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Growth marks in eyestalks of *Portunus trituberculatus*: A development of technique and evidence of molting

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Abstract

The analysis of growth bands in the eyestalk has been increasingly used for estimating crustacean ageing and molting. In this study, we developed an effective method to process and observe the eyestalk microstructure of the swimming crab (*Portunus trituberculatus*). We found that dark pigmentation as a result of boiling has an influence on the observation of the eyestalk microstructure. Choosing an unboiled eyestalk, this study compared the cross section and longitudinal section, and concluded that the cross section is suitable for the observation of growth increments with 6.1% CV (coefficient of variation), and growth bands are suitable for the observation of the longitudinal section with 5.4% CV. The width of growth increments near the edge of the endocuticle is small, and the width of growth increments of the middle part of the endocuticle is large. Relationship of number of growth bands to molting time was fitted to a linear function with the slope not significantly different from 1, indicating that growth bands are formed associated with molting. Periodicity of growth increment formation was calculated as 3.7 d, however was not verified. Our results provide a new improved technique for identification of crustacean molting and growth.

Key words: growth bands, growth increments, *Portunus trituberculatus*, eyestalk, molting

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1 Introduction

The swimming crab (*Portunus trituberculatus*) is widely distributed in Japanese, Korean and Chinese inshore waters (Miyake, 1983). It is the most important species in China and Japan because of its substantial economic value, and dominates the portunid crab fisheries of the world (Secor et al., 2002). There are three common species of *Portunus* in China, being the Herbst (*P. sanguinolentus*), Linnean (*P. pelagicus*) and swimming crab. The catch of the swimming crab is proportionally the largest, at around 80% of the total catch of the *Protunus* fishery (Dong, 2012).

There are six methods for estimating the age of Decapoda (Kilada et al., 2012; Vogt, 2012). Rearing in captivity is the most precise and reliable ageing technique (Vogt, 2012). Tagging and recapture is another direct and precise method. Several decapod species have already been marked by internal tags, released into the wild and recaptured (Bubb et al., 2002; Weingartner, 1982). In 2002, the swimming crab was tagged and recaptured using this method (McPherson, 2002). Analysis by length-frequency distribution was the most common method used in early research to identify crustacean age. This depended on the identification of modes in distribution, which could be equated with year classes or with recruitment cohorts. The lipofuscin method of ageing is based on the continuous life-long deposition of lipofuscin in ageing cells (Vogt, 2012). The lipofuscin method for estimating ageing has repeatedly shown to be superior to size or weight based ageing estimation techniques (Belchier et al., 1998). The radiometric method is suitable for determining the age of the exoskeleton, which reflects the time period which has elapsed from the last molting (Gardner et al., 2002; Vogt, 2012).

Calcified structure analysis has emerged in recent years for ageing crustaceans. Age determination in the Decapoda is severely hampered by the lack of permanent age information bearing structures such as the scales and otoliths of fish (Skurdal et al., 1985), the bivalve shells (Schöne et al., 2005) or the genital plates of sea urchins (Flores et al., 2010). Kilada et al. (2012) first

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reported the presence of clear and readable growth increments in the calcified structures of *Nephrops norvegicus* and *Cancer pagurus* that were thought to be retained through molting. It was concluded through chemical tagging of young known-age *Homarus americanus* that the gastric mill and eyestalks are retained through the molting process. More importantly, these calcified structures provide a possible mechanism for growth increments to record age in the endocuticle throughout the life of the crustacean (Sheridan et al., 2015). Growth bands within eyestalks and gastric mills were first observed in the snow crab (*Chionoecetes opilio*), northern shrimp (*Pandalus borealis*) and American lobster (*H. americanus*) (Kilada et al., 2012), and the number of growth bands was equal to the actual age in year of the sample (Kilada et al., 2012).

The microstructure of the eyestalk of a swimming crab consists of four parts, which are the epicuticle, exocuticle, endocuticle and membranous layer (Jiang et al., 2018). The epicuticle is a thin layer with uniform texture locating the outermost of the eyestalk (Jiang et al., 2018). The exocuticle, a calcified chitin layer, consists of many cylindrical objects with melanin-like pigment locating below the epicuticle. The endocuticle, the highest calcified chitin layer, accounts for the majority of the entire thickness of the eyestalk with less pigment (Jiang et al., 2018). In the endocuticle, obvious periodic growth increments parallel to the surface of the eyestalk and also some increments perpendicular to the surface of the eyestalk can be found (Jiang et al., 2018). The membranous, an thin uncalcified layer, has the highest transparency (Jiang et al., 2018).

Most of the above studies focused on the relationship of growth bands with age. In addition to this, it is uncertain which is the most appropriate pretreatment method and grinding method for the eyestalks and gastric mills. In this study, we compare different pretreatment methods and grinding sections in order to select the most appropriate method and section. Periodicities of the growth increments and bands were analyzed relating to age and time of molting.

