Songklanakarin J. Sci. Technol.
40(2), 397-401, Mar. - Apr. 2018

http://www.sjst.psu.ac.th

Original Article

Characterization of molting stages in the giant freshwater prawn, *Macrobrachium rosenbergii* using setagenesis of pleopod

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Received: 28 July 2016; Revised: 22 October 2016; Accepted: 9 January 2017

Abstract

This paper characterizes the molting stages of giant freshwater prawn, *Macrobrachium rosenbergii* under laboratory conditions. Using the distal fifth pleopod as the main reference region and applying Drach's classification system, we checked the epidermis and carapace hardness and documented major structural changes, such as the retraction of epidermal tissues from the cuticle and setal development. Our findings showed that in early postmolt, no development of seta matrix was observed. A closer examination of seta lumen during the intermolt stage showed that the epidermis was a densely granular structure and the internal cone was developed. In the premolt stage, a retraction of the epidermis from the cuticle (apolysis) and the formation of new seta were recorded. This study shows that using internal seta development changes is apparently a useful, easy, and practical method of determining molting stage in order to facilitate the study of molt-linked processes and metabolism.

Keywords: setagenesis, molting stage, *Macrobrachium rosenbergii*, metabolism

1. Introduction

The molting cycle in crustaceans is generally divided into four phases: The first phase is metecdysis (Stages A and B), the period immediately following ecdysis; the second phase is anecdysis (Stage C), a period of tissue growth and accumulation of food reserves; the third phase is proecdysis (Stage D), a period of active morphological and physiological changes in preparation for the next molt; and the fourth phase is ecdysis (Stage E), the shedding of the old cuticle (Kuballa & Elizur, 2007; Tian *et al*., 2012).

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The ability to recognize the stages of molting is essential in the study of molt-linked processes, which can benefit hatchery operations and farm management. Several methods have been used to stage the molting cycle, including histological techniques (Benhalima *et al.*, 1998; Promwikorn *et al.*, 2005), measuring gastrolith size (Teruaki *et al.*, 2000), monitoring the regeneration of automized walking limbs (Maruzzo & Minelli, 2011; Mykles, 2001), and determining the changing titer of the molting hormone via radio-immunoassay (Hopkins, 1983; Wilder *et al.*, 1991). Among these techniques, external changes in the exoskeleton and internal seta development of the appendages are the most widely used by many researchers in a variety of crustacean species (Gardner & Mills, 2013; Guerao *et al.*, 2010; Marco, 2012; Musgrove, 2000; Philp & Marteinsdottir, 2013; Tian *et al.*, 2012) because, they are fast and cause little harm and no

stress to the animals. This method was principally developed by Drach and Tchernigovtzeff (1967). The objective of this study is to provide a detailed account of the molting stages in the economically important giant freshwater prawn, *Macrobrachium rosenbergii*, with reference to the morphological characteristics of setagenesis and external changes in carapace hardness.

2. Materials and Methods

M. rosenbergii with a mean body weight of 31.03±0.75 g were acclimated to laboratory conditions for at least two weeks in a circular, polyvinyl chloride maturation tank. After the two-week acclimation period, the individual prawns were transferred into a (59x34) cm^2 Perspex aquaria, each divided into two compartments by perforated plastic, for ecdysis observation. Ecdysis, marked by the presence of exuviae from the animal's body, was observed daily. Because ecdysis occurs at night, the incidences were recorded the following morning. The temperature of the aquarium was maintained at 28°C using a centralized-electronic control system. Food was offered once per day *ad libitum*. A total of 0.3 g (five pellets) of commercial finisher pellets (42% protein) for bottom-feeding invertebrates was given per animal. The water quality parameters (ammonium, nitrite, nitrate, and pH) were recorded once weekly (0900 hours) throughout the 25 day study period. For detailed observation of the molting cycle characteristics, animals were classified according to three overall stages: postmolt, intermolt, and premolt. Each molting stage was further divided into the following sub-stages: postmolt (Sub-stages A and B), intermolt (Sub-stages C_0 and C_1), and premolt (Sub-stages D_0, D_1, D_2, and D_3). Seta morphology was observed at 0, 5, 10, 15, 20, and 25 days after ecdysis. To measure setagenesis, the distal fifth pleopod on the left-side of the prawns was excised, floated in distilled water on a microscope slide, and observed at 200X magnification for seta development and at 100X magnification for epidermis structure. To confirm the molting stage, an image was captured using a digital image system connected to a computer. External changes in degree of carapace hardness were also observed by gently touching the outer surface of the carapace and rostrum (Figure 1).

