained within ± 1.5 °C of the treatment temperatures until the end of the experiment.

The feeding rate was 1.0 - 1.2% bw day⁻¹ for the experimental formulated diets. For the fresh enriched *Ulva* sp. treatment, fresh *Ulva* sp. was harvested daily and abalone were fed to excess at a rate of 1.5% body weight day⁻¹ for the first week and this was then increased to 2.5% body weight day⁻¹ for the remainder of the experiment.

Abalone were fed daily at 16:00 h after a day of stocking. 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 μ m). The uneaten feed was then 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 correction of 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 μ m), and dried until a constant weight was achieved. To maintain stocking density, dead abalone were replaced with similar size abalone that were fed with the same diet as the dead abalone.

Feeding rates were adjusted based on the biomass changes arising from mortalities and replacements.

4.3.3. Specimen sampling and analyses

The methods for image analysis were fully described in section 2.3.3 Chapter 2

The methods of pigment extraction and analysis were previously described in section 2.3.3 Chapter 2. Astaxanthin (Sigma SML0982) was added and measured in this chapter and its retention time was 4.7 mins.

4.3.4. Statistical analysis

The data was analysed using SPSS (version 22) with the significance level set at $P < 0.05$. Two-way ANOVA was used to determine the interactive effects between dietary type (a commercial formulated diet, the commercial diet containing 30% of *Ulva* meal and enriched fresh *Ulva* sp.) and water temperature (22 and 26 °C) on colour components (hue, saturation and brightness) and pigment contents of abalone. When no significant interaction between dietary type and water temperature was observed, Tukey's HSD post-hoc test was used to detect significant differences between treatment means. In contrast, differences in dietary type are compared within 22 or 26 °C (Tukey's HSD test) when significant interaction between those two factors was observed.

4.4. Results

Water quality parameters were maintained at 21.9 ± 0.1 °C and 25.4 ± 0.6 °C, dissolved oxygen $82.5 \pm 0.19\%$ saturation (5.87 ± 0.2 mg L⁻¹) to $90.7 \pm 1.81\%$ saturation (6.49 ± 0.12 mg L⁻¹) at 22 °C, $83.2 \pm 0.41\%$ saturation (5.62 ± 0.05 mg L⁻¹) to $89.5 \pm 1.50\%$ saturation (6.04 ± 0.10 mg L⁻¹) at 26 °C, pH 8.11 ± 0.01 - 8.18 ± 0.01 at 22 °C or 8.15 ± 0.01 - 8.21 ± 0.01 at 26 °C, and salinity 36 ± 1 ppt. The abalone survival was 100% in all treatments at 22

°C, but it was only 47% in the commercial diet treatment, 78% in the diet of 30% dried *Ulva* sp. and 98% in the live *Ulva* sp. diet at 26 °C (Lange et al., 2014).

4.4.1. Shell colour properties

Dietary type (two-factor ANOVA; $P = 0.625$; Table 2; Fig. 1), water temperature ($P = 0.612$) and the interaction between these two factors ($P = 0.451$) did not significantly affect shell hue. Shell hue values ranged from 54.84 ± 2.04 to 60.35 ± 3.03 degree.

Shell saturation was only significantly influenced by the dietary type (two-factor ANOVA; $P = 0.020$; Table 2; Fig. 1). Abalone fed the commercial diet had significantly higher shell saturation than those fed enriched fresh *Ulva* sp. ($P = 0.011$), but not from those fed 30% dried enriched *Ulva* sp. meal ($P = 0.243$).

Shell brightness was only significantly influenced by water temperature (two-factor ANOVA; $P = 0.007$; Table 2; Fig. 1). Shell brightness was significantly lower at 26 °C than at 22 °C ($P = 0.007$).

4.4.2. Lip colour properties

Among lip colour components, only lip colour saturation was significantly affected by dietary type (two-factor ANOVA; $P = 0.045$; Table 2; Fig. 2) and water temperature ($P = 0.001$). Abalone fed enriched fresh *Ulva* sp. had significantly higher lip colour saturation than those fed 30% of enriched dried *Ulva* sp. meal ($P = 0.035$), but not from those fed the commercial diet ($P = 0.738$). Saturation was similar between abalone fed the commercial diet and 30% enriched dried *Ulva* sp. meal ($P = 0.218$). Abalone had significantly lower lip colour saturation at 26 °C compared to those at 22 °C ($P = 0.001$).

4.4.3. Foot colour properties

Foot hue was significantly influenced by dietary type ($P < 0.001$; two-factor ANOVA; Table 2; Fig. 2) and water temperature ($P < 0.001$), but not by their interaction ($P = 0.513$).

Abalone fed enriched fresh *Ulva* sp. had a light golden foot, while the foot was brown in abalone fed the commercial diet or 30% enriched dried *Ulva* sp. meal. The foot hue was a darker brown at 22 °C than at 26 °C.

There was significant effect from water temperature on foot colour saturation ($P = 0.02$; two-factor ANOVA; Table 2). However, dietary type ($P = 0.246$) and the interaction between these two factors ($P = 0.051$) had no significant effect on foot saturation. Abalone showed a significantly reduced foot colour saturation at 26 °C compared to those at 22 °C ($P = 0.002$).

Foot colour brightness was only significantly impacted by dietary type ($P = 0.001$; two-factor ANOVA; Table 2; Fig. 2). Abalone fed 30% enriched dried *Ulva* sp. had significantly lower foot brightness than those fed the commercial diet ($P = 0.012$) and enriched fresh *Ulva* sp. ($P < 0.001$). Foot brightness value was similar between abalone fed the commercial diet and enriched fresh *Ulva* sp. ($P = 0.318$).

4.4.4. Pigment contents in diets and abalone tissue

The pigment contents of the tissue are presented in Table 1. The content of β -carotene in the whole tissue of abalone was significantly affected by both dietary type ($P < 0.001$) and water temperature ($P < 0.01$). Abalone fed enriched fresh *Ulva* sp. had a significantly higher amount of β -carotene than those fed the commercial diet ($P < 0.001$) and 30% enriched dried *Ulva* sp. inclusion ($P < 0.001$).

4.5. Discussion

This study demonstrates that the foot hue of 3-year-old greenlip abalone was affected by both diet and water temperature, while foot saturation was influenced by temperature and foot brightness was impacted by diets only. Abalone fed fresh *Ulva* sp. had a light gold foot, while the foot was dark brown in abalone fed the commercial diet or the diet containing 30% enriched dried *Ulva* sp. meal. The foot of abalone was paler at 26 °C than at 22 °C. Foot

colour of some abalone species is one of the most important characteristics in determining abalone quality, price and attractiveness at market. Abalone with a light colour foot generally fetch a better price, while more pre-market processing is required to sell abalone with a darker foot (Oakes and Ponte, 1996; Freeman, 2001; Allen et al, 2006; Brown et al., 2008). For example, Allen et al. (2006) suggested to exclude algal stimulants from the diet of blackfoot abalone *Haliotis iris* for a period prior to sale due to producing darker foot. Although diet and water temperature had some effects on foot colour, neither had any impact on the green pigmentation of the lip, which is more important marketing trait than the colour of foot or shell in greenlip abalone in the current study.

The green pigment of lip is more relevant to the market than colour of other body parts in greenlip abalone (Bansemer, et al., 2014; Hoang et al., 2017). However, the 3-year-old greenlip abalone exhibited milky lip colour across all treatments, which was similar to results from our previous study where the lip colour of 1-year-old greenlip abalone fed enriched dried *Ulva* sp. meal and formulated diet was also milky (Hoang et al., 2017). The previous study shows that abalone developed a green lip when fed $\geq 10\%$ of red macroalgae *Glacilaria cliftonii* meal, which is similar to the colour of wild abalone (Hoang et al., 2017). The green pigment of the lip is presumably associated with red macroalgae pigments (e.g., carotenoids) or melanin pigments, which are bio-accumulated from dietary sources or endogenous biosynthesised by abalone from algae precursors (Hoang et al., 2017). Since the green pigmentation of the lip is an important factor to assess product quality in greenlip abalone, further research should investigate the role of red macroalgae pigments on lip colour of 3-year-old greenlip abalone as it closes to market size and lip colour at harvest is important to sale price.

In the present study, foot colour was dependent on diet type. The abalone fed fresh *Ulva* sp. had a light gold foot at 22 °C, which was slightly different from the light yellow foot

of 1-year-old abalone fed the same diet in our previous study (Hoang et al., 2016). The difference in foot colour between studies may have been due to size and age of the abalone because the same fresh *Ulva* sp. were used to feed greenlip abalone at the same temperature (22 °C) and under the same environmental conditions (Hoang et al., 2016).

In the current study, the 3-year-old greenlip abalone exhibited a brown foot when fed the diet containing 30% enriched dried *Ulva* sp. meal or the commercial diet at 22 °C, whereas the foot was light gold in abalone fed enriched fresh *Ulva* sp. Dietary pigment analysis showed that the contents of β -carotene, astaxanthin, β -cryptoxanthin, zeaxanthin and chlorophyll *a* in the enriched fresh *Ulva* sp. diet was higher than in the diet containing 30% enriched dried *Ulva* sp. It is likely that the pigments in the 30% enriched dried *Ulva* sp. diet was lost due to processing and therefore resulted in weak influence on foot colour. Similarly, Choubert and Heinrich (1993) reported a degradation of green alga *Haematococcus pluvialis* carotenoids during the pelleting process (-3.3%) even at a low temperature (42 °C), as well as during the 15-day storage at 7 °C ambient temperature (-5.2%).

