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Differences in Total Carotenoid Content in Tissues of the Pearl Oyster *Pinctada fucata* with Regard to Cuticle Shell Color

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Abstract

The aim of this study was to compare total carotenoid content (TCC) in tissues, and its correlation with shell color between four different shell color strains of *Pinctada fucata*. A total of 120 individuals (30 golden shells, 30 red shells, 30 black shell and 30 white shells) of *P. fucata* of similar size were evaluated. In this study, stomach, gill, adductor, and mantle were used for measuring the determination of TCC. The color measurements were taken from both the nacre shells and cuticle shells. The results showed that TCC varies significantly among four different tissues and the four different shell color strains ($P < 0.05$). A significant difference between the cuticle shell colors of the four different shell color strains of *P. fucata* was observed ($P < 0.05$), while the nacre shell colors of four shell strains were similar ($P > 0.05$). In the present study, results indicated that the TCC of *P. fucata* was significantly related to tissue and cuticle shell colors ($P < 0.001$), but not related to nacre shell colors ($P > 0.05$). TCC relating to the cuticle shell color suggested that increasing or decreasing TCC in *P. fucata* through selective breeding of cuticle shell color was feasible, and it could be significant both for food for humans, and the cultivation of high-quality pearls.

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Introduction

The Carotenoids Database (http://carotenoiddb.jp) lists a total of 1,182 natural carotenoids which have been found in 700 source organisms (Junko, 20181206). Carotenoids, also known as tetrterpenoids, are organic pigments produced by plants, algae, and some bacteria and fungi. Few animals can synthesize carotenoids. Aphids and spider mites have obtained their genes and abilities to produce carotenoids from fungi (Moran et al. 2010, Altincicek et al. 2011 and Novakova et al. 2012). Even though animals lack the ability to synthesize carotenoids de novo, they can obtain carotenoids from their diet. Many marine animals accumulate carotenoids with structural diversity (Maoka, 2011; Matsuno, 2001). In bivalves, total carotenoid content (TCC) accumulation has been observed in tissues such as hepatopancreas, gonad, gill, adductor, and mantle (Li et al., 2014[a]). TCC varies in different tissues. It is highest in the hepatopancreas, and lowest in the adductor muscle (Li et al., 2014[a]).

P. fucata is one of the best species for pearl culture and is economically important worldwide. It is native to the Indo-Pacific region, from the Red Sea, the Persian Gulf, to coastal waters of India, China, Korea, Japan, and the Western Pacific Ocean. There are shell color polymorphisms in wild and cultured populations, the different shell colors are golden, red, black, and white. From 2006 to date, Chinese scientists have tested their growth and survival rates (Deng et al., 2013; Chen et al., 2016), genetic regularity analysis (Li et al., 2017), shell prism layer, content of metal elements in the nacre layer (Zou et al., 2015), the effect of nuclear insertion breeding (Fu et al., 2012), and the candidate AFLP markers related to shell color (Zou et al., 2014). These studies have laid the theoretical foundation for the breeding of high-quality pearl oysters and the cultivation of high-quality pearls.

In many aquatic animals, tissue colors are often attributed to the presence of carotenoids that play significant roles in tissue color (Ytrestoyl et al., 2004; Li et al., 2010; Zheng et al., 2010; Kop et al., 2008; Teimouri et al., 2013). Furthermore, in *Hyriopsis cumingii*, there were significant correlations between TCC in mussel mantle and the inner-shell color intensity (Li et al., 2014[a]). There is still a lack of information regarding the relationships between the shell color and TCC in *P. fucata* therefore there is a need to learn about the difference in TCC and shell color of *P. fucata* and investigate whether TCC in the *P. fucata* is related to tissues and shell colors (nacre shell or cuticle shell colors). TCC in tissues of stomach, gill, adductor, and mantel from individuals of *P. fucata* with golden, red, black and white shells were determined by UV-1800BPC spectrophotometer, and the color of the shell (nacre and cuticle) was assessed with high-quality spectrophotometer NS820. The results of the present study may provide the basis for further studies on the four shell color strains of *P. fucata,* and the biological roles of carotenoids in shell color formation.