2 Materials and methods

2.1 *Sampling*

A total of 31 swimming crabs (with carapace length (CL) ranges from 34 mm to 79 mm, carapace width (CW) ranges from 65 mm to 161 mm, and weight ranges from 12.6 g to 197.3 g) were sampled from the East China Sea (32°10′–32°18′N, 123°22′–123°27′E) during October and December in 2016. A total of 24 known age swimming crabs (with CL ranges from 65 mm to 79 mm, CW ranges from 101 mm to 127 mm, and weight range from 111.2 g to 228.5 g) were sampled from the Jiangsu Qidong Scientific Research Base of the Shanghai Fisheries Research Institute (31°49′N, 121°39′E) in January 2017.

2.2 *Eyestalk processing*

Specimens were thawed in the laboratory. CL and CW were measured, and body weight was weighted according to the standard methodology (Dai et al., 1977). Two kinds of methods were used to remove the internal muscles and connective tissues of eyestalks. One of the paired eyestalks was using anatomical needles and pointed tweezers and the other is boiling in 100 °C water for 2 min. After that the eyestalks were stored in a 2 mL centrifuge tube with 75% alcohol.

Cross and longitudinal sections were respectively performed to the left and right eyestalks to observe their microstructure (Fig. 1). The eyeball was removed from the distal eyestalk and proximal eyestalk for the convenience of embedding the eyestalk (Fig. 2). The prepared eyestalks were placed vertically in a mold and embedded with epoxy mixed with a hardener (Fig. 3b, Fig. 4b). The embedded mold was left for 24 h to harden (Fig. 3c, Fig. 4c). The mold was grounded using 240, 600, 1 200 and 2 500 grit waterproof sandpaper in succession along the two sections to the middle of the eyestalk (Fig. 3d, Fig. 4d), and polished with 0.05 μm aluminum oxide powder (Fig. 3e, Fig. 4e). The mold was attached to a glass slide (Fig. 3f, Fig. 4f) and grounded again with grit waterproof sandpapers to the middle of the eyestalk (Fig. 3g, Fig. 4g). During the grinding process, the blocks needed to be constantly checked with a microscope to avoid overgrinding. We polished with 0.05 μm aluminum oxide powder (Fig. 3i, Fig. 4i) until the section of the growth increments was clearly visible (Fig. 3h, Fig. 4h).

Increments within the eyestalk were observed at 1 000× and 400× magnifications using an Olympus light microscope, and digital images of the whole sections were captured with a HTC3.0 Camera (Shanghai Weitu Technology Development Co.) and then processed with Photoshop7.0 software. The width of the growth increments of eyestalks were measured using Digimizer image processing software (Shanghai Weitu Technology Development Co.).

2.3 *Counting experiment*

The numbers of growth increments and growth bands for each eyestalk were counted three times, independently, by three

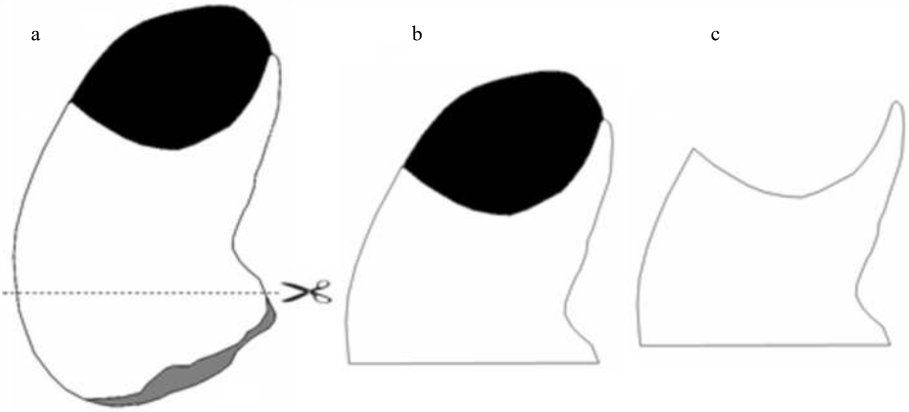

Fig. 1. Schematic of trim eyestalk production.