Water temperature, a part of feeding treatment, significantly affected the abalone foot hue. At 22 °C, abalone had lower foot hue than those at 26 °C in all treatments. The foot colour of greenlip abalone at the high water temperature was paler. Studies on the effects of water temperature on colour or pigment deposition in abalone are rare. However, water temperature may have a large influence on the intake of dietary pigments, due to its effects on feed intake (Stone et al., 2013, 2014; Lange et al., 2014). Generally, within the optimal temperature range, feed intake of greenlip abalone increases with an increase in water temperature. Nevertheless, it declines when water temperature is beyond this range as abalone may be under thermal stress (Stone et al., 2013, 2014; Lange et al., 2014; Bansemer et al., 2015). According to Lange et al. (2014) and Stone et al. (2014), the feed intake of abalone at 22 °C significantly increases, whereas it is suppressed at 26 °C.

High water temperature can also decrease digestion in aquatic animals, related to a reduction in residence time of food in the gut (Ytrestøyl et al., 2005). In greenlip abalone, gastrointestinal evacuation time has been shown to decrease with increasing water temperatures from 14 to 26 °C in 2-year-old (6.7 g) and 3-year-old (25.7 g) animals (Currie et al., 2015). The reduction of food residence time leaves less time for absorption, whereas a longer residence time may increase nutrient digestibility and absorption due to low food passing time (Ytrestøyl et al., 2005; Bansemer et al., 2016). In addition, most carotenoids are lipophilic molecules and their absorption from the gut is a relatively slow process compared to that of other essential nutrients (Ytrestøyl et al., 2005). In the present study, the content of β -carotene in abalone tissues was significantly lower at 26 °C than at 22 °C. Therefore, it is possible that high water temperature had a negative effect not only on pigment intake, but also on digestibility and absorption of carotenoids. However, the metabolism of carotenoids and other pigments at different water temperature needs further investigation to understand the pigment pathways from the diet to animal tissues.

Changes in body colour hue or pattern are crucial adaptations of aquatic animals to their environments (Yasir and Qin, 2009). According to Miura et al. (2007), temperature is likely to be the most significant factor causing shell colour change in the intertidal gastropod *Batillaria* sp. The dark shell colour was an adaptation to cold while light shell adapted to hot temperature. However, shell colour of greenlip abalone was not influenced by water temperature. It is possible that no difference in shell colour of abalone cultured at 22 and 26 °C due to short experimental period.

Colour change is subject to not only variation of pigment quantity, but also due to hormone regulation relevant to environmental stress (Van der Salm et al., 2004). In the present study, greenlip abalone were chronically exposed to an elevated water temperature (26 °C) for 38 days during the experimental period. Therefore, it is possible that the

difference in foot colour of greenlip abalone between 22 and 26 °C is a stress response to high water temperature.

In conclusion, water temperature and diets have a tangible impact on foot colour and the amount of β -carotene in the whole tissue of 3-year-old greenlip abalone, but had little influence on shell and lip colour. Foot colour was lighter and the β -carotene content in the whole tissue was lower at the higher water temperature. Three-year-old greenlip abalone fed 30% enriched dried *Ulva* sp. or the commercial diet exhibited brown foot colouration which may potentially affect product quality and consumer choice, whereas abalone fed enriched fresh *Ulva* sp. had a light golden foot colour, similar to wild greenlip abalone.

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Table 1. Ingredients and biochemical composition of experimental diets fed to greenlip abalone

Diets	Commercial diet	Enriched fresh <i>Ulva</i> sp.	30% enriched dried <i>Ulva</i> sp. meal
Ingredients¹			
<i>(g kg⁻¹ diet as fed)</i>			
Commercial diet mash	983.0	na	683.0
Enriched dried <i>Ulva</i> sp.	0.0	na	300.0
Vitamin-mineral premix	2.0	na	2.0
Salmon oil (mL kg ⁻¹)	15.0	na	15.0
Fresh <i>Ulva</i> sp.	0.0	1000.0	0.0
Sum	1000.0	1000.0	1000.0
Proximate composition¹			
<i>(g kg⁻¹ diet as fed)</i>			
Moisture	100.0	780.0	100.0
Crude protein	306.0	59.8	295.7
Lipid	45.0	9.3	44.1
Ash	62.0	36.7	93.4
Carbohydrate	518.7	42.9	421.7
NFE	587.0	114.3	566.7
Gross energy (MJ kg ⁻¹)	17.9	3.7	16.0
Diet pigments			
Astaxanthin (µg g ⁻¹)	-	5.50	1.50
β-carotene (µg g ⁻¹)	0.13	3.50	1.35
Chlorophyll <i>a</i> (µg g ⁻¹)	-	4.26	2.17
β-cryptoxanthin (µg g ⁻¹)	-	1.63	-
Zeaxanthin (µg g ⁻¹)	0.05	1.07	0.51

¹ Reported by Lange et al. (2014). “-” denotes as not detectable (Chlorophyll *a* content less than 0.001 µg g⁻¹ and carotenoids less than 0.0003 µg g⁻¹). “na” as variables not analysed.

Table 2. Pigment contents in greenlip abalone tissue, *Haliotis laevis* fed graded levels of macroalgae inclusion and colour components of shell, foot and lip of abalone fed those diets.¹

Diets	22 °C			26 °C			ANOVA (<i>P</i> value)		
	Comm. (A)	Fresh <i>Ulva</i> sp. (B)	30% dried <i>Ulva</i> sp. (C)	Comm. ² (A)	Fresh <i>Ulva</i> sp. (B)	30% dried <i>Ulva</i> sp. (C)	Diet A B C	Temp. (°C)	Interaction
The content of β-carotene in whole tissue (µg g ⁻¹)	1.43 ± 0.09	6.37 ± 0.24	3.71 ± 0.06	0.27 ± 0.05	2.63 ± 0.12	1.22 ± 0.06	< 0.001 X Z Y	< 0.001 22 °C > 26 °C	0.476
Colour components of shell									
Hue (degree)	59.96 ± 3.59	58.63 ± 2.40	57.15 ± 2.65	54.84 ± 2.04	60.35 ± 3.03	57.16 ± 1.26	0.625	0.612	0.451
Saturation (%)	55.23 ± 1.91	47.30 ± 2.31	53.19 ± 2.03	53.25 ± 2.72	45.51 ± 2.92	46.98 ± 3.49	0.020 Y X XY	0.131	0.632
Brightness (%)	48.62 ± 2.62	50.03 ± 1.01	49.43 ± 1.52	49.45 ± 1.22	43.95 ± 2.49	39.16 ± 2.86	0.122	0.007 22 °C > 26 °C	0.061
Colour components of foot									
Hue (degree)	23.19 ± 1.21	30.95 ± 1.23	20.64 ± 1.45	30.41 ± 2.23	36.77 ± 0.79	29.80 ± 2.00	< 0.001 X Y X	< 0.001 22 °C < 26 °C	0.513
Saturation (%)	66.79 ± 1.62	66.79 ± 0.94	68.98 ± 1.00	66.99 ± 1.47	60.72 ± 2.39	59.06 ± 3.04	0.246	0.002 22 °C > 26 °C	0.051
Brightness (%)	52.92 ± 2.55	60.71 ± 1.54	48.23 ± 2.01	58.93 ± 1.27	56.86 ± 3.98	49.00 ± 3.62	0.001 Y Y X	0.668	0.228
Colour components of lip									
Hue (degree)	43.86 ± 0.94	46.37 ± 0.67	44.99 ± 1.21	45.75 ± 0.45	45.43 ± 0.47	45.87 ± 0.81	0.449	0.382	0.251
Saturation (%)	53.20 ± 1.91	54.06 ± 2.05	51.08 ± 1.78	45.70 ± 2.61	49.93 ± 2.66	41.18 ± 2.79	0.045 XY Y X	0.001 22 °C > 26 °C	0.458
Brightness (%)	56.40 ± 1.50	59.93 ± 0.87	54.68 ± 1.70	58.30 ± 2.10	54.86 ± 3.10	51.99 ± 2.76	0.103	0.279	0.299

¹ Data is presented as mean ± SE, n = 3, ² indicated n = 2. A 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; Tukey's HSD test; *P* < 0.05). X, Y, Z: for variables with a significant effect of diet (X indicates the lowest value; *P* < 0.05).

