Pakistan J. Zool., vol. 51(5), pp 1655-1661, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1655.1661

Carotenoids in Pearl Oyster *Pinctada fucata*: The Tissue Distribution and Correlation to Color Parameters

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Article Information
Received 15 April 2018
Revised 22 May 2018
Accepted 31 May 2018
Available online 12 June 2019

Authors' Contribution
BZ and ZM did the experiments. BZ wrote the manuscript. CZ, BL, LZ, GH, JS, and SF assisted in the experitments. DY designed the experiments, provided financial support and revised the manuscript.

Key words
Carotenoids, Color parameter, Pearl oyster, *Pinctada fucata*, Tissues.

ABSTRACT

The pearl oyster, *Pinctada fucata*, is an economically important and carotenoid-containing bivalve shellfish that is cultured for pearls and acts as a source of seafood. To investigate the distribution of carotenoids in *P. fucata* and establish a more efficient method to assess carotenoid contents, we measured the carotenoid levels in selectively bred *P. fucata* individuals of different colors and analyzed the correlations between TCC (total carotenoid content) and color parameters. The percentage of total carotenoids in the adductor, gill, mantle, and visceral mass was 6.50%, 10.79%, 15.11% and 67.61%, respectively. Generally, the tissue-specific carotenoid distribution is ranked in the order being adductor < mantle < gill < visceral mass. Significant correlations were found between TCC and the color parameters, with the highest fit (r = 0.908) in the Pearson's correlation between the color parameter, *a** (red degree), and TCC of the adductor. Measuring the *a** is, therefore, likely to be an appropriate, rapid, reliable, and nondestructive method to estimate TCC in bivalves.

INTRODUCTION

Carotenoids are photosynthetic pigments that animals are deficient of de novo synthesis. Carotenoids, also as a nutrient, play multiple biological roles in morphology, behavior, immunology and fitness (Diler and Dilek, 2002; Maoka, 2011), which attracts great interests of the aquatic animal researchers and further deepens our understanding of the bioactivity of natural pigments (Rowe *et al*., 2012). In particular, dietary intake of carotenoids contributes to avoiding skin oxidative damage (Palombo *et al*., 2007) and macular degeneration (Chew *et al*., 2013). It's reported that carotenoids ameliorates the increased serum phospholipid oxidation in Alzheimer's disease patients (Ademowo *et al*., 2017). Besides, carotenoids are considered as an important dietary supplement for the skin pigmentation or body color (Meilisza *et al*., 2017; Liu *et al*., 2014), improve the anti-oxidative capabilities, hepatic HSP70 levels, and resistance to acute crowding stress (Liu *et al*., 2016), and higher levels of carotenoids in eggs associated with improved survival to zoea III (Palacios *et al*., 2001).

Carotenoids from Marine animals show different structures and many can be derived from β-carotene, fucoxanthin, peridinin, diatoxanthin, alloxanthin, and astaxanthin, *etc.* (Maoka, 2011). Carotenoids can give rise to bright color in the skin or muscle of some fish species and in the soft parts of some edible mollusks (Choubert *et al*., 2009; Li *et al*., 2010; Meyers, 1994; Tejera *et al*., 2007), which influences the quality, acceptance, taste, and flavor of these aquatic products, as well as the purchasing desire of consumers (Tunio *et al*., 2013; Yanar *et al*., 2007). Previous studies of carotenoids in mollusks were focused on the localization, sources, functions, and prospective biotechnological usage of these pigments, and concluded that the carotene amount in mollusks depends on factors such as sexual maturity, seasonal variation, sources of dietary algal, and whether the animal is artificially reared or not (Kantha, 1989). However, the carotenoid contents and its distribution patterns in edible mollusks have gained great attention recently since the benefits of carotenoids are not limited in shell and the soft parts coloration (Ji *et al*., 2013; Zheng *et al*., 2010), *e.g.* their capability of anti-oxidation (El-Agamey *et al*., 2004; Gostyukhina *et*

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0030-9923/2019/0005-1655 $ 9.00/0

al., 2013; Soldatov *et al*., 2007; Ji *et al*., 2013) and even gene expression regulation (Li *et al*., 2014; Wang *et al*., 2014; Zheng *et al*., 2012a). The industry application is also proposed as carotenoids are inclined for pearl color and bleaching (Han *et al*., 2011; Zhang *et al*., 2001).