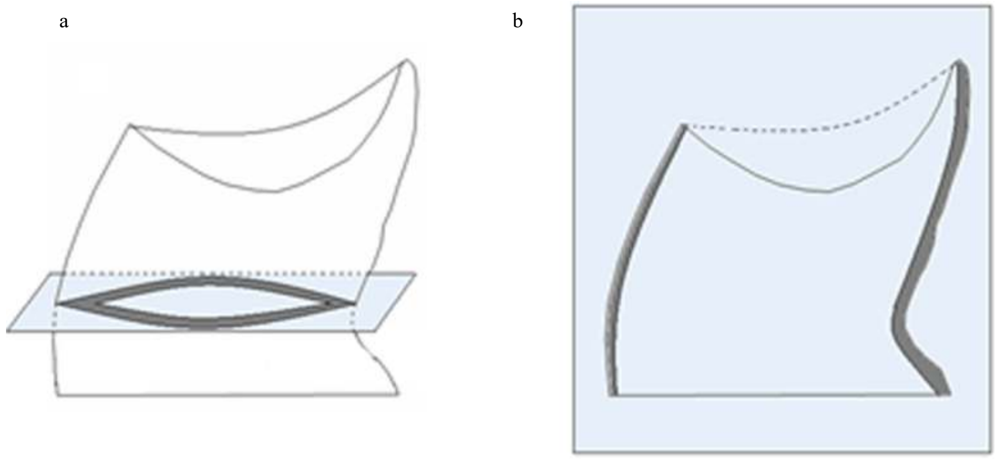

Fig. 2. Cross section (a) and longitudinal section (b) (dark part).

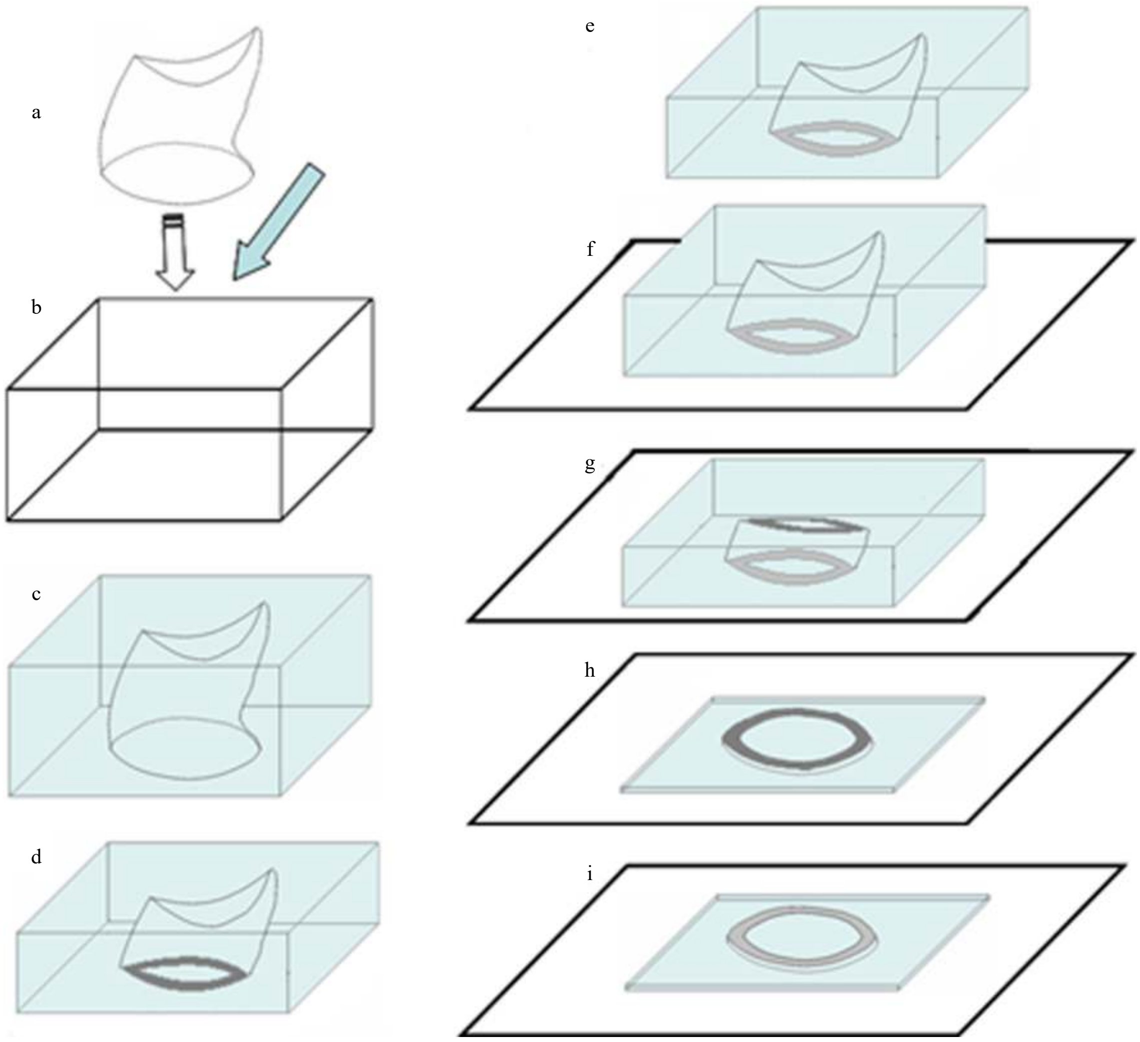

Fig. 3. Schematic of eyestalk cross section slices production (a. eyestalk, b. embed, c. harden, d. grind, e. polish, f. fix, g. rough grinding, h. fine grinding, and i. polish).