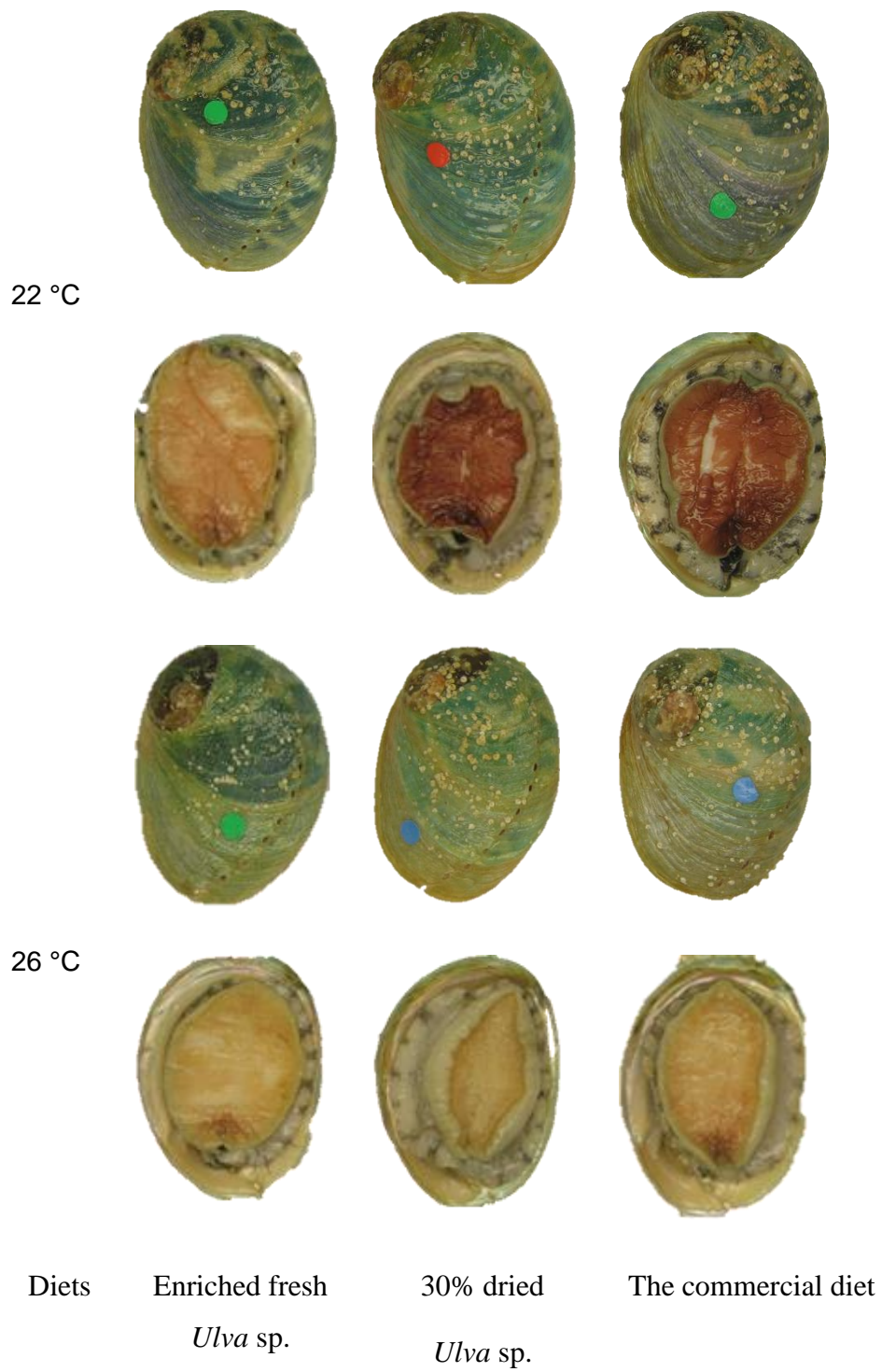


Fig. 1. Shell and foot colour of greenlip abalone (*Haliotis laevis*) fed different diets at 22 or 26 °C

Chapter 5

The limited impact of dietary supplementation of synthetic astaxanthin and tyrosine on the colour of greenlip abalone (*Haliotis laevis* Donovan)

In preparation as

Hoang, T.H., Qin, J.G., Harris, J.O., Duong, D.N., Stone, D.A., 2016. Dietary supplementation of synthetic astaxanthin and tyrosine had limited impact on the colour of greenlip abalone (*Haliotis laevis* Donovan). In preparation.

5.1. Abstract

Greenlip abalone, in land-based farming systems, often lose their natural lip colour and may become less attractive to consumers at market. It is proposed that abalone colour is related to carotenoids in the diet and melanin production. This study evaluates the effect of dietary supplementation of synthetic astaxanthin and L-tyrosine on the colour of greenlip abalone. One-year-old greenlip abalone (shell length 27.27 ± 0.05 mm, weight 2.47 ± 0.01 g, $n = 280$) were fed a control diet (0% pigment addition), or three levels of astaxanthin (0.05, 0.1 and 0.2 g kg^{-1}), or three levels of tyrosine (10, 15 and 20 g kg^{-1}) in formulated control diets. The experiment was conducted in tanks with four replicates of each treatment, over 72 days. Supplementation of synthetic astaxanthin in the diet did not significantly influence the colour components (hue, saturation and brightness) of abalone body parts or the pigment content in the whole abalone tissue, compared to the control diet. Similarly, no significant differences were found in the shell, foot and lip colour or in the tissue pigment content of abalone fed the three treatments of tyrosine. The melanin content in the lip was independent of dietary tyrosine inclusion level. Greenlip abalone exhibited a light green shell, brown foot and milky lip in all treatments. This study indicates that greenlip abalone had little capacity to absorb and metabolise synthetic astaxanthin or tyrosine to change body colour, and supplementation of these products in the diet is not recommended for farming.

5.2. Introduction

Abalone are important for commercial and recreational fisheries, inhabiting shallow, subtidal rocky waters, in tropical and temperate regions (Gordon and Cook, 2013). In the past few decades, due to declines in catch and an increase in global demand, abalone aquaculture in land-based systems has rapidly developed (Gordon and Cook, 2004, 2013). The production of farmed abalone has increased from 50 mt in 1970 to 103,464 mt in 2013 (Cook, 2014). Abalone farming is currently challenged by global warming (Lange et al., 2014; Stone et al.,

2014; Duong et al., 2016), nutrition supply (Stone et al., 2013; Bansemer et al., 2015) and disease (Nicolas et al., 2002; Hooper et al., 2007). More recently, the effect of colour fading in cultured abalone fed formulated diets, has become a concern, as abalone colour can affect market acceptability (Qi et al., 2010; Ju et al., 2015; Hoang et al., 2016a,b; 2017).

In Australia, greenlip abalone *Haliotis laevis* are mainly cultured in land-based systems (Stone et al., 2013). Recent studies show that the colour of abalone fed a formulated diet differs from their wild counterparts (Hoang et al., 2016a; 2017). Specifically, abalone fed a formulated diet display a milky lip, light green shell and brown foot, whereas wild greenlip abalone have a distinguished green lip, light yellow foot and a variety of shell colours (Mottet, 1978; Department of Fisheries, 2011; Mayfield et al., 2014). The colour difference between wild and farmed abalone is either attributed to the range and concentration of pigments in the natural algae diet; or compounds that are being biosynthesized by the abalone themselves, most likely from precursors obtained from the natural diet (Hoang et al., 2016a; 2017).

Carotenoids are produced in a variety of algae including Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyta (brown algae). The colour of wild abalone is derived mainly from dietary algal sources, as they are unable to synthesise carotenoids *de novo* (Shahidi and Brown, 1998; Maoka et al., 2010; Maoka, 2011). To overcome the colour difference between farmed and wild abalone, supplementation of pigments that would be found in the natural diet is recommended to achieve natural, desirable coloration (Horiguchi et al., 1987; Lim and Lee, 2003; Canales-Gómez et al., 2010; Hoang et al., 2016a). Some studies have reported that natural sources such as micro- and macroalgae are effective for enhancing the natural colour or manipulating pigments in a farming condition (Lim and Lee, 2003; Hoang et al., 2017). For example, the shell of Pacific abalone *Haliotis discus hannai* fed a diet containing 2% *Porphyra*, became yellow-red and orange, which is similar to the

shell colour of the wild population (Lim and Lee, 2003). Similarly, the shell colour of greenlip abalone was also changed when they were fed 3% dried *Spirulina* sp., to yellow-brown and the shell and lip of greenlip abalone fed *Gracilaria cliftonii* meal changed to red/brown and green, respectively (Hoang et al., 2016a; 2017). Despite the promising results of using ingredients containing natural pigment supplementation to improve abalone colour (Horiguchi et al., 1987; Lim and Lee, 2003; Hoang et al., 2016a; 2017), the availability and high cost has hindered the chance of widespread use in the aquaculture industry, compared to the synthetic forms. The use of commercialised, synthetic products to enhance colour has been well documented in various species, including fish and shrimp (Torrissen, 1989, Bjerkgeng et al., 1992; De la Mora et al., 2006; Higuera-Ciapara et al., 2006; SajjadMirzaee et al., 2012; Teimouri and Keramat Amirkolaie, 2015). For example, the inclusion of 0.08 or 0.10 g kg⁻¹ astaxanthin (Lucantin Pink[®], BASF, Ludwigshafen, Germany) in the diet was recommended to enhance pigmentation of rosy barb *Pethia conchonius* (Teimouri and Keramat Amirkolaie, 2015). The combination of 0.2 g kg⁻¹ synthetic astaxanthin (Carophyll pink, 5% astaxanthin) and canthaxanthin (Carophyll red, 10% canthaxanthin) from Hoffmann-La Roche, Basle, Switzerland significantly increased total carotenoid deposition in rainbow trout *Salmo gairdneri* flesh than both astaxanthin or canthaxanthin alone (Torrissen, 1989). Supplementation of 0.1 - 0.2 g kg⁻¹ Carophyll Pink (synthetic, 5% astaxanthin, Roche Co. Ltd.) has been shown to be the most effective treatment for achieving desired pigmentation in kuruma prawn *penaeus japonicus* (Chien and Jeng, 1992). Since few reports have been found on the application of synthetic carotenoids to the diet to enhance colour in abalone, supplementation with similar levels of synthetic astaxanthin, which have been recommended in other species, may also change greenlip abalone colour.