Materials and Methods

Animals and tissue sample

Pearl oysters *P. fucata* were collected from a cultured population in the sea port of Xincun village, Hainan Province, China, in July 2018. A total of 120, 10 month old pearl oysters *P. fucata* (30 golden shell, 30 red shell, 30 black shell, 30 white shell individuals) with a similar size (length: 54.87 ± 3.70 mm, height: 54.47 ± 3.55 mm, width: 21.06 ± 1.88 mm, body weight: 28.17 ± 6.52 g) were selected. Stomach, gill, adductor, mantle, and shells of each group were selected and stored at -20℃ individually for the further analysis. All the shells were cleaned and kept for the color measurement.

Carotenoids extraction and total carotenoids content determination

Carotenoids extraction was performed according to the report by Yanar Y., with some modifications (Yanar et al., 2004). The extraction solutions consisted of anhydrous sodium sulfate and acetone. To avoid degradation and isomerization of carotenoids, 10 mL test tubes wrapped in tin foil were used. Extractions were performed in a dark environment. Approximately, 0.1 g tissue sample was mixed with 0.1 g anhydrous sodium sulfate and 10 mL acetone to fully homogenate using homogenizer (N153 SGSEA/120117 made in UK). After being incubated at 4℃ in the dark for 3 days, the mixtures were centrifuged at 4000 rpm for 10 min using a centrifuge machine (Kait TG16G, China). The resulting supernatant (i.e., acetone phase) was separated, and measured at 480nm in a UV-1800BPC spectrophotometer (Mapada, Shanghai, China).

The computational formula of TCC was described by the following equation,

$$TCC\ (\mu g/g) = A_{480} \times y \times 10^4 / (E^{1\%}_{1cm} \times w)$$

where A_{480} is the light absorption value at 480nm, y is the extraction liquid volume (mL), W is the sample mass (g), and TCC was calculated using an extinction coefficient $E^{1\%}1$ cm of 1,900 (Yanar et al., 2004; Foss et al., 1984).

Color measurement

Identifying color differences of the shell with the CIE L*a*b* (Sun et al., 2012) and CIE L*C*h coordinates. CIE L*a*b* color space is a color space defined by the International Commission on Illumination in 1976. The space itself is a three-dimensional real number space, allowing an infinite number of possible representations of colors. L*, the lightness value, represents the darkest black at L* = 0, and the brightest white at L* = 100. The color channels, a* and b*, represent true neutral gray values at a* = 0 and b* = 0. The a* axis represents the green–red component, with green in the negative direction and red in the positive direction. The b* axis represents the blue–yellow component, with blue in the negative direction and yellow in the positive direction. The CIE L*C*h color space, similar to CIEL*a*b*, it has the same diagram as the L*a*b* color space but uses cylindrical coordinates instead of rectangular coordinates. In this color space, L* indicates lightness, C* represents chroma, $C^* = (a^{*2} + b^{*2})^{1/2}$, and h is the hue angle, h=arc tan (b*/a*) (Hunt, 1977).

In this study, shell color was assessed with a spectrophotometer (high-quality spectrophotometer NS820, 3nh, Shenzhen, china). Color measurements of the cuticle and nacre were all taken at three zones (Fig.1). The tested part was cut out with a glass rotor and washed with deionized water to dry and then measured in a dark environment. Each measurement zone (3 mm) was measured three times and the average value was taken, the average value of the three positions was used.