skilled people. The precision of the counted data was assessed with a coefficient of variation (CV), calculated as the ratio of the standard deviation over the mean (Chang, 1982). The formula for the CV is as follows:

$$\mathrm{CV}=\frac{\sqrt{\dfrac{(R_1-R)^2+(R_2-R)^2+(R_3-R)^2}{2}}}{R}\times 100\%, \qquad (1)$$

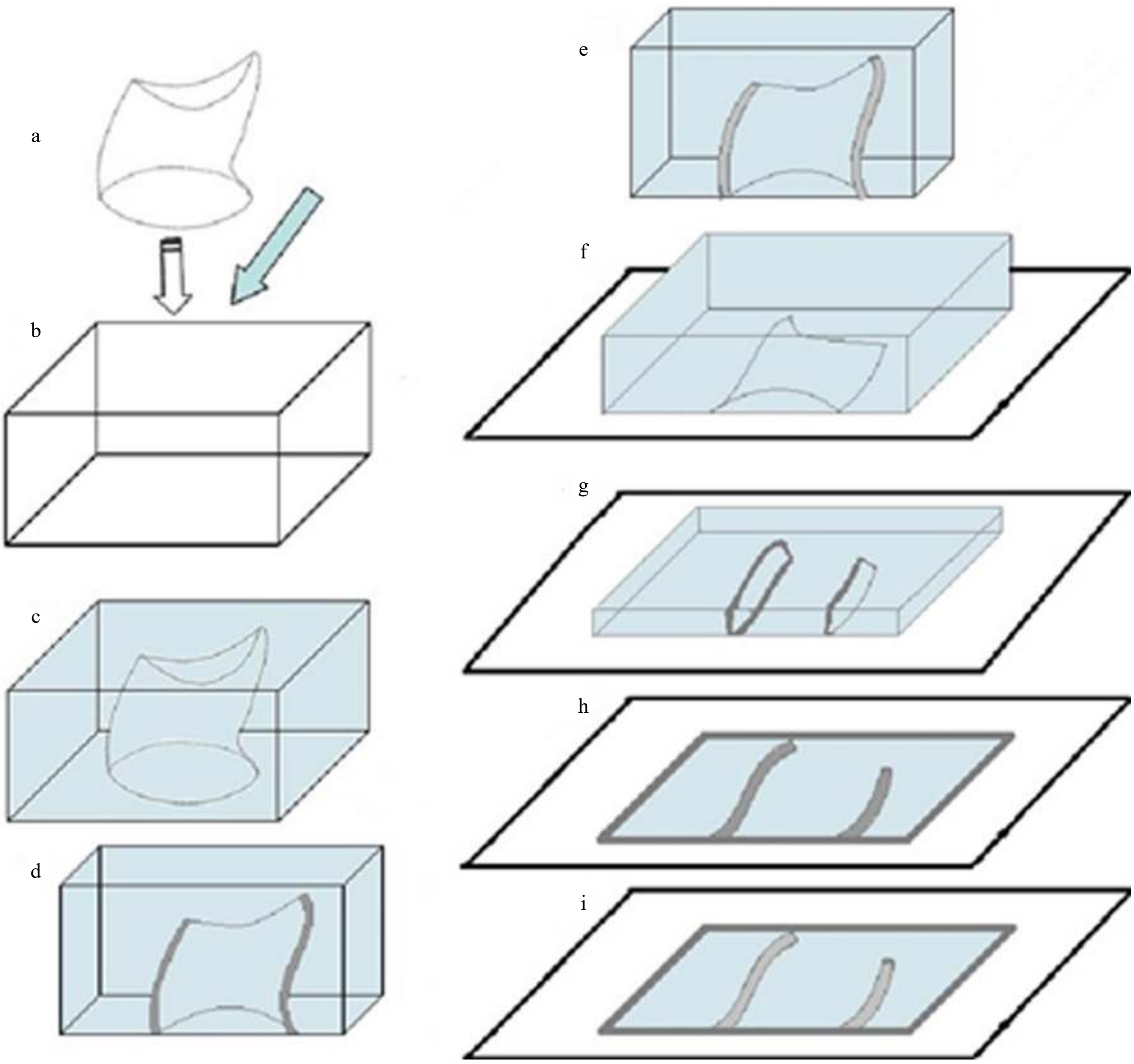

Fig. 4. Schematic of eyestalk longitudinal section slices production (a. eyestalk, b. embed, c. harden, d. grind, e. polish, f. fix, g. rough grinding, h. fine grinding, and i. polish).

where R_1, R_2 and R_3 represent the independent three counts of each sample, and R represents the average of the three counts.

In general, in the age identification study, the cumulative critical number of the growth pattern count is: independent repeat count 2 to 3 times, the number of the coefficient of variation between every time should be not more than 10% (Jackson et al., 1997; Oosthuizen, 2004).