The current methods used to evaluate carotenoid contents are overpriced and time-consuming, making it difficult to assess the carotenoid level of mollusks in a high-throughput way. Furthermore, an understanding of the level change and distribution of carotenoid in mollusks during their growth may facilitate the selective breeding. Hence, the establishment of a rapid and reliable method to quantify the carotenoids in bivalves will not only considerably optimize the commercial effectiveness in aquaculture but also advance shellfish genetics and breeding for research. Until now, a few studies have correlated reflectance color measurements with pigment content in different fruits and vegetables (Humphries *et al*., 2004; Fratianni *et al*., 2005; Meléndez-Martínez *et al*., 2003, 2007). Color values and carotenoid concentrations have been shown to be correlated (Arias *et al*., 2000; Ruiz *et al*., 2005, 2008). Color measurement has, therefore, been considered as a potential and effective method for the rapid estimation of carotenoid content, but the method has never been translated into metazoans, like bivalves.

The pearl oyster, *Pinctada fucata*, is one of the main oyster species that are cultivated in south China to produce seawater pearls. In recent years, an increasing number of cultivators have also turned to farming this edible marine bivalve as a source of food. Recently, it has been reported that some individuals of this species contain a high concentration of carotenoids (Wu *et al*., 2016; Meng *et al*., 2017), which in turn have been found to play a beneficial role in the survival rates of *P. fucata* exposed to high temperature stress (Meng *et al*., 2017). Thus, carotenoid content could serve as a preferred index to breed stress-resistant lines in selective breeding practices. In this study, we investigated the tissue distribution of carotenoids and the relationship between the color parameters and total carotenoid content (TCC) in *P. fucata*, with aim of establishing a rapid and simple method to estimate TCC in bivalves.

MATERIALS AND METHODS

Animal collection

Pearl oysters were collected from Lingshui, Hainan Province, China, and were estimated to be approximately 18 months old. To ensure that the sample population covered a wide range of colors, individuals were classified based on the coloration of the soft parts. Adductor, gill, mantle, and visceral mass were sampled and immediately frozen at -20°C.

Determination of color parameters

The adductor of each individual was sectioned laterally with a special slicer to obtain slices of the same thickness. Each adductor slice was then placed into a groove cushioned by a tin foil. The depth of the groove was a little less than the thickness of the adductor slice. The groove and slice were covered with a thin, square piece of colorless glass, and the edge of the glass was sealed by foils. Care was taken to ensure that was no air was trapped between the adductor slice and the glass, and that their contact area had a diameter greater than 4 mm. Color parameters were measured using a chroma meter tristimulus colorimeter (NS-820, 3NH, China) that was calibrated with a white porcelain reference plate. The visible reflectance spectra were obtained through a silicone photocell and a Pulsed Xenon Lamp as a source of illumination (illuminant D65, 2° view angle, illumination area diameter 4 mm). The apparatus calculated and returned the color parameters from the spectra. The color coordinates of the uniform color space CIELAB L^*, a^*, b^*, H, E, and chroma (C^*) were determined for the slice-glass contact area.

Determination of TCC

All samples were dried in a vacuum freeze-dryer and then ground to a fine powder with a pestle and mortar. TCC was determined following the method of Li *et al*. (2010) and Zheng *et al*. (2012b). The extraction solutions contain acetone and anhydrous sodium sulfate. To avoid degradation and isomerization of carotenoids, dark brown tubes were used and the extractions were performed in an aphotic environment. Approximately 0.1 g of homogenized sample was mixed with 0.1 g anhydrous sodium sulfate, diluted with 2 mL acetone, and incubated at 20°C in a dark room for 3 h at 200 rpm/min. Then the mixtures were centrifuged at 5000 rpm for 5 min. The resulting supernatant was separated, and the insoluble residue was treated as the way described above. TCC was determined by spectrophotometry (at 450 nm) and calculated using the following formula:

$$\mathrm{TCC}\left(\mathrm{mg}/\mathrm{g}^{1}\right)=\frac{A_{450}\times V\times n\times 10000}{E\times m}$$

Where, A_{450} is the optical density value at 450 nm, 10000 is a constant, V is the volume of the extracting solution (ml), n is the number of dilution times, E is the extinction coefficient (mean value $A^{1\%}_{1cm}$ 2500 of colored carotenoids), and m is the mass (g) of the sample.