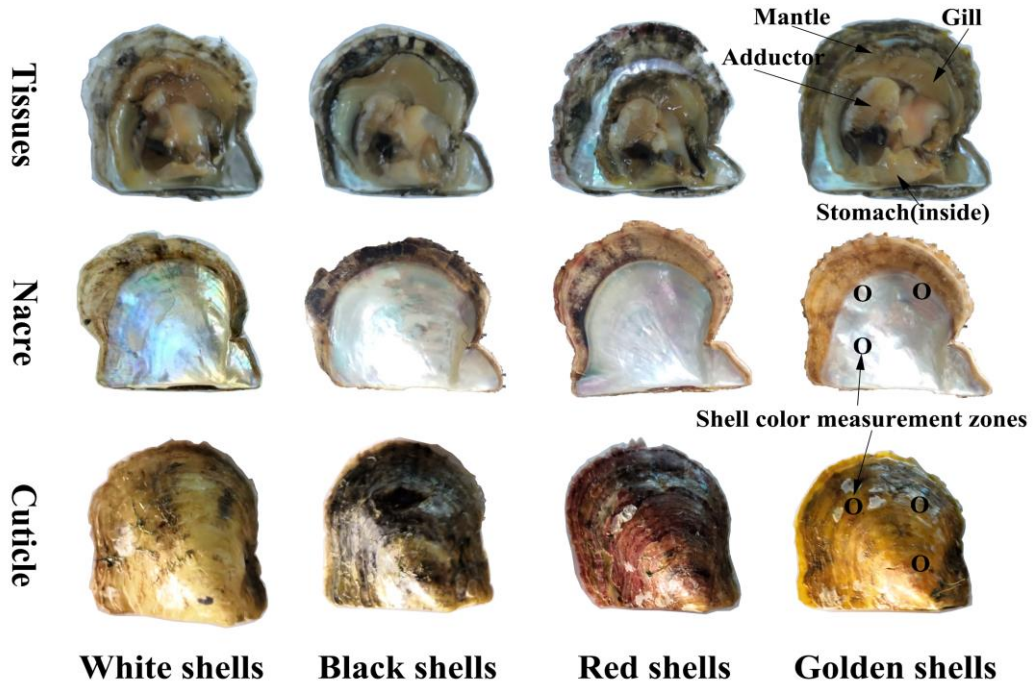

Fig.1 Shell color measurement zones and the tissues of the four shell color strains, *Pinctada fucata*

Statistical analysis

Statistical analyses were performed using SPSS18.0 software (SPSS Inc., Chicago, IL, USA). The differences in nacre and cuticle shell among different shell color strains of *P. fucata* and the differences in TCC among different tissues or groups were assessed by Duncan of multiple comparisons. The analysis of variance (general linear model, ANOVA) was carried out to examine TCC with respect to different shell colors and tissues.

Correlation analysis was carried out to determine the relationship between TCC among different tissues, and the relationship between TCC and the shell colors. Data were expressed as mean ± standard deviation. Statistical significance was set at $P < 0.05$, and extremely significance was set at $P < 0.01$.

Results

TCC difference between tissues

As shown in Fig.2, TCC in the stomach was significantly higher than in the other three tissues (gill, adductor, and mantle, $P < 0.05$), but there was no significant difference between the TCC in gill, adductor and mantle ($P > 0.05$). TCC ranged from 6.47 ± 3.93 μg/g to 53.01 ± 28.03 μg/g in white shell strains, from 9.47 ± 4.49 μg/g to 126.94 ± 73.25μg/g in black shell strains, from 3.62 ± 2.88 μg/g to 69.52 ± 34.62μg/g in red shell strains, from 3.55 ± 1.60 μg/g to 238.20 ± 2.29 μg/g in golden shell strains. In order of decreasing TCC in four tissues, TCC in stomach >TCC in gill > TCC in mantle > TCC in adductor. However, in black shell color strains of *P. fucata*, TCC in gill is lower than TCC in mantle.