2.4 *Potential periodicity verification*

According to the study of Gao et al. (2016), the molt times of swimming crabs were calculated by using weight data. In order to clearly determine an individual's growth stage, a unit step function (Yang and Bai, 2012) was used to build the relationship of weight and molt times of swimming crabs:

$$\begin{aligned} f(t) =& u(t) + u(t-0.014) + u(t-0.033) + u(t-0.104)+ \\ & u(t-0.411) + u(t-1.4) + u(t-3.89) + u(t-11.56)+ \\ & u(t-32.77) + u(t-66.28) + u(t-114.02)+ \\ & u(t-177.84) + u(t-256.61), \end{aligned} \tag{2}$$

where t is weight of swimming crab; $u(t)$ is the fuction of molt numbers of swimming crab, $u(t)=0, t<0; u(t)=1, t>0$.

A linear regression was conducted to fit the relationship of growth bands and molt times, and an analysis of covariance (ANCOVA) was used to test whether the slope is significantly different from 1 or not. Difference was considered significant when P<0.05.

3 Results

3.1 *Microstructures of eyestalk sections*

Among all boiling treated eyestalks, both growth increments and bands of 70% sections were unclear, while all of unboiled eyestalks had clear view in microstructure (Fig. 5). It is because the view microstructure in boiled eyestalks was affected by black substance. Four layers, i.e., the epicuticle, exocuticle, endocuticle and membranous layers, 148 were observed in both the cross and longitudinal sections (Fig. 6). Clear growth increments were observed in 96.2% cross-section slices and 93.3% longitudinal section ones.

Growth increments and bands were observed in the endocuticle layer, but rarely in the other three layers (Fig. 7). Growth increments have a light color, small width, close arrangement,

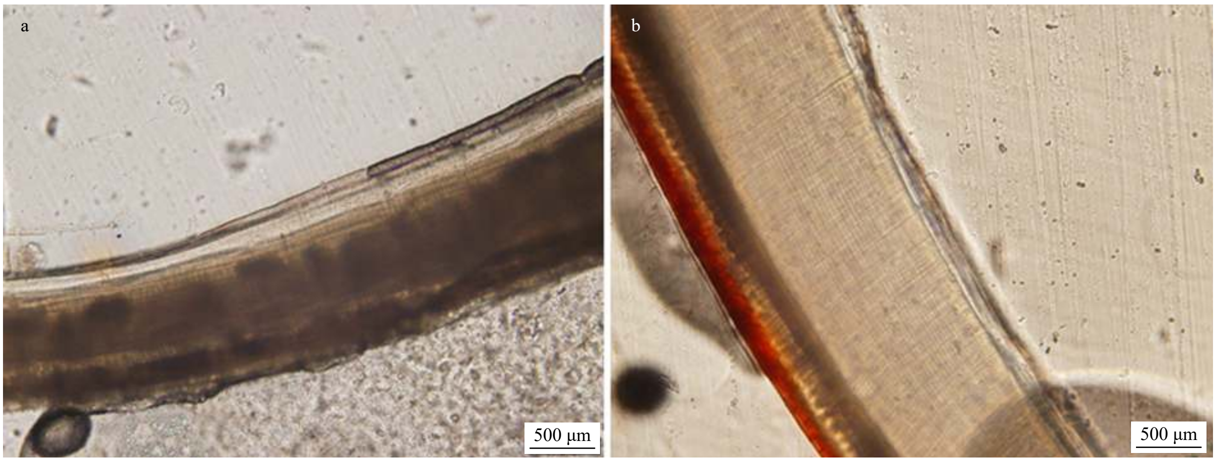

Fig. 5. The boiled eyestalk slices (a) and unboiled eyestalk slices (b).

and are easy to be observed. Growth bands have a dark color, large width, sparse arrangement, and are difficult to be observed. A growth band could contain a few or more growth increments. Clear growth bands were observed in 68.3% longitudinal section particularly in the upper part of the eyestalks (Fig. 8), while only 46.7% can be observed in cross-section ones (Table 1). The growth increments counting CV of the cross-section and longitudinal section slices were 6.1% and 7.5%, respectively. The growth bands counting CV of the cross-section and longitudinal section slices were 9.6% and 5.4%, respectively.

3.2 *Growth bands and its relationship to molting*

Within endocuticle, the widths of growth increments are wider in the middle than near the edge (Fig. 9). Growth bands counted on the eyestalks of swimming crabs are similar to the molt times (Fig. 10). The relationship of the number of growth bands to the estimated molt times was fitted significantly to a lin-

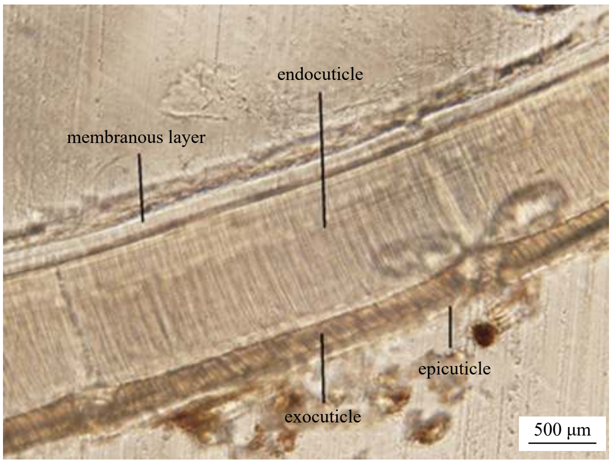

Fig. 6. Four parts of eyestalks.