Data analysis

The percentage of the total carotenoid (TC) quantity, the percentage of fresh weight (FW), and the TCC of the collective soft parts (marked as TCC_S) were calculated for each individual using the formulas below:

$$\%\ \text{of TC} = \frac{TCC_i \times m_i}{TCC_A \times m_A + TCC_G \times m_G + TCC_M \times m_M + TCC_V \times m_V}$$

$$\%\ \text{of FW} = \frac{m_i}{m_A + m_G + m_M + m_V}$$

$$TCC_S = \frac{TCC_A \times m_A + TCC_G \times m_G + TCC_M \times m_M + TCC_V \times m_V}{m_A + m_G + m_M + m_V}$$

Where, TCC_i and m_i are the carotenoid content and weight of one of the four tissues (i = adductor, gill, mantle, or visceral mass); TCC_A, TCC_G, TCC_M, and TCC_V represent the TCC of the adductor, gill, mantle, or visceral mass; and m_A, m_G, m_M, and m_V represent the mass of them, respectively.

It was difficult to directly analyze and compare the distribution characteristics of carotenoids in different parts of the collected soft parts. So the relative TCC was employed according to the following formula:

$$\text{Relative } TCC_i = \frac{TCC_i}{TCC_S}$$

Statistical analysis

The results were subjected to a one-way analysis of variance (ANOVA). Correlations between TCC and the color values were analyzed using Pearson's correlation. All statistical analyses were conducted using SPSS Software for Windows (SPSS, 19.0, IBM, USA). Statistical significance was set at $P < 0.05$ for all analyses unless noted otherwise.

RESULTS

Distribution of carotenoids in P. fucata

The percentage of carotenoids in the adductor, gill, mantle, and visceral mass was 6.50%, 10.79%, 15.11% and 67.61%, respectively, with significant differences ($P < 0.05$) between the tissues (Table I). The percentage of fresh weight in different tissues were ranked as adductor (17.38%), gill (17.29%), mantle (25.84%) and visceral mass (39.49%) with significant differences except the adductor-gill pairwise comparison. Carotenoids were unevenly distributed in the different parts of soft tissues. The relative TCC of the visceral mass, gill, mantle, and adductor were 1.73, 0.64, 0.59, and 0.36, respectively (Table I). The average TCC of the visceral mass was 1.73 times than that of the collective soft parts. The TCC in *P. fucata* can be generally summarized in the order of visceral mass > gill > mantle > adductor, with significant differences in pairwise comparisons, except the gill-mantle pairing.

Table I.- Mean (standard error) of percentage of total carotenoid amount, percentage of fresh weight and relative TCC (Mean ± SD) in different tissues.

Combination	Tissues			
	Adductor	Gill	Mantle	Visceral mass
Percentage of total carotenoid amount	6.50 ± 4.01^{d}	10.79± 3.46^{c}	15.11± 4.25^{b}	67.61± 9.49^{a}
Percentage of fresh weight	17.38± 3.08^{c}	17.30± 4.48^{c}	25.84± 3.58^{b}	39.49± 5.79^{a}
Relative TCC	0.36± 0.15^{c}	0.64± 0.21^{b}	0.59± 0.17^{b}	1.73± 0.26^{a}

Means with different letters indicate significant difference ($P < 0.05$).

Table II.- Pearson correlation between TCC of the four tissues and soft part.

	TCC_A	TCC_G	TCC_M	TCC_V	TCC_S
TCC_A	1	0.795**	0.959**	0.790**	0.891**
TCC_G		1	0.728**	0.652**	0.759**
TCC_M			1	0.791**	0.886**
TCC_V				1	0.972**
TCC_S					1

**, $P < 0.01$; A, adductor; G, gill; M, mantle; V, visceral mass; S, softpart.

Pairwise correlation of TCC among tissues

The Pearson's correlation coefficients of TCC among the four tissues (TCC_A, TCC_G, TCC_M and TCC_V) and the collective soft parts(TCC_S) are listed in Table II. The correlation coefficient between each pair of TCC_A, TCC_G, TCC_M, TCC_V and TCC_S were found to be significant ($P < 0.01$). The TCC of all tissues had a high correlation with the TCC of the collective soft parts, especially that of the visceral mass (r = 0.972) and the adductor (r = 0.891). Moreover, the correlation coefficients between the TCC of the adductor and of other three tissues were also found to be relatively high, with the highest being between the TCC of the adductor and the mantle (r = 0.959).

The linear correlation between the TCC of these four tissues and the TCC of the collective soft parts were found to be different (Fig. 1), and the linear correlation between the TCC of the visceral mass and that of the collective soft parts was found to be the highest ($R^2 = 0.9453$). The second highest correlation of TCC was seen between the adductor and the collective soft parts ($R^2 = 0.7938$), and the lowest was between the gill and the collective soft parts ($R^2 = 0.5755$).