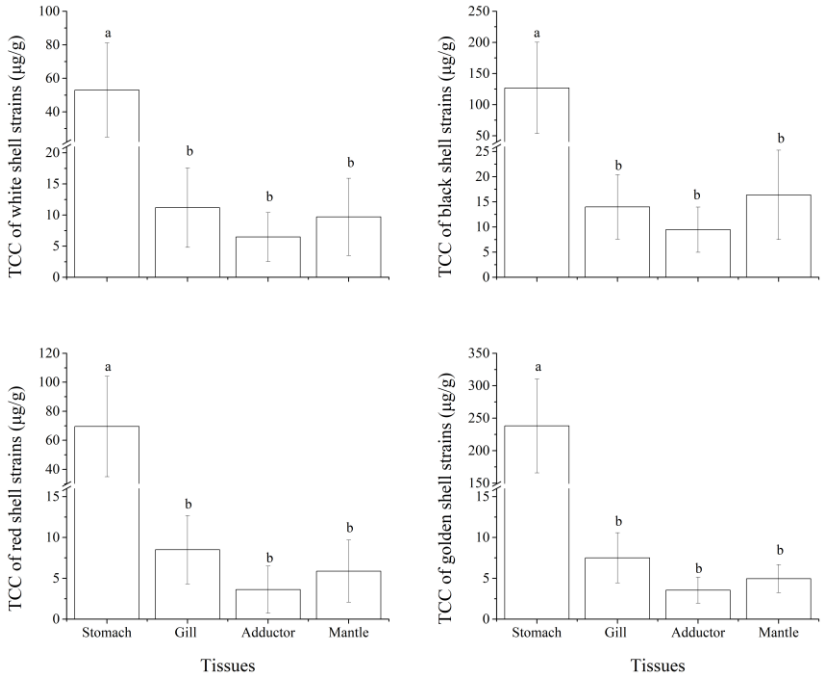

Fig.2 Difference of TCC between four tissues of *Pinctada fucata*

Note: Data are expressed as mean ± SD. Different lowercase letters in the same panel indicate that the difference is significant (P <0.05), the same below.

TCC difference between the four different shell color strains of P. fucata

In the four shell color strains of pearl oysters *P. fucata*, TCC was significantly different. It ranged from 53.01 ± 28.03 μg/g to 238.20 ± 72.29 μg/g in stomach, from 7.51 ± 3.07 μg/g to 13.99 ± 6.41μg/g in gill, from 3.55 ± 1.60 μg/g to 6.47±3.93 μg/g in adductor muscle and from 4.96 ± 1.72 μg/g to 9.70 ± 6.20 μg/g in mantle (Fig.3). In order of decreasing TCC in the same tissue of *P. fucata* with different shell colors, the results showed that TCC in black shell strains > TCC in white shell strains > TCC in red shell strains > TCC in golden shell strains. However, in the stomach, TCC in golden shell strains was significantly higher than the TCC in black shell strains ($P < 0.05$), the TCC in black shell strains was significantly higher than that in red shell strains and white shell strains ($P < 0.05$).