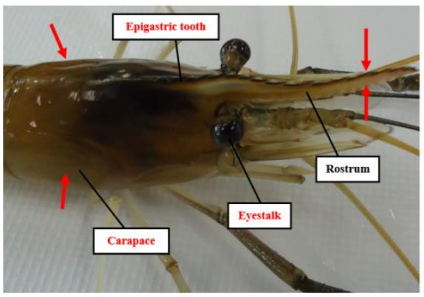

Figure 1. Positioning of animals for observing degree of carapace and rostrum hardness. Arrow indicates the point of examination.

3. Results and Discussion

The characteristics and duration of the molting stage after ecdysis in laboratory-maintained broodstocks of female *M. rosenbergii* are shown in Table 1.

Table 1. Characteristics of molting stages and approximation of duration after ecdysis in laboratory-maintained broodstocks of giant freshwater prawn, *M. rosenbergii*.

Molting stage	[1]Body weight (g)	[2]Approximation of duration after ecdysis	Exoskeleton texture	Epidermis structure	Seta development
A	31.10±1.71	<24 hour	Soft	Light blue pigmentation, granular	Clear seta matrix
B	31.58±3.04	1-3 days	Hardened	Granular	The internal matrix developed within the seta
C_0	30.95±2.29	4-9 days	Hard	Granular	The internal matrix cones develop within the seta
C_1	29.11±2.13	10-16 days	Hard	Granular very dense	Matrix in the internal cones of the seta begins to retract
D_0	34.37±1.70	17-20 days	Hard	Retraction of epidermis, pigmentation	Very fine structure of internal cones of seta
D_1	25.90±2.14	21-23 days	Hard	New setal-forming regions appear in the epidermis surface	New seta begin to develop
D_2	29.40±2.26	24-27 days	Hard	Developing new seta can be seen within the setal-forming regions in the epidermal surface	New seta form barbules
D_3	29.05±2.20	28- 29 days	Soft	Complete apolysis	Old setal organs disappear, new setae fold

*Total number of prawns observed, N = 73.[1]The values are expressed as (mean±S.E.M).[2]The values are expressed (in range).

Stage A is the first stage immediately after the prawn flicks clear of the old exuvia. At this stage, the entire body and the exoskeleton were found to be very soft, slippery to the touch, and pale in color, with a flesh-like appearance. Due to these characteristics, the prawns exhibited hiding behavior to avoid predation by other intermolt prawns. The epidermis had a light blue pigmentation and was less granular than in the previous stage (Figure 2-1a). The granular protoplasm was continuous throughout the setae filling, and an absence of internal cones was observed (Figure 2-1b). The duration of Stage A was short; it took only a few hours, ending less than 24 hours after ecdysis.

The beginning of cuticular node development marked the B stage, which generally took place 1-3 days after ecdysis. The setal lumen of the pleopod appeared more granular than in the previous stage, and the setal walls thickened, as shown in Figures 2-2a and 2-2b. The cuticle between the seta bases was still thin, and it did not extend below the seta bases. The exoskeleton became relatively hard, but it was easily depressed.