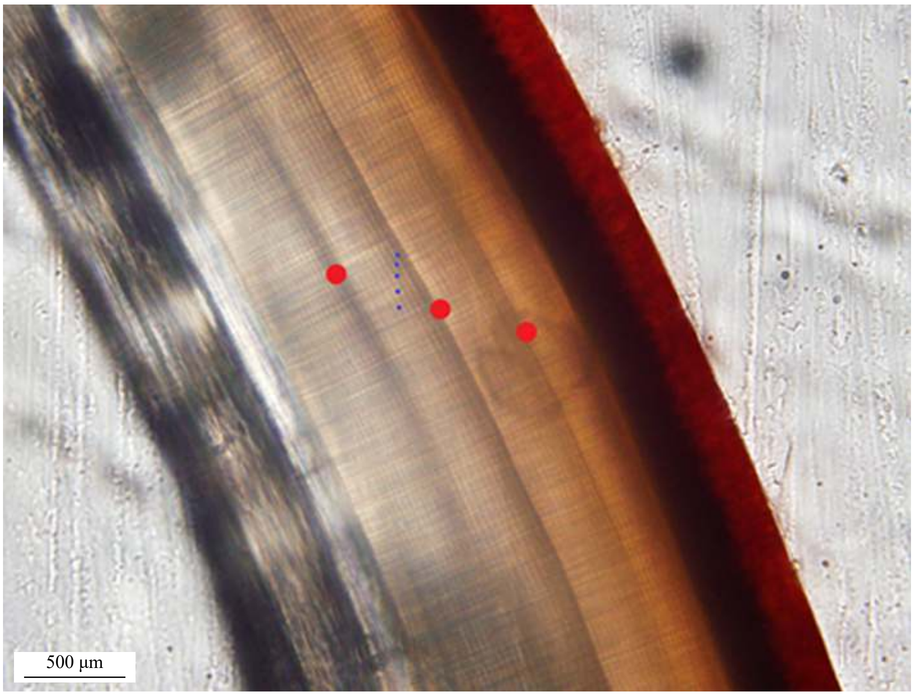

Fig. 7. The growth bands (red points) and growth increments (blue points) of eyestalks.

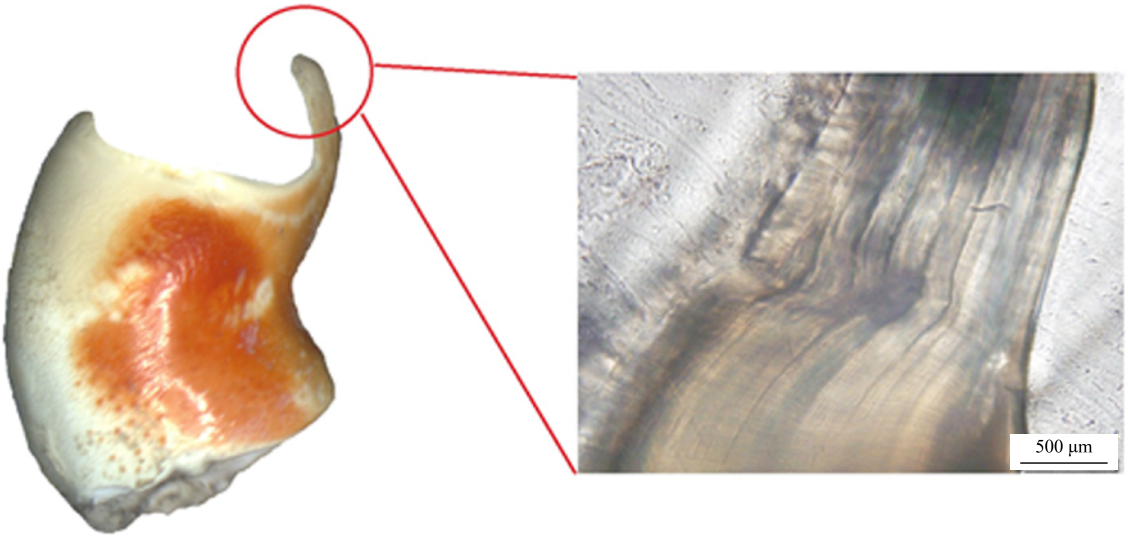

Fig. 8. clear growth bands in slices of eyestalks upper part.