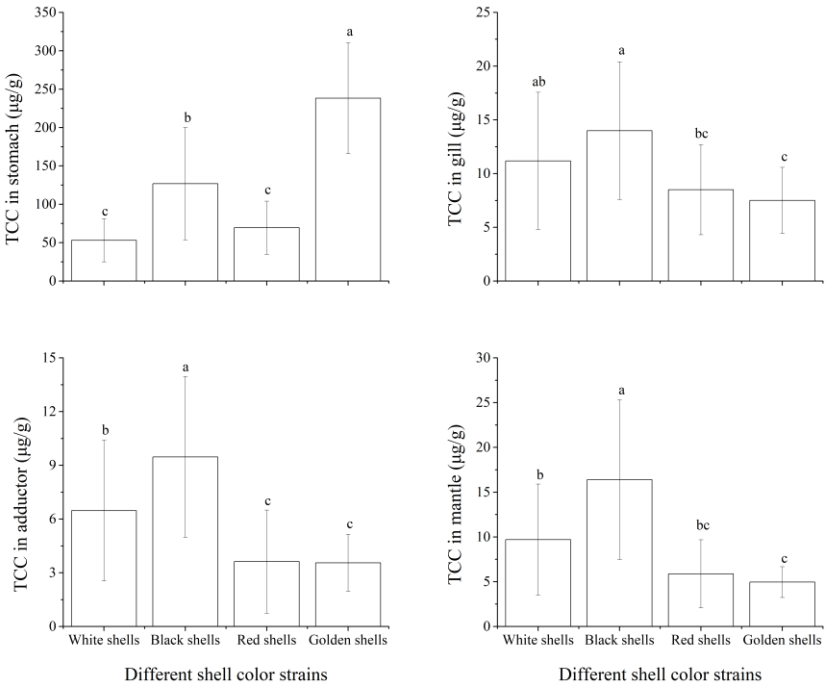

Fig.3 Difference of TCC between four shell color strains of *Pinctada fucata*

Color differences between the four different shell color strains of P. fucata

The characteristic color of the four shells of pearl oysters was defined in absolute terms using the CIE L*a*b* and CIE L*C*h system of color notation. The color measurements were taken from the cuticle shells and the nacre shells. The results are shown in Fig.4 (cuticle shells) and Fig.5 (nacre shells). There were significant differences in the colors (L*, a*, b*, C* and h) of cuticle shells between four group pearl oysters ($P < 0.05$, Fig. 4), except the differences of L* between red shell strains and golden shell strains ($P > 0.05$), the differences of a* and h between white shell strains and black shell strains ($P > 0.05$). For the cuticle shell color of four different shell color strains, L* ranged from 39.13 ± 5.61 to 53.45 ± 3.71, a* ranged from 1.04 ± 1.31 to 7.18 ± 1.49, b* ranged from 8.05 ± 2.07 to 18.81 ± 2.99, C* ranged from 9.17 ± 2.12 to 20.16 ± 3.12, h ranged from 61.49 ± 6.36 to 84.82 ± 6.65. There were significant differences in the nacre shell color (L* and h) between four differently shell color strains ($P < 0.05$, Fig. 5), but the difference of the nacre shell color (L*, a*, b*, C*, h) between black shell color strains and red shell color strains was not significantly ($P > 0.05$, Fig. 5). For the nacre shells color of four different shell color strains, L* ranged from 77.27 ± 2.62 to 80.24 ± 2.72, a* ranged from -5.33 ± 2.81 to -4.43 ± 2.11, b* ranged from -1.54 ± 3.01 to 0.34 ± 2.35, C* ranged from 5.47 ± 2.30 to 6.21 ± 2.30, h ranged from 175.62 ± 32.82 to 196.65 ± 33.75.

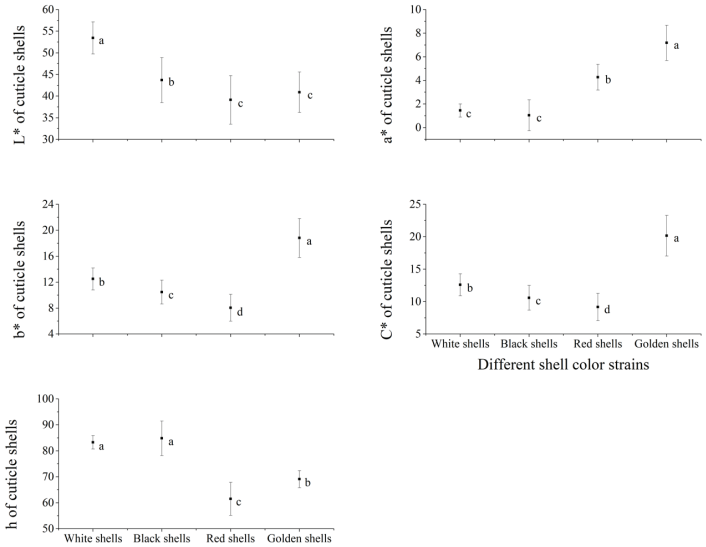

Fig.4 Color differences of the cuticle shells between four shell color strains of *Pinctada fucata*

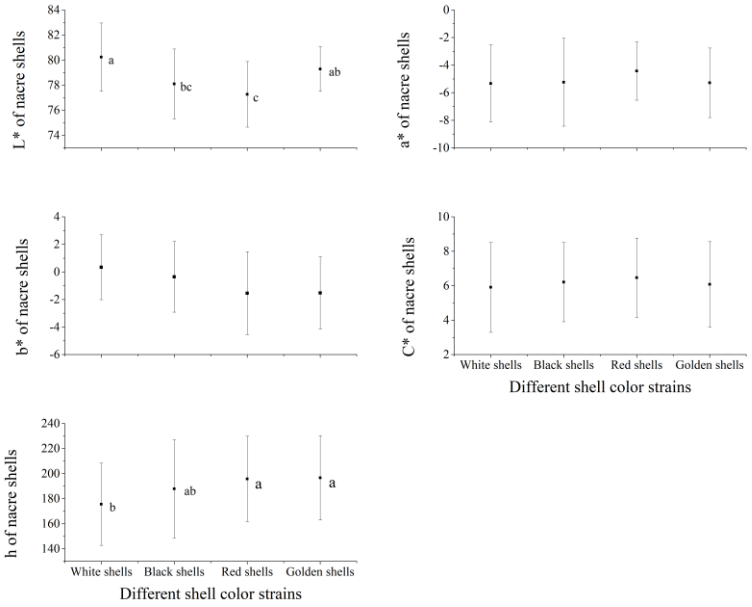

Fig.5 Color differences of the nacre shells between four shell color strains of *Pinctada fucata*

Differences in TCC of P. fucata between different shell colors and tissues

Analysis of variance (general line model, ANOVA) result shows that TCC was significantly different ($P < 0.001$) with respect to shell colors (nacre shell colors or cuticle shell colors) and tissues, and there were significant ($P < 0.001$) interactions (Table.1).