Melanin is a group of pigments contributing to black, brown, red, orange and yellow colours on animals (Chew, 1973; Fox, 1983; Bandaranayake, 2006). In molluscs, melanin

exists in soft tissues, especially in the mantle and hepatopancreas (Williams, 2016). Melanin is also credited with the dark and blue pigments in molluscan shells, the ink of cephalopod molluscs and dark pigments in the integument gastropods (Palumbo, 2003; Miyashita and Takagi, 2011; Miyamoto et al., 2013; Lemer et al., 2015; Sun et al., 2015; Williams, 2016). In the green ormer *Haliotis tuberculata*, the black lines along the mantle edge contain melanin and the intensity of the black line is affected by the food that the abalone consumes (Bravo et al., 2001). Additionally, based on the results of various chemical analyses, Chew (1973) reported that the green lip pigment of greenlip abalone *Haliotis laevis*, the black pigment of foot and lip of blackfoot *Haliotis iris* and blacklip *Haliotis rubra* was associated with melanin production. Our recent study also indicates that melanin may play an important role in the formation of the green colour in the lip of *H. laevis* but the precursor leading to melanin synthesis is not clear (Hoang et al., 2017).

Tyrosine is an amino acid used by cells as a precursor for melanin synthesis (Fox, 1979; Boonanuntanasarn et al., 2004; Li et al., 2009; Williams, 2016). According to Li et al. (2009), the level of dietary tyrosine could profoundly influence pigmentation development in fish. In molluscs, melanin has been shown to contribute to shell pigmentation in the pearl oyster *Pinctada fucata*, the black-lipped pearl oyster *Pinctada margaritifera* (Miyamoto et al., 2013; Lemer et al., 2015) and the yesso scallop *Patinopecten yessoensis* (Sun et al., 2015). The gene tyrosinase has also been identified in the mantle tissue of these species. Miyashita and Takagi (2011) reported that tyrosinase proteins are responsible for the formation of blue pearls in the Akoya pearl oyster *Pinctada fucata*. Since melanin synthesis is related to tyrosine, it is possible that supplementation of this amino acid may affect the colour of greenlip abalone. Recent studies show that greenlip abalone fed fresh *G. cliftonii* or graded levels of dried *G. cliftonii* up to 20%, showed brown shell and green lip colours (Hoang et al., 2016a; 2017). According to Basemer et al. (2016), dried *Gracilaria cliftonii*

meal contained 5.4 g kg⁻¹ of tyrosine and a commercial formulated diet supplemented with 5 - 20% dried *G. cliftonii* contained 10.9 - 12.3 g tyrosine kg⁻¹ diet. This was the diet used in Hoang et al. (2017) which resulted in brown shell and green lip in abalone. So, it is proposed that the supplementation of up to 20 g kg⁻¹ free tyrosine may induce a colour change in greenlip abalone. The aim of study was to evaluate the effect of feed additives (commercial synthetic astaxanthin and free L-tyrosine) and the level of supplementation on the colour properties of the shell, foot and lip and the melanin content of the lip and whole tissue pigments of greenlip abalone.

5.3. Materials and methods

5.3.1. Experimental animal and system

One-year-old greenlip abalone were purchased from South Australian Mariculture at Boston Point, Port Lincoln, SA, Australia. They 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) prior to the trial and had not been used previously for any other experiments.

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).

5.3.2. Experimental design, diets, stocking and feeding

Experimental design and diets

A commercial, synthetic astaxanthin (CAROPHYLL® Pink 10% CWS; supplied by DSM Nutritional Products Australia Pty Limited, NSW, Australia) was formulated into Abgrow premium mash (provided by Eyre Peninsula Aquafeeds Pty. Ltd. Lonsdale, South Australia) at three doses (0.05, 0.1 and 0.2 g kg⁻¹) and L-tyrosine powder (Thermo Fisher Scientific Australia Pty Ltd, 5 Caribbean Dv, Scoresby Vic 3179, Australia) supplementations were 10, 15 and 20 g kg⁻¹. The Abgrow premium control diet comprised of 34% crude protein, 5.1% crude lipids, 42.8% carbohydrates, 4.8% ash, 12.7% moisture and 17.5 MJ g⁻¹ gross energy. The supplements were dissolved in water prior to adding to the diets.

To manufacture the diets, the required amounts of dry Abgrow premium mash, vitamin/mineral premix and fish oil were weighed out and mixed in a Hobart mixer (Hobart Corp., Troy, OH, USA) for 5 min. The vitamin/mineral premix and fish oil inclusions were kept constant in all diets at 2 and 15 g kg⁻¹, respectively. Water (~ 30%) and sodium alginate binder (0.36%) were then added to the diet mash and mixed for a further 3 min. The diets were cold pelleted using a TR110 pasta machine (MacchinePer Pasta SRL, Molina Di Malo, VI, Italy) to produce a 5-mm flat sinking pellet. Diets were then dried at 50 °C for approximately 48 h until the diets had <10% moisture. The dietary pigments and L- tyrosine contents of the experimental diets are presented in Table 1.

Stocking and feeding

Greenlip abalone (2.47 ± 0.01 g; shell length 27.27 ± 0.05 mm) were selected, weighed, measured, and randomly stocked (10 abalone tank⁻¹) into each of the 28 tank. Four replicate

tanks per treatment combination were used. Abalone were acclimatised to the system for one week and were fed the respective experimental diet. After 7 days, the water temperature was slowly raised from 19 °C to the final temperature of 22 °C. Water temperature was then maintained within a target level ± 1.0 °C throughout the remainder of the 65-day experiment.

The feeding rate was 4% biomass tank⁻¹ day⁻¹, which was in excess of the abalone's daily food ration under experimental conditions. Feeding occurred at 16:00 daily. Cleaning and collection of uneaten food took place at 08:30 daily by straining the entire tank contents through a fine mesh (500 μ m). The uneaten food was weighed, stored frozen at -20 °C and then dried in an oven at 105 °C for 16 h. The difference between the amount of dry uneaten food and feed offered was used to calculate daily feed consumption. For corrected feed consumption, leaching loss was taken into account by immersing the experimental diets in water at 22 °C for 16.5 h without the presence of animals, collected through a fine mesh net (500 μ m) and dried to a constant weight. To maintain stocking density, dead abalone were replaced with similar size abalone. Feed rates were adjusted based on the biomass changes arising from mortalities and replacements.

5.3.3. Specimen sampling and analyses

The methods for image analysis were fully described in section 2.3.3 Chapter 2 of this thesis.

The methods of pigment extraction and analysis were also previously described in section 2.3.3 Chapter 2. Astaxanthin (Sigma SML0982) was added and measured in this chapter and its retention time was 4.7 mins.

Determination of the melanin content in the abalone lip was based on a modified method of Szisch et al. (2002). Briefly, freeze-dried abalone lip tissue was ground to homogenous and incubated with 1% HCl at 60 °C for one hour to decalcify. Samples were

then washed three times with distilled water and boiled in 0.2% NaOH for 1 h. Samples were then centrifuged at 16 000 g for 5 min and the solutions were transferred to UV-transparent 96 well plate. The melanin concentration was determined using SPECTROstar Omega. The absorbance of the solution was set at 340 nm. The melanin standard (M2649, Sigma Aldrid) was solubilized in 1 ml 1 M NaOH and 10 μ l 3% H₂O₂ by heating in a water bath (100 °C) for 30 min.

5.3.4. Statistical analysis

Statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) for Windows (version 22, IBM Corp., Armonk, NY, USA). Two-factor ANOVA was used to determine the interactive effects between two types of feed additives (astaxanthin and L - tyrosine) and three levels of supplementation (low, medium and high) on colour components (hue, saturation and brightness) and the pigment content in whole abalone tissue. In order to perform two-factor ANOVA, the different levels of supplementation between astaxanthin (0.05, 0.1 and 0.2 g kg⁻¹) and L-tyrosine (10, 15 and 20 g kg⁻¹) were encoded as low, medium and high. When no significant interaction between feed additive and level of supplementation was observed, Tukey's HSD post-hoc test was used to detect significant differences between treatment means. The differences in levels of supplement were compared within each feed additive at where significant interactions between those two factors were observed. Dunnett's post-hoc test was used to compare colour components and pigment deposition in the whole tissue between abalone fed the control diet (0%) and those fed different feed additives.

5.4. Results

Water quality parameters were 22.1 \pm 0.0 °C for temperature, 81.5 \pm 0.19% saturation or 5.67 \pm 0.2 mg L⁻¹ for dissolved oxygen, 8.11 \pm 0.01 for pH and 36 \pm 1.0 ppt for salinity.

The survival was 100% in all treatments. For growth data, there was no significant difference in final weight of abalone in all treatments. The final weights of greenlip abalone (mean \pm SE) were 11.32 ± 0.11 g and 44.15 ± 0.3 mm shell length.