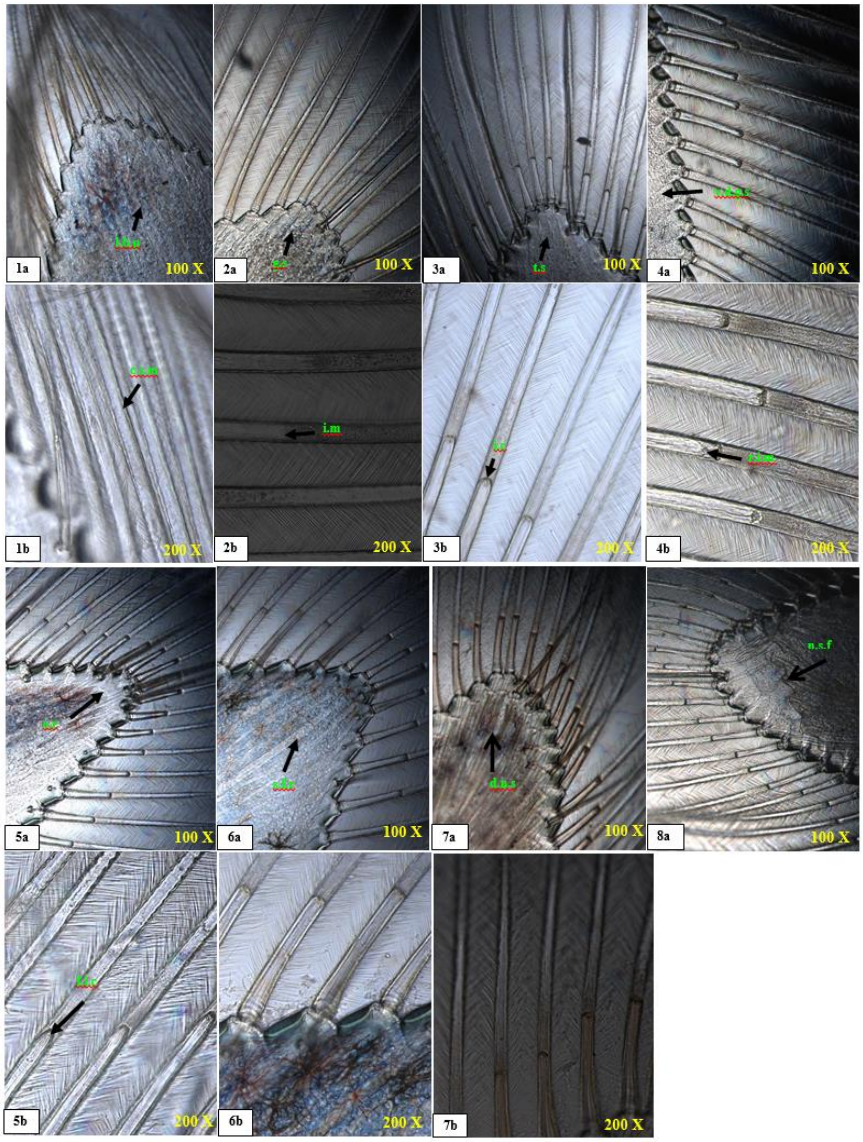

Figure 2.Morphological characteristics of setagenesis in the giant freshwater prawn, *M. rosenbergii* during (1) early postmolt A stage: light-blue color pigmentation (l.b.p) of epidermis, less granular structure (1a), and clear seta matrix (c.s.m.), no internal cones (1b); (2) late postmoltB stage: granular structure (g.s.) of epidermis (2a), and the formation of internal matrix (i.m) inside seta lumen as marked by two contrast area (2b); (3) early intermolt C_0 stage: transparent structure (t.s) of epidermis (3a), and the formation of internal cones (i.c) inside the seta lumen (3b); (4) late intermolt C_1 stage: epidermis forms a very dense granular structure (v.d.g.s)(4a), and retraction of matrix in the internal cones (r.i.m.); (5) early premolt D_0 stage: retraction of epidermis (e.r.) (5a), and fine-structural of internal cones (f.i.c) (5b); (6) earlypremolt D_1 stage: new setal-forming regions (s.f.r) appear in the epidermis surface (6a), and new seta (n.s) begin to develop (6b); (7) late premolt D_2 stage: developing new seta (d.n.s) with tube in a tube with well-defined, blunt proximal end (7a), and new seta forms barbules structure (b.s) (7b); (8) late premolt D_3 stage: Old setae organs disappear, new seta fold (n.s.f). Complete apolysis (8a). Arrow indicates point of focus.

The most significant observation of Stage C_0 was that of the fully developed cuticular nodes and the formation of internal cones in the seta of the pleopods. The distal parts of the seta appeared clear and transparent, due to the retraction of the setae protoplasmic matrix, as shown in Figures 2-3a and 2-3b. The cuticle was thick and generally extended below the seta bases. The rostrum could be bent easily, and the carapace became firm and rigid. This stage occurred approximately 4-9 days after ecdysis. Figure 2-4a shows that in stage C_1, there was a very dense granular structure in the epidermis zone. It was further observed that the seta protoplasmic matrix retracted from the tip of the internal cones, as shown in Figure 2-4b. This particular stage generally occurred 10-16 days after ecdysis.