Table.1 Differences in TCC of four shell color strains between different shell colors and tissues

Source	*Sum of squares (type Ш)*	*Degree freedom*	*Mean square*	*F Values*	*P Values*
Model	1.795E6	15	119695.906	148.870	<0.001
Intercept	650274.422	1	650274.422	808.768	<0.001
Shell colors	148993.308	3	49664.436	61.769	<0.001
Tissues	1160327.444	3	386775.815	481.046	<0.001
Shell color × Tissues	486117.839	9	54013.093	67.178	<0.001
Error	373070.492	464	804.031		
Total variation	2818783.504	480			
Corrected total variation	2168509.082	479			

Correlation analysis of TCC in tissues relative to shell color

In order to determine whether the difference and interactions in TCC were caused by nacre shell colors or cuticle shell color, analysis of variance was calculated for difference in TCC of black shell strains and red shell strains (Since there was no significant difference between black shell color and gold shell color strains in nacre shell color ($P > 0.05$), but not in cuticle shell color ($P < 0.05$) this was measured. Furthermore, the correlation analysis between TCC in tissues and shell color (nacre shell color and cuticle shell color) was measured. Results indicated that TCC was significantly different ($P < 0.001$) with respect to cuticle shell colors and tissues, and there were significant ($P<0.001$) interactions (Table.2).The result of correlation analysis revealed that TCC of tissues exhibits no positive or negative significant correlation ($P > 0.05$) with the color of nacre shell (Table.3). For the color of cuticle shells, TCC in hepatopancreas exhibited highly significant negative correlation with L*($r = -0.316$, $P < 0.01$), and exhibited extremely significant positive correlation with a*($r = 0.571$, $P < 0.01$), b*($r = 0.617$, $P < 0.01$) and C*($r = 0.641$, $P < 0.01$, Table.4). TCC in gill, adductor, and mantle exhibited negative correlation with a*, b* and C*, especially for the adductor, exhibited the highest negative correlation with a*($r = -0.436$, $P < 0.01$), and TCC in adductor showed no significant correlation with b*($r = -0.17$, $P > 0.05$). According to the above results, we concluded that TCC in tissues of *P. fucata* with white, black, red, and golden shell colors were related to tissues and cuticle shell colors, but not nacre shell colors.

Table.2 Differences in TCC of black and red shell strains between different shell colors and tissues (n=240)

Source	*Sum of squares (type Ш)*	*Degree freedom*	*Mean square*	*F Values*	*P Values*
Model	1.540E6	7	220040.848	163.815	<0.001
Intercept	664708.481	1	664708.481	494.858	<0.001
Shell colors	28663.707	1	28663.707	21.339	<0.001
Tissues	1351471.819	3	450490.606	335.379	<0.001
Shell color × Tissues	160150.408	3	53383.469	39.743	<0.001
Error	311629.434	232	1343.230		
Total variation	2516623.649	240			
Corrected total variation	1851915.368	239			

Table.3 Pearson correlation of TCC in tissues and the nacre shell color of *P. fucata* (N=120).

TCC of tissues	*Color parameter*				
	*L**	*a**	*b**	*C**	*h⁰*
stomach	0.064	-0.114	-0.100	0.148	0.055
gill	0.063	-0.130	0.088	0.142	-0.106
adductor	0.038	-0.173	0.077	0.160	-0.105
mantle	-0.020	-0.098	0.072	0.121	-0.103

Table.4 Pearson correlation of TCC in tissues and the cuticle shell color of *P. fucata* (N=120). "A" means significant at 0.05 level, "a" means extremely significant at 0.01 level.