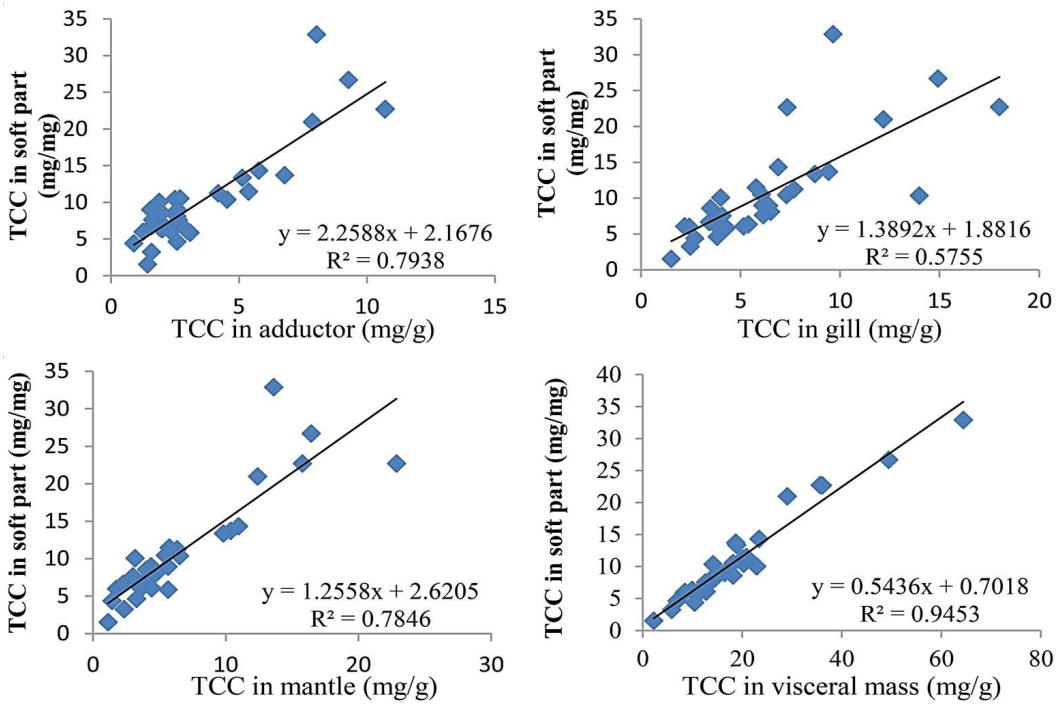

Fig. 1. Linear correlation between TCC of the four tissues and TCC of the soft part.

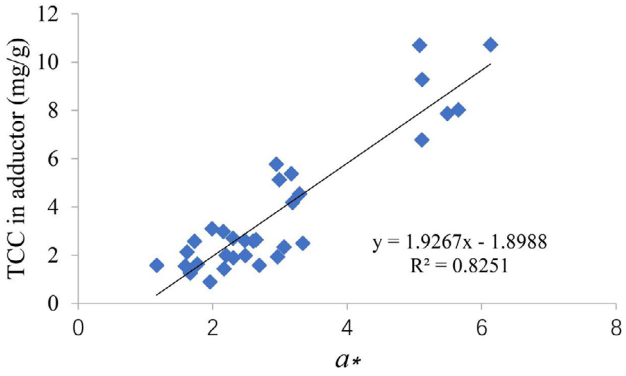

Fig. 2. Linear correlation between TCC and *a** in adductor.

Table III.- Pearson correlation between TCC and color measurements of the adductor.

	*L**	*a**	*b**	*C**	*H*	*E*	*a*/b**
r	-0.645**	0.908**	0.692**	0.728**	-0.773**	0.747**	0.817**

**, $P < 0.01$.

Relationship between TCC of the adductor and the color parameter

Correlation coefficients between the TCC_A and the color parameters were assessed (Table III). Significant correlations were observed between TCC_A and all color parameters ($P < 0.01$). The color parameter, *a**, showed the highest correlation coefficient with TCC_A (r = 0.908), indicating a highly positive correlation, which is also in agreement with previous studies (Meléndez-Martínez *et al*., 2003, 2007). The linear correlation between TCC_A and *a** ($R^2 = 0.8251$) is shown in Figure 2.

DISCUSSION

Pinctada fucata is a multipurpose bivalve that is cultured in the south of China for pearl production and as a source of food. The soft part tissues of this species are rich in carotenoid pigments and exhibit a wide range of coloration. Carotenoids showed potential in improving the survival rates of *P. fucata* under high-temperature stress by enhancing the antioxidant system (Meng *et al*., 2017). Investigations into the genetic features of carotenoids in this bivalve may provide basic support and guidance for genetic and breeding practices. Attempts to correlate color parameters and the TCC began in plant-based research many years ago in order to develop more effective and efficient methods of carotenoid content quantification (Arias *et al*., 2000; Ruiz *et al*., 2005, 2008), but similar research has never been carried out in animals.