Table 1. The proportion of growth increments and bands in cross section and longitudinal section slices

	Cross section slices proportion/%	Longitudinal section slices proportion/%
Growth increments	96.2	93.3
Growth bands	46.7	68.3

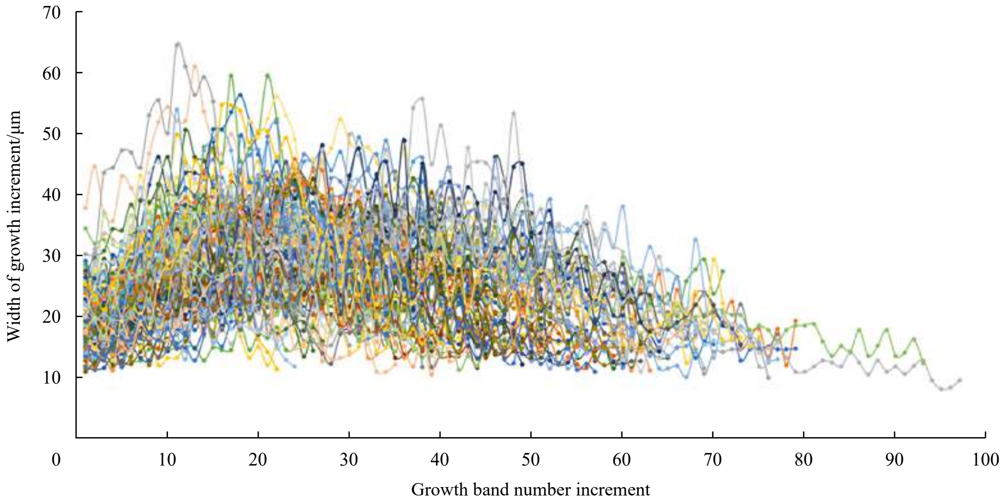

Fig. 9. The width of growth increment vs. growth band increment of *Portunus trituberculatus*.

ear function: y=0.961 5x+0.102 6 (R^2=0.463 8, n=24, P<0.05), and the slope was not significant difference from 1 (ANCOVA, P< 0.05).

3.3 *Growth increments and potential periodicity*

Samples of swimming crab were reared in the Jiangsu Qidong Scientific Research Base of the Shanghai Fisheries Research Institute, 24 known age samples of 9 months old were used, with CL ranging from 6.5 cm to 7.9 cm, CW ranging from 12.49 cm to 15.35 cm, and weight ranging from 111.17 g to 221.98 g. By dividing the number of counted growth increment with known age, the formation periodicity of one growth increment was 3.7 d (Table 2).

4 Discussion

With a typical crustacean shell (Zhao, 2006), this study also observed epicuticle, exocuticle, endocuticle and membranous

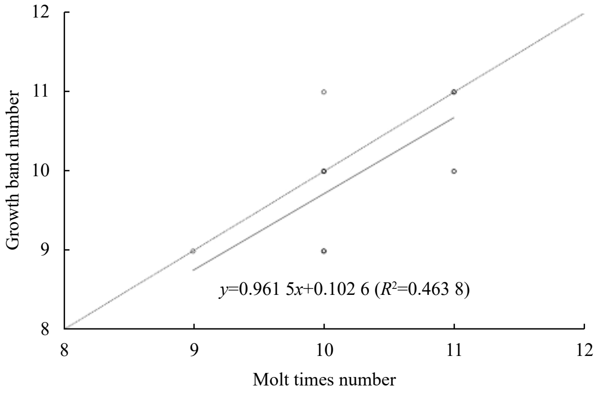

Fig. 10. The relationship between number of growth bands and molt times.

Table 2. Information of reared swimming crab

CL/ cm	CW/ cm	Weight/ g	Growth increment count	Growth band count	Molting time
6.90	13.30	143.97	60	9	10
7.40	14.33	172.94	79	9	10
7.90	15.35	221.18	81	11	11
6.90	13.30	141.89	65	10	10
7.90	15.35	216.25	83	11	11
6.80	13.10	144.93	66	10	10
7.20	13.92	169.60	60	10	10
6.60	12.69	139.75	60	10	10
6.80	13.10	139.27	62	9	10
6.90	13.30	151.06	73	9	10
6.50	12.49	111.17	76	9	9
7.00	13.51	155.41	63	9	10
6.60	12.69	121.31	85	10	10
7.50	14.53	181.83	64	11	11
7.90	15.35	228.52	90	10	11
7.70	14.94	218.16	68	10	11
6.90	13.30	149.66	64	10	10
7.90	15.35	221.98	95	11	11
7.20	13.92	176.12	69	11	11
7.20	13.92	173.59	63	10	10
7.20	13.92	158.32	82	10	10
6.70	12.90	136.12	65	9	10
6.50	12.49	123.92	65	11	10
7.80	15.15	124.85	64	10	10