TCC of tissues	*Color parameter*				
	*L**	*a**	*b**	*C**	*h⁰*
stomach	-0.316^{A}	0.571^{A}	0.617^{A}	0.641^{A}	-0.184^{a}
gill	0.172	-0.386^{A}	-0.194^{a}	-0.233^{a}	0.354^{A}
adductor	0.154	-0.436^{A}	-0.170	-0.222^{a}	0.450^{A}
mantle	0.088	-0.425^{A}	-0.201^{a}	-0.248^{A}	0.417^{A}

Discussion

The composition and content of carotenoids in marine animals are influenced by genetic and environmental factors. The main source of carotenoids in marine animals is through the ingestion of algae or other organisms containing carotenoids, which are metabolized, transported, and finally deposited in the body. Therefore, food carotenoids have a direct impact on the composition and content of carotenoids in animal bodies. For example, shrimp-eating storks can accumulate astaxanthin in their body (Negro et al., 2000). Carotenoid content in the gonads of sea urchin is directly dependent on the carotenoid content in food (Shpigel et al., 2006), and if *Pomacea canaliculata* are fed with different carotenoids, the carotenoid products detected in the body are also different (Tsushima et al., 1997). Different species have different metabolic pathways for carotenoids, and the absorption of carotenoids by animal bodies is selective. At the same time, the demand for carotenoids is different in different physiological stages and environmental conditions, which will inevitably lead to the variety and content of carotenoids changing with species, physiological factors and environmental changes. For example, astaxanthin is the main carotenoid composition in tiger shrimp (Dall et al., 1995). The species of carotenoids differ greatly among shellfish, mainly β-carotene, lutein, zeaxanthin, diatoxanthin, fucoxanthin, and isoflavin etc. The composition of carotenoids in the body of the same species varies greatly due to their different metabolic pathways to carotenoids, just as the red family of aphid contains the carotenoid torulene while the green family does not (Moran et al., 2010). The yellow phenotype of pearl oysters *Pinctada fucata martensii*, is characterized by higher TCC than the black phenotype of pearl oysters, which may reflect differences in melanogenesis, retinal, and rhodopsin metabolism, biomineralisation and calcium signaling pathways (Xu et al., 2019). In this study, the culture environment and management of all experimental animals (four different shell color strains of *P. fucata*)

were all the same. We selected the experimental animals with similar size, so the TCC differences between the four shell color strains of *P. fucata* were genetic.

In this study, TCC in *P. fucata* varied among tissues and individuals with different shell colors. The stomach is the main tissue of *P. fucata* which absorbs and stores carotenoids, and TCC in the stomach is significantly higher than that in the gill, mantle, and adductor of the four shell color strains. For the black and white shell strains of *P. fucata*, TCC in gill and mantle are significantly higher than in adductor; for the golden and red shell strains of *P. fucata*, there are significant differences between the four tissues, and stomach > gill > mantle > adductor. TCC in the four different shell color strains of *P. fucata* varies among body tissues, which is consistent with results reported in other mollusks. For example, TCC in *Chlamys nobilis* (Bivalve: Pectinidae) was in the order of gonad > mantle> adductor > gill (Zheng et al., 2010). In *Paphia undulate*, the order of TCC was: foot > gonad > mantle > gill > adductor (Li et al., 2017), and in *Paphia textzle*, TCC was higher in the order of foot > gonad > gill > mantle > adductor (Deng et al., 2018). The TCC in the same tissue varies between the four shell color strains of *P. fucata*, such as in the stomach, it ranged from 53.01±28.03μg/g(white shell strains) to 238.20 ± 72.29 μg/g(golden shell strains), and it ranged from 7.51 ± 3.07 μg/g (golden shell strains) to 13.99 ± 6.41 μg/g (black shell strains) in gill. Similar results have been found in *Chlamys nobilis* (Zheng et al., 2010) and *Hyriopsis cumingii* (Li et al., 2014[a]). In addition, for the single tissue of the single shell color strains, the results of this study showed that there was a large difference between the TCC in different individuals. For example, TCC in adductor of red shell strains was 3.62 ± 2.88 μg/g, and the TCC in stomach of black shell strains was 126.94 ± 73.2 5 μg/g. The individuals of red and black shell strains are all from the same natural sea area, and the growth environment is basically the same. Therefore, it's reasonable to believe that TCC varies from individuals, and it provides a basis theoretical for breeding new varieties of *P. fucata* with high TCC or low TCC (Meng et al., 2016).