The present study found a significant correlation between the TCC and the color parameter, *a**, in *P. fucata*, suggesting that the measurement of this color parameter

may offer the most appropriate, rapid, reliable, and nondestructive method to estimate the carotenoid content in bivalves. This linear correlation may, however, be improved to some extent in further research by improving the accuracy of the TCC determination in the adductor.

The distribution of carotenoids in *P. fucata* was found to be uneven and higher in specific tissues, which has similarly been reported in other mollusks (Zheng *et al*., 2010, 2012a). In our study, the visceral mass was found to have the most total carotenoids (67.61%) and fresh weight (39.49%), and had the highest relative TCC (1.73 times as much as the TCC of the collective soft parts). The relative TCC of the adductor was found to be the lowest (0.36) with 6.50% of the total carotenoid amount and 17.38% of the fresh weight. This variation in the TCC among the tissues is consistent with the findings reported for other marine mollusks (Zheng *et al*., 2010, 2012a; Wu *et al*., 2016). For example, TCC followed the order of gonad > mantle > adductor > gill in the noble scallop (Zheng *et al*., 2010), and Wu *et al*. (2016) found the TCC in *Pinctada martensii* to be significantly different among the adductor, gill, hepatopancreas, mantle edge, and mantle center of different cultured groups.

In these studies, the highest correlation coefficient between the TCC of the four tissues and that of the collective soft parts was obtained for the visceral mass, which may be due to there being high-TCC tissue in the visceral mass. For example, the gonad is reported have the highest TCC in noble scallop (Zheng *et al*., 2010), and hepatopancreas is likely to be an important tissue in the transformation and accumulation of carotenoids in *P. martensii* (Wu *et al*., 2016). In addition, the present study found the correlation coefficient between each pair of the TCC of the different tissues to be significantly different, and that the high linear correlations among them revealed the possibility that the TCC of the collective soft parts could be evaluated by that of other tissues, such as the adductor and visceral mass. To our knowledge, our results are the first of their kind reported for mollusks.

This difference in the correlation coefficient may due to the metabolism of carotenoids: a result of the absorption, conversion, transfer, and/or storage of these pigments. Previously, the work of Li *et al*. (2010) revealed that the main pigment isolated from the muscle tissues of the Yesso scallop (*Patinopecten yessoensis*) was identified as the carotenoid, pectenolone, implying a certain level of selectivity in the absorption or conversion of carotenoids in bivalves. Another study showed that the TCC in the noble scallop (*Chlamys nobilis*) was related to body tissue, shell color, and gender (Zheng *et al*., 2012b). This latter study demonstrated that carotenoids might be transferred from other tissues to gonad during maturation in mollusks, particularly in the female individuals (Zheng *et al*., 2012b). In fact, earlier report of Kantha (1989) had indicated that sexual maturity, seasonal variation, sources of dietary algal, and whether the animal is artificially reared or not may all impact on the carotene amount in mollusks. And similar research in wild marine shrimps demonstrated that the carotenoid contents were considerably higher during spring and summer than that in winter and autumn seasons (Yanar *et al*., 2004), too. What's more, recent research on the expression pattern of *PySCD* in two types of Yesso scallops detected a significantly greater number of *PySCD* transcripts in the carotenoid-rich scallops, implying that *PySCD* might be involved in carotenoid accumulation (Li *et al*., 2015). So, the high-level TCC in pearl oysters in our research probability due to the sample season and the expression of some genes which need further studies.

Although our findings indicate that the high level of correlation among the TCC of different tissues makes it possible to estimate the TCC of every individual just by evaluating the TCC of one tissue, further research is required to better understand the correlations among the different tissue types in *P. fucata*.

ACKNOWLEDGEMENTS

This work was supported by the Special Fund for Agro-scientific Research in the Public Interest (2015TS08), Guangdong Provincial Science and Technology Project (2015A020209045), Special Fund by Sanya Government (2014KS04), Special Fund for Marine Fisheries Research and Extension of Guangdong Province (Z2014006, Z2015006, Z2015013, Z2015009, B201601-Z03 and Z2014003) and the Key Science and Technology Plan Projects of Hainan Province (ZDYF2016086), and Earmarked Fund for China Agriculture Research System (Grant No. CARS-48).

Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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