5.4.1. Shell colour properties

Feed additive type (two-factor ANOVA; $P > 0.05$; Table 2; Fig. 2), supplement level ($P > 0.05$) or the interaction between these two factors ($P > 0.05$) did not significantly affect any colour components of shell. Abalone had a light green shell across all treatments. The values of shell hue, saturation and brightness ranged from 74.0 ± 5.72 - 85.0 ± 7.34 degrees, 19.5 ± 2.47 - $22.5 \pm 2.47\%$ and 47.8 ± 0.64 - $49.8 \pm 1.13\%$, respectively. Abalone fed the control diet had a similar shell colour to those fed diets with the different levels of pigment supplementation (Dunnett's post-hoc test; $P > 0.05$; Table 2; Fig. 2).

5.4.2. Foot colour properties

Colour components of the foot were also not significantly influenced by feed additive (two-factor ANOVA; $P > 0.05$; Table 2; Fig. 3), supplement level ($P > 0.05$) or their interaction ($P > 0.05$). Abalone had a brown foot in all treatments. Foot hue, saturation and brightness values ranged from 21.3 ± 0.90 - 24.4 ± 0.72 degree, 55.0 ± 1.41 - $59.0 \pm 1.88\%$ and 50.3 ± 0.97 - $55.9 \pm 0.29\%$, respectively. Abalone fed the control diet also had dark brown foot which was similar to those fed the diets with different levels of pigment supplementation (Dunnett's post-hoc test; $P > 0.05$; Table 2; Fig. 3).

5.4.3. Lip colour properties

Lip hue, saturation or brightness were not significantly impacted by feed additive (two-factor ANOVA; $P > 0.05$; Table 2; Fig. 3), supplement level ($P > 0.05$) or their interactive effects ($P > 0.05$). The lip colour of abalone was milky in all treatments. Lip colour hue, saturation and brightness values ranged from 47.2 ± 1.75 - 52.6 ± 1.86 degree,

24.3 ± 2.83 - 31.1 ± 3.67% and 51.2 ± 1.20 - 55.1 ± 1.84%, respectively. There was no significant difference in lip colour between abalone fed the control diet and the diets with differing levels of pigment supplementation (Dunnett's post-hoc test; $P > 0.05$; Table 2; Fig. 3).

5.4.4. Pigments and melanin contents in diets and abalone

Pigment content in diets and the whole tissue of abalone

The results of the dietary pigment analyses showed that the level of astaxanthin in the diets reached the target levels of expectation (Table 1). However, the concentration of astaxanthin, β -carotene, chlorophyll *a*, β -cryptoxanthin and zeaxanthin in the whole abalone tissue were below the level of HPLC detection (Table 2).

Melanin content in the lip of abalone

The results of dietary L-tyrosine analysis showed that the amount of tyrosine in the diets were much higher than the target levels of the manipulation. This was due to the contribution of protein-bound tyrosine in the diets. However, the levels of melanin in the lip of abalone were not significantly boosted by the type of pigment additive (two-factor ANOVA; $P = 0.673$; Table 2; Fig. 3), levels of supplement ($P = 0.548$) or their interaction ($P = 0.508$). Abalone fed the L-tyrosine supplement diets contained a similar amount of melanin in the lip with those fed the control diet (Dunnett's post-hoc test; $P = 0.697$; Table 2; Fig. 3). Greenlip abalone exhibited a milky lip across all diet treatments.

5.5. Discussion

The colour of abalone, like many other marine animals, is associated with the type and amount of pigments in the diet. Under farming conditions, in land-based systems, abalone need a diet supplementation to maintain the body colour similar to that seen in the wild (Hoang et al., 2016b; 2017). Ingredients containing a natural sources of carotenoids have been used in

aquaculture to improve the quality and marketability of products in terms of flesh colour and body pigments (García-Chavarría and Lara-Flores, 2013). However, commercial synthetic carotenoids have been recently received more attention due to lower costs, ease of access and higher stability in chemical composition (De la Mora et al., 2006; SajjadMirzaee et al., 2012; García-Chavarría et al., 2013; Teimouri and Keramat Amirkolaie, 2015). The supplementation of synthetic astaxanthin in the diet has successfully improved the skin colour of ornamental fish, flesh of salmonids, and epidermis of crustaceans (Sommer et al., 1992; Choubert and Heinrich, 1993; Higuera-Ciapara et al., 2006). However, in the present study, the inclusion of commercial synthetic astaxanthin had no impact on the colour of greenlip abalone. This is similar to the results reported by Canales-Gómez, et al. (2010) who found that supplementation of a commercial synthetic astaxanthin did not significantly change the shell colour of juvenile red abalone *Haliotis rufescens*.

The amount of synthetic astaxanthin, in the diet, that has an impact on the colour of aquatic animals varies among species. For example, the threshold concentrations of astaxanthin resulting in colour change ranged from 0.04 - 0.1 g kg⁻¹ for salmon, 0.05 - 0.1 g kg⁻¹ for trout, 0.02 - 0.03 g kg⁻¹ for gilthead sea breams, 0.05 - 0.1 g kg⁻¹ for gold fish, 0.04 - 0.2 g kg⁻¹ for shrimps (e.g., giant tiger prawn *Penaeus monodon*, Pacific white shrimp *Litopenaeus vannamei*), 0.2 g kg⁻¹ for hermit crab *Clibanarius erythropus*, 0.22 g kg⁻¹ for American clawed lobster *Homarus americanus* and 0.12 g kg⁻¹ for the tropical spiny crayfish *Panulirus ornatus* (Diler and Dilek, 2002; Wade et al., 2015). It was initially expected that the supplementation of synthetic astxanthin with a similar dose found in other aquatic animals could result in colour change in greenlip abalone. However, despite the use of a relatively high dietary inclusion of astaxanthin (0.2 g kg⁻¹) which has been reported to work on other species, no measurable colour change was detected. This suggests that greenlip

abalone may have different pathways to transfer dietary pigments to body colour, or they may reject this carotenoid through faeces.

The processes of pigment absorption, utilisation and metabolism are also species-specific. For instance, salmon and red sea bream (*Pagrus major*) are not able to convert xanthophylls to canthaxanthin or astaxanthin, but penaeid shrimp (*Penaeus monodon*) have the ability to convert β -carotene to astaxanthin (Chatzifotis et al., 2005). Synthetic astaxanthin is utilised by salmonid and crustacean to intensify their pink colour flesh and shell colour, respectively (Higuera-Ciapara et al., 2006). In contrast, dietary carotenoids have not been shown to effect body colour in juvenile abalone *Haliotis rufescens* abalone (Canales-Gómez et al., 2010) or in greenlip abalone in the current study. The differences in the digestive and metabolic systems between molluscs, fish and crustaceans may explain these discrepancies.

The absorption of carotenoids in marine animals is also dependent on other ingredients in the diet (Barbosa et al., 1999). As carotenoids are fat-soluble compounds, a diet with a high lipid content may increase carotenoid absorption, while a diet with low fat can reduce absorption after ingestion (Fox, 1983; Canales-Gómez et al., 2010). It has been reported that the high dietary lipid level (24%) increased concentration of astaxanthin in the serum of rainbow trout *Oncorhynchus mykiss* fed green algae *Haematococcus pluvialis* or a commercial astaxanthin (8% CAROPHYLL® Pink), compared to those fed a low fat diet (9%) (Barbosa et al., 1999). The abalone diet used in this study contained ~3.6% lipid as recommended by Dunstan et al (2000). Thus, it is possible that the low dietary fat level may have affected carotenoid absorption in abalone. Unfortunately, greenlip abalone do not tolerate high levels of dietary lipid (Dunstan et al., 2000), therefore, research may be required to alter the fat/water soluble molecular structure of the pigment in an attempt to alter and improve uptake rates.

The low efficacy of dietary synthetic astaxanthin in this study may also be related to the molecular size of natural and synthetic astaxanthin (Higuera-Ciapara et al., 2006; Canales-Gómez et al., 2010). In green sea urchins *Strongylocentrotus droebachiensis* and purple sea urchin *Paracentrotus lividus*, the natural algal diet or addition of natural β -carotene from *Dunaliella salina* resulted in an intensification of gonad colour. But including synthetic formulas of β -carotene or astaxanthin did not improve gonad colour (Robinson et al., 2002; Shpigel et al., 2006). Similarly, our previous studies show that greenlip abalone fed fresh or dried red algae *Gracilaria cliftonii*, that contain astaxanthin as the major carotenoid (Banerjee et al., 2009; Stone et al., 2014), had a brown shell, light yellow foot and more green pigments in the lip compared with the control diet (Hoang et al., 2016a; 2017). However, no significant colour change was observed in abalone fed the synthetic carotenoid additive in the present study. It is likely that abalone cannot effectively use the commercial synthetic carotenoid astaxanthin to change body colour, or this carotenoid is not responsible to colour of any body parts of abalone.

Colour of wild greenlip abalone is associated with dietary pigments found in macroalgae, particularly in red macroalgae (Hoang et al., 2016a). Farmed greenlip abalone fed formulated diets do not normally have a high enough intake of pigments compared to the same species living in nature. When these pigments are not included in the formulated feed, the colour of the abalone foot, shell and lip will fade. In the current study, the amount of synthetic astaxanthin supplementation reached 0.2 g kg^{-1} , but little impacts on colour were detected. Therefore, the use of a commercial synthetic astaxanthin product in the abalone diet is not recommended to manipulate colour in land-based systems. On the other hand, red macroalgae contain a variety of other carotenoids such as lutein and zeaxanthin (Schubert et al., 2006; Chandini et al., 2008; El-Baky et al., 2008; Banerjee et al., 2009), their addition may be more effective than the addition of astaxanthin alone for changing abalone colour.