There was a significant difference between the cuticle shell color of the four different shell color strains of *P. fucata*, and the nacre shell color of the four different shell color strains of *P. fucata* was similar. It has been reported that shell color varies and is inherited in several species including *P. fucata* (Zou et al., 2014), bay scallop *Argopecten irradians* (Qin et al., 2007; Du et al., 2017), noble scallop *Chlamys nobilis* (Zheng et al., 2013), clam *Meretrix petechialis* (Zhang et al., 2018), *Hyriopsis cumingii* (Li et al., 2014[a]), Chilean scallop *Argopecten purpuratus* (Winkler et al., 2001), and Pacific oyster *Crassostrea gigas* (Evans et al., 2009; Song et al., 2017).

Carotenoid is one of the main shell pigments found in Mollusca (Williams, 2016). The spectra of most shell pigments exhibit a skeletal signature typical of partially methylated polyenes, possibly modified carotenoids (Wade et al. 2019). In this study, TCC was significantly different with respect to shell colors (nacre shell colors or cuticle shell colors) and tissues ($P < 0.001$), and there were significant interactions ($P < 0.001$). This result is similar to the results in *Chlamys nobilis* (Zheng et al., 2010), and *Hyriopsis cumingii* (Li et al., 2014[a]; Li et al., 2014[b]). TCC was significantly different with respect to cuticle shell colors and tissues ($P < 0.001$), and there were significant interactions ($P < 0.001$), but due to the significant difference in cuticle shell color of four shell color strains, analysis of variance could not be taken to confirm whether TCC is significantly different with respect to nacre shell color and tissues or not. Our results showed that TCC was not significantly related to nacre shell color, suggesting that TCC was not significantly different with respect to nacre shell color and tissues. For *P. fucata* with different shell colors, our most important finding was that TCC was related to tissues and cuticle shell colors, but not to nacre shell colors. Inversely, in *Hyriopsis cumingii,* TCC was significantly different with respect to inner shell colors and tissues. There are two possible reasons for the differences: color measurements were taken at two individual zones of the *Hyriopsis cumingii*, fringe inner shell zone and middle inner shell zone, but the color of the cuticle shell was not measured, so the conclusion drawn was that TCC in tissues of *Hyriopsis cumingii* was related to the fringe inner shell and middle inner shell; another possible reason is that the inner shell color varies among *Hyriopsis cumingii,* and the cuticle shell color varies among *P. fucata* (due to the difference in selective breeding) ((Li et al., 2014[a]; Li et al., 2014[b]).

Results also showed that TCC in tissues exhibits highly significant positive or negative correlation with the outer shell color of *P. fucata*. For example, TCC in the stomach exhibits positive correlation with C*(r = 0.641**), and TCC in adductor exhibits negative correlation with a*(r = -0.436**). This proved that TCC in tissues was significantly related to cuticle shell color of *P. fucata*. Furthermore, the author tried to devise an equation for TCC and the outer shell color:
TCC in stomach = 148.240 - 3.807L* + 11.620a* + 46.237b* - 36.116C*, R^2 = 0.497. Perhaps this was because TCC in tissues was not only related to the shell color, but also related to gender (Borodina, 2018), food (Borodina, 2016), genetics (Lu et al., 2016), and other environmental factors (Zheng et al., 2012). The results of this study show that it is possible to get the approximate amount of TCC based on cuticle shell color. In the future, more accurate equations can be devised by taking gender, diet, genetic and environmental factors into account.

Carotenoids are widespread as feed additives, use in cosmetic, and food colorants (Ye et al., 2008), and they also play roles similar to the protective roles pigments play in plants and microalgae. Carotenoids also provide a protective role for humans (Gong et al., 2016) and mollusks (Meng et al., 2017). TCC in *P. fucata* was significantly related to cuticle shell colors, which suggests that it was feasible to increase or decrease the TCC in *P. fucata* through the selection of cuticle shell colors. Furthermore, improving TCC in the *P. fucata* through selective breeding can be promising for culturing stress resistant *P. fucata* with higher levels of beneficial carotenoids for humans.

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