Note: CL represents carapace length, and CW represents carapace width.

layers in the eyestalk of the swimming crab, and this is consistent with the result provided by Kilada et al. (2012) and Sheridan et al. (2015). Feng (1984) classifies shells into two layers: a non-chitin layer and a chitin layer. The chitin layer is divided into three parts, namely the outer-chitin layer, middle-chitin layer and inner-chitin layer, which is identical with the observation of this research. Feng (1984) argues that under observation, using an electron microscope, it can be seen that the fibrillose crystal structure of the chitin layer is arranged horizontally, forming wide (light) -narrow (dark) bands. Fibrils are densely ranged in the dark band; in the related dark band, parts of the fibril curve are upward, and parts curve are downward. Parts of the fibrils are arranged vertically, which accords with the few growth increments perpendicular to the epishell of the eyestalk observed in this research. Apart from this, the light and dark bands are also identical with the growth bands observed in the study. Lin and Lin (1993) found a stratiform parallel to the surface of the shell in a crustacean shell, with the main part being chitin and protein. This study found that the spacing of growth bands at the endocuticle edge is small, and the spacing of growth bands at the central part of the endocuticle area is large. The growth rates at different growth stages may lead to different widths of growth increments. The growth increments near the exocuticle represent the early growth stage, and near the middle of the endocuticle represent the larvae stage, and the membranous layer represents the adult phase. The trend of the width of growth increment is consistent with the trend of the growth rate of the swimming crab (Sun et al., 1984), which shows that the greater the width of growth increment, the faster the growth rate at different growth stages.

Some previous studies believed that boiling the calcified structures make it easier for any adhering tissue to be removed from the structures, resulting in a much better embedding in epoxy resin (Sheridan et al., 2015). However, on comparing the boiled and unboiled eyestalk slices, this study considered that the boiling pretreatment of the eyestalk is not conducive to observation. Although boiling the eyestalk is convenient for removing any organisms attached to the eyestalk, a large amount of black substance will be produced, blocking the inner part of the eyestalk. On the other hand, despite being attached to some organic tissue, the unboiled eyestalk slices could be observed clearly. This tissue was only present in the outer layer of the membranous layer and did not affect the observation. Therefore, boiling the eyestalk may not be an appropriate procedure for pretreatment.

Clore (2014) used a cross section plane for identifying *Procambarus clarkii* growth increments. Krafft et al. (2016) and Sheridan et al. (2015) used a longitudinal section plane for identifying northern shrimp (*P. borealis*), Antarctic krill (*Euphausia superba*) and *N. norvegicus* growth bands. No study takes both cross and longitudinal sections into consideration in order to find a suitable section plane. As the result of comparing these two sections, we have found and developed a new way of using eyestalks to determine crustacean age. Experiments show that the clearest and most readable growth bands were observed in the longitudinal sections of the eyestalk (CV is 5.4%), and the clearest and most readable growth increments were observed in the cross sections of the eyestalk (CV is 6.1%). The results show that the cross section is suitable for the observation of growth increments, and the longitudinal section is suitable for the observation of growth bands.

Kilada et al. (2012) found in their experiments that the age estimated by growth bands in the eyestalk of the American lobster (*H. americanus*) is consistent with their real age in year. However, in the research of *Sclerocrangon boreas* and *Chionoecetes opilio*, the number of the growth bands were found to be independent of age. In this study, in the eyestalks of 24 swimming crab samples, that had been reared for 9 months, there were 7–11 growth bands, which were apparently independent of age. As a consequence, this study assumes that the number of the growth bands is dependent on the number of molts. The fitting result of the estimated number of molting and the associated number of growth bands potentially verified this assumption. Because the number of molts used in this study is an estimated number based on the weight of the samples, compared with the actual number of molts, there may be some small error. In order to verify the assumption, the actual number of molts are needed.

Previous study also found growth increments in the eyestalks of *Procambarus clarkii* (Clore, 2014). However, without specific ages for the *Procambarus clarkii* samples, it is difficult to determine the exact nature of the growth increments. In this study, 24 samples that were known to be 9 months old were used. The range of the number of growth increments was from 60 to 95. Therefore, the formation of a single growth increment took 2.9–4.5 d, average value (3.7 ± 0.8) d. However, the potential physiological mechanism for the formation of growth increment is still unknown, and also how to identify the first increment is required. There are many efforts need to be made in the future to verify the time when growth increments are formed on the eyestalks of swimming crabs.

In order to verify the assumption of the relationship between the number of growth bands and molt times, and the relationship between the number of growth increments and days, this study observes and analyses the microstructure of the swimming crab. An understanding of the microstructure and its relevance to the composition of the eyestalk is also stated in this study. This study provides an improved and reliable method for the molting identification of crustaceans. The results of this study, in concert with those of Kilada et al. (2016) and Sheridan et al. (2015), indicate that it may soon be possible to routinely age, a range of economically important crustacean species for age-based stock assessments.

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