The green pigment on greenlip abalone may also be biosynthesized from the precursors obtained from dietary algae (Hoang et al., 2017). Melanin is an end-product from the catabolism and oxidative breakdown of the amino acid tyrosine (Fox, 1979, 1983). The oxidative degeneration of tyrosine and related phenolic compounds are expressed as dirty-yellow, orange, ruddy, tan-coloured, dark brown, or black. The existence of melanin in abalone has not gained enough attention in past literature. The idea of supplementing L-tyrosine in the diet to manipulate the colour of abalone was based on the results of Chew (1973) who reported that the green lip pigment of *H. laevigata* is related to the production of melanins. In the present study, three levels (10, 15 and 20 g kg⁻¹) of free tyrosine were added to the abalone diet based on the concentrations of tyrosine in a diet with 5 - 20% of dried *G. cliftonii* (12.0 - 12.3 g tyrosine kg⁻¹) used in Bansemer et al. (2016). However, in the current study, the melanin content in the abalone lip was not affected by adding free tyrosine and no significant colour change was detected in the lip, shell or foot of abalone, regardless of tyrosine levels.

The efficiency of using free amino acids depends on not only the type of amino acids but also their form. It has been reported that fish and prawns can utilise crystalline amino acids less efficiently than the protein-bound form due to rapid absorption, uncoordinated assimilation in tissues, quick degradation and excretion (Andrews et al., 1977; Yamada et al., 1981; Schuhmacher et al., 1997; Murai et al., 1982; Teshima et al., 1992; Chen et al., 1992; Zarate and Lovell, 1997; Nunes et al., 2014). The protein-bound lysine in dehulled soybean meal is more efficiently utilised than free lysine in the channel catfish *Ictalurus punctatus* (Zarate and Lovell, 1997). According to Teshima et al. (1992), soybean plastein has a higher bioavailability than crystalline methionine as a dietary methionine supplement for *Penaeus japonicus*. In abalone, Chew (1973) found that the epidermis of three *Haliotis* species, including greenlip abalone, contain the polymers of melanin. However, it worth noting that

those abalone species in the study by Chew (1973) were collected from the wild where macroalgae are the main food source. The difference between the molecular size of L-tyrosine and that of tyrosine bound in macroalgae proteins, may be the reason for our observation of no significant colour changes in abalone. This study suggests the poor ability to utilise L-tyrosine in greenlip abalone towards body colour change.

In conclusion, the supplementation of commercial astaxanthin and free tyrosine had no beneficial effects on the content of pigments and colour change of greenlip abalone. Abalone were unable to utilise the synthetic carotenoid astaxanthin as well as the free form of an amino acid to change their colour. We do not recommend the use of these products as colour enhancers in commercial abalone feeds.

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Table 1. The amount of astaxanthin and L - tyrosine of experimental diets fed to greenlip abalone (*Haliotis laevis*).

Diet	The control diet	Tyrosine			Astaxanthin		
		Supplementation level	Low	Medium	High	Low	Medium
Diet supplement							
Astaxanthin (g kg ⁻¹) ¹	-	-	-	-	0.046	0.08	1.89
L-tyrosine (g kg ⁻¹) ^{2,3}	12.4	25.9	33.9	39.7	--	--	--

-- denotes as not detectable. "--" denotes variables not analysed .

¹ Low, medium and high level of the commercial synthetic astaxanthin were 0.05, 0.1 and 0.2 g kg⁻¹, respectively.

² Low, medium and high level of L - tyrosine supplement were 10, 15 and 20 g kg⁻¹, respectively.

³ Sum of L - tyrosine residue mass in protein (molecular weight minus H₂O) and free L - tyrosine molecular weight.

Table 2. Two-factor ANOVA results for shell, foot and lip colour and the pigment contents in whole tissue of greenlip abalone (*Haliotis laevis*) fed (n = 4)¹.

Pigment	The control diet	Tyrosine ⁴			Astaxanthin ⁵			2 factor ANOVA (<i>P</i> value) ²			Dunnett's test ³
		Supplement level	Low	Medium	High	Low	Medium	High	Additives	Inclusion level	
Melanin content in lip (mg g ⁻¹)	1.07 ± 0.38	1.25 ± 0.43	1.23 ± 0.36	1.23 ± 0.38	1.13 ± 0.26	1.49 ± 0.12	1.25 ± 0.19	0.673	0.548	0.508	0.697
Colour components of shell											
Hue (degree)	84.3 ± 8.20	78.0 ± 4.31	74.0 ± 5.72	79.1 ± 1.85	80.5 ± 5.87	75.0 ± 4.57	85.0 ± 7.34	0.470	0.365	0.895	0.770
Saturation (%)	21.2 ± 2.12	22.5 ± 2.47	19.5 ± 2.47	20.8 ± 2.75	22.1 ± 1.40	20.4 ± 1.59	19.7 ± 1.69	0.904	0.491	0.879	0.932
Brightness (%)	48.9 ± 1.75	48.1 ± 1.32	49.2 ± 0.66	48.2 ± 1.89	47.8 ± 0.64	48.3 ± 0.67	49.8 ± 1.13	0.884	0.658	0.512	0.914
Colour components of foot											
Hue (degree)	22.9 ± 1.36	24.4 ± 0.72	21.4 ± 2.08	21.3 ± 0.90	23.5 ± 1.55	22.2 ± 1.13	23.6 ± 0.74	0.472	0.248	0.470	0.551
Saturation (%)	56.1 ± 3.42	57.8 ± 1.11	57.4 ± 0.74	55.8 ± 1.55	56.1 ± 1.36	59.0 ± 1.88	55.0 ± 1.41	0.801	0.214	0.531	0.782
Brightness (%)	52.6 ± 1.79	55.9 ± 0.29	51.2 ± 1.96	50.3 ± 0.97	53.6 ± 1.98	53.7 ± 2.38	54.6 ± 0.77	0.221	0.234	0.101	0.199
Colour components of lip											
Hue (degree)	49.2 ± 0.72	49.1 ± 1.74	52.6 ± 1.86	52.1 ± 1.25	50.9 ± 1.98	47.2 ± 1.75	50.4 ± 0.52	0.193	0.646	0.111	0.216
Saturation (%)	27.7 ± 1.55	31.1 ± 3.67	26.6 ± 1.80	24.3 ± 1.90	25.5 ± 2.64	30.4 ± 3.64	24.3 ± 2.83	0.781	0.279	0.279	0.428
Brightness (%)	53.9 ± 1.17	55.1 ± 1.84	53.9 ± 0.91	51.2 ± 1.20	52.3 ± 1.16	54.6 ± 1.76	53.5 ± 1.31	0.937	0.405	0.210	0.487

¹ Data presented as mean ± SE; n = 4.

² 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 (Tukey's HSD test; *P* < 0.05).

³ Dunnnett's post-hoc test, *P* < 0.05 was used to compare abalone fed the control against those fed dietary supplement (*n* = 4 treatment⁻¹).

⁴ Low, medium and high level of the commercial synthetic astaxanthin were 0.05, 0.1 and 0.2 g kg⁻¹, respectively.

⁵ Low, medium and high level of L - tyrosine supplement were 10, 15 and 20 g kg⁻¹, respectively.

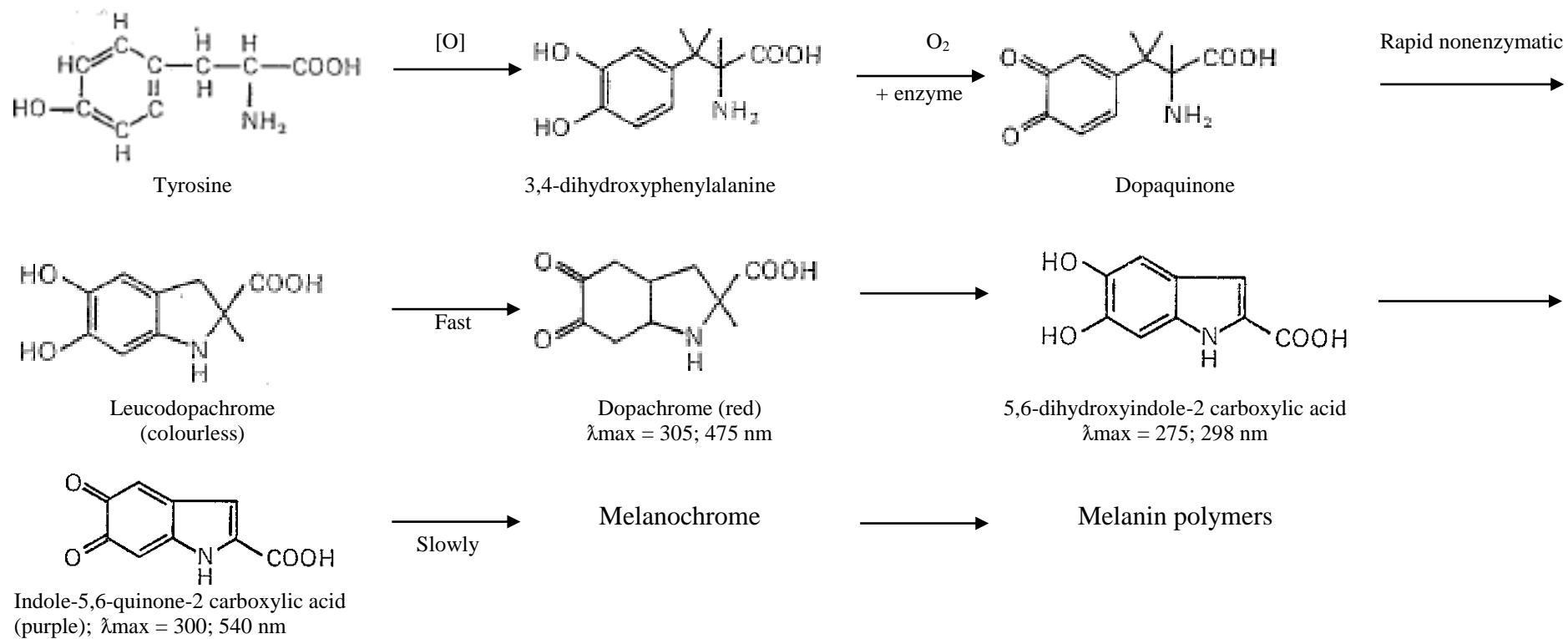


Fig.1. The melanin chemical pathway (Fox, 1979).