The onset of the premolt period was marked by a separation of the cuticle at the base of the seta due to the withdrawal of the epidermis and subsequent development of the new seta. An enlargement of the epidermal cells and retraction of the epidermis were observed during the premolt D_0 stage in the cuticle between the seta bases. The diagnostic feature of this stage was the retraction of the pigmented epidermis, which left a transparent gap between the cuticle and the epidermis (Figures 2-5a and 2-5b). This stage generally occurred 17-20 days after ecdysis. The shafts of the developing seta were entirely visible above the epidermis surface during the D_1 stage (Figure 2-6a). The presence of fully developed seta with ill-defined proximal ends was characteristic of this stage, as shown in Figure 2-6b. The prawns generally entered this stage 21-23 days after ecdysis. In stage D_2, the fully developed new seta had the appearance of a tube within a tube with a well-defined, blunt proximal end (Figure 2-7a). As shown in Figure 2-7b, the development of the seta was completed, with shafts bearing barbules. This stage generally occurred 24-27 days after ecdysis. Morphological observations of the D_3 stage revealed the disappearance of old seta organs and the new seta beginning to fold, marked by complete apolysis, as shown in Figure 2-8a. The carapace became soft and spongy. This stage generally occurred 28-29 days after ecdysis.

The morphological changes associated with seta development in pleopods and the degrees of carapace hardness in the *M. rosenbergii* were found to be good indicators for identifying the different sub-stages of the molting cycle. Pleopods were used in the present study because the removal of other appendages results in trauma or death, and the relatively thin cuticle of the pleopod facilitates observations of seta development. The different staging methods used by various researchers was not the area of concern in this study; being able to determine the molting stages of each experimental animal to facilitate studies on molting was the subject of interest.

We observed that the duration of the molting cycle, as well as the length of individual molting stages, showed a high degree of variability in *M. rosenbergii* individuals, even under laboratory conditions. These findings are corroborated by Granados *et al.* (2012), who observed the molting cycle of *Macrobrachium tenellum* in the laboratory and, suggested that the length of a molting stage provides a close approximation of proper staging, but not a fully accurate one, because experimental conditions and food are different from natural environmental conditions. Long intermolting periods in *M. rosenbergii* take up an average of 50-60% of the total molting cycle duration; thus, it would be arbitrary to define the transitions between intermolt and premolt stages. In this regard, it is very difficult to estimate the expected time scale for individual molting stages because frequency of molting is highly dependenton factors closely related to hormonal control, developmental stages (Guerao *et al.*, 2010; Hayd *et al.*, 2008) and environmental conditions such as temperature (Gong *et al.*, 2015), food (Kumulu & Kir, 2005; Wang *et al.*, 2016), water chemistry, and photoperiod (Guo *et al.*, 2011, 2012a, 2012b, 2013; Talghavi *et al.*, 2013). This study also shows that the water quality parameters are generally suitable for the *M. rosenbergii* culture, with measurements of temperature ranging from 28-30ºC, ammonium levels (≤0.03 ppm), nitrite levels (≤1 ppm), nitrate levels (≤60 ppm), and pH values ranging from 7.0-8.3.The range of water temperature in this study (28-30ºC) were found to coincide with the study conducted by Gong *et al.* (2015), where they found that the temperature ranging from 26-32ºC showed higher molting success rates(90.7-94.7%), and the molting interval shortened to within 3.7-4.9 days in the early juvenile mud crab, *Scylla paramamosain*.

Degree of carapace hardness provides a basic external clue to the general molting stages i.e., post-, inter-, or pre-molting; however, the confirmation of these sub-stages would require detailed examination of the seta. Therefore, we suggest a quantitative measurement of carapace hardness using a hand-held durometer, as reported by Chung *et al.* (2012), in order to calculate the molt-associated somatic increments. In our observation, premolt animals possess a carapace that is relatively harder than that of postmolt animals. This phenomenon draws attention towards evolutionary studies on how cuticle synthesis and calcium deposition influence the degree of carapace hardness in the molting process of *M. rosenbergii*. To our knowledge, no studies have yet been conducted in this species, only in terrestrial isopods, such as *Titanethesalbus*. Vittori *et al.* (2012) incorporate sternal calcium deposits and ultra-structural characteristics of epidermal cells to understand the relationship between calcium dynamics and molting.

4. Conclusions

The findings of this study provide a basis for further study of molt-linked processes and proper assessment of the impact of environmental changes on the molting cycle and metabolism of *M. rosenbergii*. The characterization of molting stages based on setagenesis was found to be a simple, easy, and practical method to assist aquaculture practitioners and researchers in identifying the individual molting stages.

Acknowledgements

Authors would like to thank Thirukanthan Chandra Segaran, Institute of Marine Biotechnology, Universiti Malaysia Terengganu, for his assistance in English proof-reading of this manuscript. Our appreciations also to all individuals involved in this study.

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