L - tyrosine					
Astaxanthin					
Supplement levels	0%	Low	Medium	High	wild greenlip abalone

Fig.2. Shell, foot and lip colour of greenlip abalone (*Haliotis laevigata*) fed graded level of the commercial astaxanthin (low, medium and high as 0.05, 0.1 and 0.2 g kg⁻¹, respectively) and L - tyrosine supplement (low, medium and high as 10, 15 and 20 g kg⁻¹, respectively). Note: The picture of South Australia wild greenlip abalone foot and lip is provided by Dr. Matthew Bansemer.

Chapter 6

General discussion and conclusion

6.1. Introduction

The colour of greenlip abalone is of great importance for their commercial value. It is a crucial factor for grading product quality and is directly associated with consumer preference. In Australia, greenlip abalone are predominantly cultured in the land-based system and, in this case, are totally reliant on a formulated diet. Wild-caught greenlip abalone ordinarily have a green lip, light yellow foot and a variety of shell colours, but farmed greenlip abalone usually have a milky coloured lip, dark brown foot and light green shell, which may influence market acceptance by consumers. Body pigments in wild abalone are derived from natural food sources, such as algae by either direct accumulation in the body or biosynthesis from precursors since its inability to synthesize some pigments or carotenoids *de novo*. Water temperature can also influence the colour of greenlip abalone as it affects pigment intake, absorption and digestion.

The overall objective of this thesis was to explore the effects of diet and water temperature on the colour of abalone shell, lip and foot and pigment deposition in the body tissue. Such knowledge will help maintain of the colour of abalone on farms through manipulation of diet and the environment. Four trials were carried out to address the research objectives. The first experiment was to investigate the colour change of abalone fed enriched vs non-enriched fresh red algae (*Gracilaria cliftonii*) and green macroalgae (*Ulva* sp.) or a diet with a dried algae meal inclusion (3% *Spirulina* sp. or 10% *Ulva* sp.). The second trial evaluated the effects of including dried *G. cliftonii* and *Ulva* sp. Meal, in formulated diets, at graded levels (0, 5, 10 and 20%), on body colour and pigment deposition in the whole tissue. In the third trial, abalone were fed three different diets (a commercial diet, fresh *Ulva* sp., and

a 30% enriched dried *Ulva* sp. inclusion) at two water temperatures (22 or 26 °C). The fourth experiment studied the effects of feed additives, in the forms of a commercial synthetic carotenoid, astaxanthin, and a free amino acid, L-tyrosine, on the colour and pigment deposition in the whole tissue of greenlip abalone. The general findings of the above studies are summarised below.

6.2. Summary of major findings

- 1) One-year-old abalone developed a brown shell when fed fresh *G. cliftonii* or a light yellow-brown shell when fed 3% *Spirulina* sp. meal. However, abalone exhibited a light green shell when fed fresh *Ulva* sp. or a commercial diet. Greenlip abalone fed either fresh *Ulva* sp. or fresh *G. cliftonii* had a yellowish foot.
- 2) One-year-old abalone developed a red/brown shell and a green lip when fed the diet containing $\geq 5\%$ and $\geq 10\%$ dried *G. cliftonii* meal inclusion in formulated diet. The increasing level of *G. cliftonii* meal in the formulated diet resulted in a darker brown shell, but did not intensify the green colour of the lip.
- 3) The foot colour of abalone fed enriched fresh *Ulva* sp. was light gold, whereas abalone fed the 30% *Ulva* sp. meal or the commercial diet had a dark brown foot. The foot colour of abalone cultured at 26 °C was paler than those at 22 °C.
- 4) β -carotene was the dominant pigment in the whole tissue of abalone fed fresh or dried macroalgae in the diet and its content proportionally increased with the level of macroalgae inclusion up to 20%, but decreased at high water temperature.
- 5) The supplementation levels of a commercial synthetic carotenoid, astaxanthin, or free amino acid, tyrosine, in the formulated diet, had no effect on the body colour or pigment deposition in the whole abalone tissue.

6.3. General discussion

6.3.1. Abalone colour positively respond to the type of fresh algae in the diet

The response of abalone colour to algal diets depends on the type of algae. It appears that the shell colour of abalone fed red algae is normally red/brown while it is light blue/green when fed brown or green algae. For example, the tropical abalone *H. asinina* has a brownish shell by feeding red algae *Gracilariopsis bailinae* (Gallardo et al., 2003). The shell is dark brown when the Pacific abalone *H. discus hannai* feeds on red Pacific dulse *Palmaria mollis*, but it becomes green when fed brown kelp *Laminaria japonica* (Qi et al., 2010; Ju et al., 2015). In addition, the colour of abalone fed the same algae species also varies greatly. Chew (1973) reported that shell of *H. cracherodii*, *H. rufescens* and *H. corrugata* fed brown algae *Macrocystis* sp. were a dark-blue shell, a brick-red or dull-green to reddish-brown, respectively. The results of the current study support the findings of previous studies reporting that the shell colour of greenlip abalone is brown when fed fresh *G. cliftonii*, but light green when fed *Ulva* sp., a formulated diet or the commercial diet. Furthermore, the foot colour of greenlip abalone fed fresh red and green macroalgae was light yellow, which is similar to the foot colour in the wild. These findings are useful for abalone farming as they provide new knowledge on the effects of dietary macroalgae on the colour change of greenlip abalone. The results of this study have also provided fundamental information that can be used for future research to improve or alter greenlip abalone colouration through dietary manipulation.

6.3.2. The change of abalone colour depends on the type and dose of dried algal meal in the diet

The colour response of abalone to the supplementation of algae meals in the diet is species-specific. The shell colour of greenlip abalone fed 3% *Spirulina* sp. or $\geq 5\%$ *G. cliftonii* meal was yellow/brown or red/brown, respectively, but was light green when fed the diet

including enriched dried *Ulva* sp. meal or the control diet. Among the three algae meals tested, only the inclusion of $\geq 10\%$ dried *G. cliftonii* produced the desirable green colour on the lip. In some abalone species, the colour of the shell and foot are relevant to the market value, but the green pigment on the lip is the most important feature for greenlip abalone in terms of product quality and identity (Brown et al, 2008; Qi et al., 2010; Bansemer et al., 2014; Ju et al., 2015). Since farmed greenlip abalone fed the commercial diet exhibited a milky-coloured lip, the inclusion of $\geq 10\%$ *G. cliftonii* and intensified green colour of the lip from this study may result in improved market value of this species.

The dose dependent effect of algae meal supplementation on abalone colour varied between algal species in the meal and among body parts. The shell colour was yellow-brown in abalone fed a diet containing 3% *Spirulina* sp. and became red/brown when fed the diet with the inclusion of $\geq 5\%$ *G. cliftonii* inclusion, increasing the colour intensity with the level of algae, up to 20%. However, the green pigment of the lip was only intensified in the diet containing $\geq 10\%$ dried *G. cliftonii* meal. A higher level of inclusion (20%) did not further increase the intensity of the green pigment. In contrast, the inclusion of up to 20% of *Ulva* sp. had little impact on the colour of abalone. To date, little research had tested the dose effect of dried algae meal on abalone colour. According to Lim and Lee (2003), the shell colour of *H. discus hannai* became yellow/red and orange when fed a diet containing 2% *Porphyra*, which is lower than the inclusion level in the present study, further demonstrating a dose-dependent effect of dietary algae on the body colour of abalone species.

6.3.3. Water temperature can influence abalone colour and pigment contents in whole abalone tissue, but depend on diet and abalone size

Water temperature could affect the foot colour of 3-year-old abalone, developing a paler foot at 26 °C than at 22 °C, regardless of dietary fresh or enriched dried *Ulva* sp. inclusion. The pale foot of abalone at high water temperature (26 °C) may be attributed to a

lower intake of pigments associated with reduced feed intake or reduced pigment absorption in the gut (Stone et al., 2014; Currie et al., 2015). Since there was no tangible effect on the colour of abalone at 26 °C, it is not recommended to feed greenlip abalone with fresh or enriched dried *Ulva* sp. for the sake of colour improvement at a high water temperature (26 °C or above).

At the laboratory optimal temperature for greenlip abalone, 22 °C, the foot colour is affected by animal size and diet type. The foot of 3-year-old abalone fed fresh *Ulva* sp. was light gold, but it was light yellow in 1-year-old abalone fed the same diet. However, feeding 30% enriched dried *Ulva* sp. meal to 3-year-old abalone resulted in a dark brown foot, similar to when the commercial formulated diet was given. A degradation of pigments was also observed in the formulated diet. The amount of β -carotene in the diet with a 30% *Ulva* sp. inclusion was lower than that in the fresh *Ulva* sp. The β -carotene content in the whole tissue of abalone fed 30% *Ulva* sp. meal was significantly lower than those fed fresh *Ulva* sp. These findings agree with Choubert and Heinrich (1993), that the degradation of carotenoids in the green algae *Haematococcus pluvialis* during the pelleting process and storage may cause lower retention and colour expression in the muscle of rainbow trout, *Oncorhynchus mykiss* compared to those fed synthetic carotenoids. This study suggests that feeding enriched fresh *Ulva* sp., but not enriched dried *Ulva* sp. meal, to greenlip abalone at 22 °C, may result in a similar foot colour to what is found in the wild.

Pigment content in the whole abalone tissue depended on diet type and water temperature. The amount of β -carotene in the whole tissue of abalone fed fresh *Ulva* sp. was significantly higher than those fed fresh *G. cliftonii*. It significantly increased up to the level with a 20% dried macroalgae inclusion (*G. cliftonii* or *Ulva* sp.) in the diet. Abalone fed fresh *Ulva* sp. had a higher β -carotene content than those fed 30% enriched dried *Ulva* sp. However, the amount of β -carotene in the abalone tissue was significantly reduced at higher

water temperature (26 °C). β -carotene also has been found as a major pigment in other abalone species in the wild (Tajima et al., 1980a,b; Kantha, 1989), but this present study has experimentally tested the effects of diet type and water temperature on the content β -carotene in the whole abalone tissue. The findings indicate that the amount of pigments/carotenoids, such as β -carotene, in the whole tissue of abalone can be improved by including fresh or dried macroalgae meal in the diet.

6.3.4. Limited effect on abalone colour when supplementing synthetic carotenoids or amino acid, L-tyrosine in the diet

It is unlikely abalone colour can be altered by adding synthetic carotenoids into a formulated diet, as abalone had little capacity to utilise this product. The supplementation of commercial astaxanthin in the diet, despite its wide use in salmonid and crustacean aquaculture for colour improvement, did not change the colour of the lip, shell or foot in greenlip abalone. These findings are consistent with Canales-Gómez, et al. (2010) which that the supplementation of commercial synthetic astaxanthin had a limited effect on the colour of juvenile red abalone *Haliotis rufescens*. Astaxanthin was not detected at all in the whole tissue of abalone, in the present study suggesting it may have also been interconverted to another unmeasured pigment, or an inability of abalone to digest and store this commercial carotenoid in the whole tissue. It is possible that after ingesting synthetic astaxanthin, greenlip abalone may pass it all through the gut, to become faeces, without absorption as it has been reported in some other invertebrates (Fox 1979). Additionally, astaxanthin naturally occurring in algae is in an esterified form, but it is in an unesterified form in the synthetic product. The difference in chemical forms may be a reason for the low efficacy observed in the current study (Higuera-Ciapara et al., 2006).

The colour of abalone, especially the green pigment in the lip, was also not affected by the addition of a free amino acid, L-tyrosine. The green pigment was assumed to be

related to melanin production based on reporting by Chew (1970) who suggested the possible link between melanin synthesis and green pigment in the abalone lip. The unpublished data of Li et al. (personal communication) also showed that greenlip abalone colour is dependent on melanin pigments in the epidermal layer. It is assumed that tyrosine is a precursor for melanin synthesis in molluscs (Fox, 1979; Williams, 2016) so the last experiment in Chapter 5 was performed to investigate the relationship between melanin production and abalone colour by supplementing graded tyrosine into the diet. However, the results show that the melanin content in the abalone lip was not affected by the amount of free tyrosine in the diet and no significant colour change was detected in the lip, shell or foot. Similar to the addition of synthetic astaxanthin, greenlip abalone may not be able to utilise the free form of tyrosine. This is possibly due to the difference between the protein-bound form and the free form of tyrosine used in this study. It has been reported that fish and prawns can utilise crystalline amino acids less efficiently than the protein-bound forms due to rapid absorption, fast degradation and excretion of crystalline amino acids (Zarate and Lovell, 1997; Nunes et al., 2014). Since greenlip abalone exhibited a milky colour on the lip across all dietary treatments, the inclusion L-tyrosine in the diet is not recommended to achieve an intensified green colour in the lip.

6.3.5. *Significance of abalone colour manipulation in seafood market and fisheries*

Abalone consumers have different preferences when it comes to the colour of abalone body parts. For example, a purple or dark-brown shell is preferred over a light green shell in *H. discus hannai*, while lighter foot pigments in blackfoot paua *H. iris* usually fetch a higher price on the market (Allen et al., 2006; Qi et al., 2010; Ju et al., 2015). In greenlip abalone, the most vital colour is the green pigmentation of the lip. The milky coloured lip of farmed abalone fed formulated diet may negatively affect the market value and product identity of this species. This study shows that the green pigmentation of the lip can be improved through

dietary manipulation as demonstrated by feeding dried *G. cliftonii* in the current study. Although not as important, the shell colour can also be manipulated. The brown-red shell induced by feeding fresh or dried *G. cliftonii* meal can be considered as a harmless shell marking method for sea ranching, stock enhancement or growth monitoring in the wild. It is also possible for abalone growers to produce a unique shell colour to establish their own product identity or brand of abalone on the market.

6.4. Conclusions

- 1) The colour of greenlip abalone can be enhanced or altered through dietary manipulation.
- 2) Fresh macroalgae or dried micro/macroalgae meal inclusion in the diet is a potential solution to change the colour of abalone in a farming situation.
- 3) Water temperature together with diet can have an impact on the foot colour of abalone although dietary manipulation had a larger effect.
- 4) Supplementation of commercial synthetic astaxanthin or free tyrosine has no desirable effects on the colour or pigment content in the whole tissue of abalone.

6.5. Recommendations for management

- 1) Feeding fresh *G. cliftonii* or the inclusion of $\geq 5\%$ dried *G. cliftonii* meal in the formulated diet is recommended to produce red/brown shell, which can be used as a harmless method to mark abalone shells for the purpose of ranching, stock enhancement or growth monitoring in the field. It also may serve as a tool for abalone growers to manipulate shell colour and to develop a consistent product between sites.
- 2) The inclusion of 10% dried *G. cliftonii* meal in the formulated diet can be used to intensify the green pigment in the lip of greenlip abalone cultured in land-based systems.

- 3) Feeding fresh *Ulva* sp. and *G. cliftonii* can lead to a light-yellow foot which is closer to the foot colour of wild greenlip abalone and more attractive to consumers.
- 4) Feeding fresh or dried algae inclusion ($\leq 20\%$) in the formulated diet can increase the content of β -carotene in the whole tissue of abalone, which may improve the product quality in terms of pigment content and may have human health benefits.
- 5) Supplementation of synthetic astaxanthin or free L-tyrosine in the diet is not recommended to feed farmed abalone for the purpose of abalone colour improvement.

6.6. Future research directions

Overall, this thesis has improved our knowledge on colour change of abalone in response to diet and water temperature manipulation. Some results of this study are beneficial to improve or manipulate colour of greenlip abalone in land-based cultured systems. However, there are still some issues identified but remain unsolved by the end of this study. Thus, future research on abalone colour should be directed to the following areas.

- 1) There is a need to further explore the dose-dependent effect of dried *G. cliftonii* meal on the colour of 3-year-old greenlip abalone. This size is close to the market size and the resultant abalone colour after dietary manipulation is important to consumers. A longer feeding period is also recommended to secure the optimal green colour in the lip. It is also necessary to evaluate how abalone colour fades after a withdrawal of the diet supplemented with the macroalgae meal.
- 2) The result reveals that water temperature had a significant effect on foot colour of greenlip abalone. However, only two water temperatures (the preferred water temperature 22 °C vs a high temperature 26 °C) were investigated. Thus, further research should further identify the range of optimal water temperatures suitable for colour improvement under an optimal feeding regime.

- 3) Although no positive effects on abalone colour were observed by supplementing astaxanthin and L-tyrosine in the diet, further research should investigate the effect other dominant carotenoids in red algae, such as canthaxanthin and zeaxanthin, may have on colour change.
- 4) Although feeding $\geq 10\%$ dried *G. cliftonii* intensified the green pigment of the lip, the chemical pathways leading to this change remain unresolved. It is also possible that the green pigment could be biosynthesized by abalone themselves, using precursors obtained from the algae. Thus, further research is strongly recommended to identify the compounds that contribute to colour change.
- 5) Greenlip abalone developed a red/brown shell by feeding a fresh or dried *G. cliftonii* supplement, but the pigments responsible to the shell colour change are still unclear. Identification of the chemical pathway or pigments involved in the red/brown shell formation would be beneficial to produce particular shell colours and establish brand identities for marketing purposes.

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